A Comparative Electrophysiological Study of the Effects of Paralytic Shellfish Poisons on Nerves, Nerve-Muscle, and Neuronal Cell Types.

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A thesis submitted for the degree of Doctor of Philosophy
to the Faculty of Science of the University of Glasgow

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December 1997

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DECLARATION:

I declare that this thesis represents, except where a note is made to the contrary, work carried out by myself. The text was composed by myself.

Linda Jane Tierney

December 1997

"Red Tide" formed by the dense proliferation of the dinoflagellate *Noctiluca* in a Japanese harbour. Even non-toxic species such as this algae can kill marine animals by depleting the oxygen in shallow waters (reprinted courtesy of Don Anderson).

ACKNOWLEDGEMENTS

Many people have contributed in so many ways towards the ultimate existence of this thesis. To begin with, I would like to thank my supervisor, Dr Douglas Neil for his continued patience, support and stimulating discussions (which have contributed greatly to the quality of this thesis). I would also like to sincerely thank Dr Eddie Rowan for his generous supervision at the University of Strathclyde, Prof Alan Harvey for allowing me extended use of his laboratory and Dr John Dempster, for use of his software and useful discussions. Special thanks also go to Dr Thomas Weiger who taught me the essentials of patch clamping and to Prof Anton Hermann who allowed me to use the facilities at the University of Salzburg.

At CSL, Torry, Aberdeen, I am indebted to Dr Margaret Donald, for supplying the PSP samples for this study and for her supervision and friendship. Special thanks go to Mr. Charlie Murray for allowing me to tap his knowledge. Thanks also go to Dr Don Gibson for his initial supervision at Torry.

Thanks also are extended to Dr Harry Birkbeck for the supply of the N2a cells and other essentials, and his interest in the investigations. For maintaining the cells at the University of Glasgow, I have to thank Mrs Pat Ritchie, who was also of great moral support, and Dr Pauline Munro. I would also like to thank Dr Susan Gallacher for her discussions in the early stages of this study. Within the neurobiology laboratory at Glasgow I wish to thank Dr Maria Denheen and Dr Abdul Chrachri for their experimental expertise to get me started. I must also sincerely thank Dr John Leftley for the supply of dinoflagellate cultures, a continuous supply of reference material and background knowledge and Paul Auty for his generous help with the reproduction of the micrographs.

A large number of people have contributed through their friendship and support in a manner of ways without which I could not have completed this work with such a brave heart. These include at Strathclyde, Drs. Mohammad and Zahara Faheti, Cleane Medeiras, Louise Young, and at Glasgow, almost everyone at some stage in the "Zoology Department". Thanks to them all.

Last, but certainly not least, I have to say thanks to my family, Lawrence, Kerry and David and my parents, for all their patience and support and finally, to Jane Lee my "growth buddy" for many years.

This study was supported by a SERC grant in collaboration with CSL (MAFF), Torry Aberdeen.

SUMMARY

This study has used a variety of electrophysiological techniques to investigate the effects of crude shellfish extracts containing paralytic shellfish poisons (PSP) on the sodium (Na^+) channel behaviour and on the electrical excitability of nerves, nerve-muscle systems, and cultured and dissociated neuronal cells.

In one series of experiments, four novel *in vitro* tissue preparations were investigated. A crustacean nerve-muscle system, a crustacean mechanoreceptor-nerve preparation, an avian nerve-muscle system and an amphibian sciatic nerve, were exposed to the marine toxins saxitoxin (STX), tetrodotoxin (TTX), and PSP samples prepared under AOAC procedure by MAFF, Torry, Aberdeen.

The nerve-muscle preparation from the Norway lobster, *Nephrops norvegicus (L),* was chosen as an apparently simple yet robust system. Extracellular electrical stimulation of the motor nerve (R3) produced excitatory post-synaptic muscle potentials (epsp's). The preparation was highly sensitive to STX and TTX, with a minimum inhibitory threshold of 10^{-12} M. The inhibitory effect was not dose-dependent, toxin concentrations of less than 10^{-8} M resulted in a variable inhibitory effect on the epsp amplitude, whilst values above this concentration resulted in a sudden and complete loss of the epsp. Several concentrations of toxin could be applied to the same preparation.

The nerve preparation from the crab, *Carcinus maenas*, was also chosen for the simplicity of the system. Extracellularly-recorded action potentials induced by natural stimuli were reduced in amplitude by Na^+ channel blocking toxins present in the PSP samples.

The chick biventor cervicis (CBC) nerve-muscle preparation was highly sensitive to the sodium channel blocking activity of PSP samples. An inhibitory effect on muscle twitch amplitude could be detected at values as low as 0.4 ng/ml, making it more sensitive than the mouse bioassay, and the inhibition increased in a dose-dependent manner from 400 ng/ml. IT_{50} values (i.e. the time required for the twitch control amplitude to be reduced by 50%) of the PSP samples tested yielded a correlation of $r^2 = 0.63$.

The isolated partially desheathed sciatic nerve of *Rana temporaria* proved to be a highly robust, simple and reliable assay system for PSP samples with a detection sensitivity of 2 ng/ml. Using a Vaseline gap method of stimulation, inhibition of the compound action potential (CAP) was found to be dose-dependent with mean IT_{50} values for PSP samples yielding a correlation of $r^2 = 0.696$, and for recovery times a correlaion of $r^2 = 0.918$. A single preparation could be used for measurement of several samples, with a complete recovery of the CAP after each toxin exposure.

In another series of experiments the electrical properties of three neuroblastoma (tumour) tissue culture lines: Neuro 2a (N2a), NG108-15, S.K.N.SH, and also acutely dissociated primary cells of dorsal root ganglia (DRG) and superior cervical ganglia (SCG), from new born rat pups were examined.

Sodium channel properties of the three neuroblastoma lines were investigated at various stages of differentiation. Cell resting membrane potentials became significantly more hyperpolarized after differentiation. Patch clamp techniques revealed an inconsistency in Na^+ channel expression before differentiation. After differentiation, the IC_{50} values of the N2a and NG108-15 cells for STX were 7.8 nM and 15.5 nM respectively, with correlations of $r^2 = 0.938$ and $r^2 = 0.979$. The effect of STX was concentration-dependent. PSP samples tested on these two cell lines also had an inhibitory effect.

At least two types of Na^+ current can be recorded in the DRG and SCG cells: an STX-sensitive (STX_S) current and an STX-resistant (STX_R) current. Exposure to STX blocked >92% of the STX_S currents, whilst the mean block of STX_R currents was 18%.

Intracellular and patch clamp methods were used to examine the principle underlying the use of veratridine (VER) and ouabain (OUB) in the neuroblastoma cell bioassay. Both compounds caused a large depolarisation of cell resting membrane potential. In addition, VER reduced the size of the early transient Na^+ current and increased a persistent tail component of the Na^+ current. STX could inhibit the effect of VER but had little or no effect on the action of OUB.

Photographic studies of the N2a cell line revealed morphological changes during a four day period, including an increase in neurite outgrowth, which was dependent on the concentration of serum in the growth medium. Scanning electron micrographs revealed retraction of neurites, cell membrane damage and a decrease in cell survival rate, especially after exposure to VER.

A tetrazolium salt 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect the cytotoxic action by PSP extracts. Samples containing STX eq values almost equivalent to, or less than, the AOAC safety level (80 μg) caused a greater decrease in cell survival rates than samples with a higher STX value. VER and OUB caused significant reductions ($p = < 0.05$) in cell survival rates.

The data obtained from these investigations are discussed in relation to our existing knowledge of the action of PSP toxins, and to the development of future detection and screening methods.

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GLOSSARY

AOAC	Association of Official Analytical Chemists
CAP	compound action potential
CBC	chick biventor cervicis
CF	conversion factor
CSL	Central Science Laboratory
ELISA	enzyme-linked immunosorbent assays
epp's	endplate potentials
epsp(s)	excitatory post synaptic potential(s)
IMS	indirect muscle stimulation
ipsp(s)	inhibitory post synaptic potential(s)
Ca^{2+}	calcium ion
Cl^-	chloride ion
K^+	potassium ion
mepp's	miniature endplate potentials
MAFF	Ministry of Agriculture, Fisheries and Food
MU	mouse units
Na^+	sodium ion
OUB	ouabain
PSP	paralytic shellfish poison
RMP	resting membrane potential
RT_{50}	time required for a response to recover by 50%
SF	superficial flexor muscle
STX	saxitoxin
STX_R	saxitoxin resistant
STX_S	saxitoxin sensitive
TTX	tetrodotoxin
IT_{50}	time required to inhibit a response by 50%
VER	veratridine

Chapter 1

General Introduction

"To distinguish the fatal spot where the mussels were eaten, I have called it Poison Cove, and the branch leading to it Mussel Channel."

G. Vancouver (1798)

1.1. Algal Blooms and Paralytic Shellfish Poison

Saxitoxin (STX) and its derivative heterocyclic guanidines are produced by dinoflagellates of the genera *Alexandrium (Gonyaulax), Pyrodinium,* and *Gymnodinium* (Table 1.1), and are now known to be the causative agents of Paralytic Shellfish Poison (PSP). In an excellent review, Kao (1966) describes the history, clinical aspects, occurrence, distribution, chemistry and pharmacological actions of both STX and the related guanidine toxin, tetrodotoxin (TTX).

Microscopic, unicellular, planktonic algae (eukaryotic, mainly autotrophic, plant cells) provide a critical food source for filter-feeding bivalve shellfish such as oysters, scallops, mussels and clams, as well as the larvae of commercially-important crustaceans and finfish. When they reach densities of several million of cells per litre, proliferation of these algae is referred to as a "bloom". In most cases algal blooms are harmless in nature, but under certain circumstances they can produce potent phytotoxins which exert a major impact on the environment, cause severe economic losses in the shellfishing and aquaculture industry and threaten human health (Table 1.1).

These harmful algal blooms are natural phenomena which discolour the surface of the sea, producing "red-tides". These have been recorded in the Bible as far back as the ancient Eygptians, ...'all the waters that were in the river were turned to blood...the fish died...the river stank...the Eygptians could not drink of the water...' (Exodus 7: 20-21). A survey of fossil dinoflagellate cysts dates some species at 50 million years ago (McMinn 1989). There are some 5000 species of exant marine phytoplankton, approximately 300 of which are known to form blooms. Of these species, only about 40 are thought to produce toxins that pass through the food chain to humans (Hallegraeff 1993) causing a variety of neurological and gastro-intestinal illnesses. Those species whose toxins cause major world-wide health concern are listed in Table1.2.

Medical records for several centuries have described cases of fatal food poisoning involving PSP. In 1609, Lescarbot, a well-travelled French lawyer, noted that it was taboo for local North West American Indian tribes to eat mussels when the sea surface became bioluminescent due to dinoflagellate blooms. When they were starving they would eat their dogs or even the bark of trees, but would not touch the shellfish (Prakash *et al.*, 1971; Dale and Yentsch 1978). One of the first recorded cases of a human fatality from eating contaminated shellfish was that described by Captain George Vancouver in 1793 (see Kao 1966, for detailed description of the event).

PSP was for many years a puzzling phenomenon. It is unpredictable and sporadic in its occurrence, but always seems to be related to the consumption of mussels, scallops or clams. Toxicity of these shellfish usually appears suddenly, and yet within a week or two of severe cases of poisoning, shellfish from the same location can be eaten again without harm. It was only after a severe epidemic of mussel poisoning around San Francisco in 1927, when 102 people were affected, that detailed investigations into the cause began. Meyer *et al.* (1928) speculated that certain dinoflagellates eaten by mussels could be a contributory factor. This was later confirmed in 1932 when a "new" dinoflagellate species, *Gonyaulax catenella,* was discovered in large numbers, which caused non-toxic shellfish to became highly poisonous when fed to them. This was corroborated when acidic water extracts of these dinoflagellates and the mussels were injected into mice and killed them after evoking the same symptoms as in humans (Schantz 1960). This effect formed the basis for the most widely used detection method for PSP, the mouse bioassay (**section 2.4.1**).

There appears to be no clear correlation between algal concentrations and their harmful effects. The toxigenic dinoflagellates of the genus *Alexandrium* (alternatively known as *Protogonyaulax catenella/tamarensis* and formerly as *Gonyaulax* spp.) (Plate 1.1) can contaminate shellfish at very low cell densities. Mussels (*Mytilus californianus*) can become toxic when cell counts are as low as 200-400 cells/ml sea water, and counts as low as 20 cells/L have caused shellfish toxicity (Halstead 1984). During the last two decades algal blooms associated with human intoxication appear to have increased not only in frequency, but in their intensity and geographical distribution (Krogh 1987;

Wyatt 1995). Dinoflagellate species once considered endemic to particular geographic regions are now identified in areas previously unaffected (Figure 1.1.a) (O'Hara 1993).

A number of theories have been put forward to account for the significant global epidemic spread of these harmful algal blooms (HABs) during the past 20 years. Increased eutrophication of coastal waters and inland seas has been suggested to contribute to changes in nutrient contents of river discharges and estuaries (Smayda 1990), which have influenced decline in the Si:N and Si:P ratios favouring an increase in HABs (Provasli 1979; Anderson 1987; Sohet *et al.*, 1995). Changes in climate conditions, such as El Niño (Taylor 1992; Heath 1993) have also been implicated, and ocean currents can also transport HABs (Anderson 1994).

Increased utilization of coastal waters for aquaculture both for finfish and shellfish has occurred (Halstead 1984; White 1984), and the movement of shellfish stocks from one area to another is regarded as another possible vector (Dale *et al.*, 1978; Bricelj *et al.,* 1991). The transport of toxic dinoflagellates and their resting cysts in ships' ballast water (Hallegraeff and Bolch 1991; Rigby *et al.*, 1995), may also be responsible for the translocation and subsequent establishment of non-indigenous planktonic species to new areas. However, increased scientific awareness and the surveillance of toxic species has also increased (Levin 1992; Smith *et al.*, 1993, Anderson 1994), and the global expansion of aquaculture means that more areas are monitored closely.

Although PSP intoxication has long been associated with a variety of molluscan bivalve species, STX (sometimes together with TTX) has been isolated from marine species of finfish (e.g., mackerel), octopus and various zooplankton, and from intertidal species including the primary consumers such as barnacles, tube worms, flatworms, and annelids, and secondary consumers such as crabs, lobsters and starfish (Jonas-Davies and Liston 1985; Tamplin 1990; Desbiens and Cembella 1995; Tsai *et al.*, 1996). PSP intoxication has also been reported to be responsible for deaths in birds and marine mammals such as whales (Anderson 1994). PSP has now been reported in fresh water puffer fish (Zaman *et al.*, 1997) and the detection of new STX analogues continues to be

reported in marine species already known to become toxic (Arakawa *et al.*, 1995; Tsai *et al.*, 1996; Grzebyk *et al.*, 1997).

1.2. Biosynthesis of PSP

Variability of toxin profiles in both composition and toxicity has been identified within genetically identical dinoflagellate isolates in the same population as well those from different geographical regions (Cembella and Taylor 1985; White 1986; Ogata *et al.*, 1987; Lassus *et al.,* 1989; Anderson 1990; Oshima 1995). This appears to be dependent on many factors, including growth conditions, such as light necessary for photosynthesis (Ogata *et al.*, 1987; Kodama and Ogata 1988; Usup *et al.*, 1995); temperature (Usup *et al.*, 1995); salinity (White 1978); nutrients, especially the carbon:nitrogen ratio (Anderson *et al.*,1990; Flynn *et al.*, 1995), as well as the stage of cell cycle (Boczar *et al.*, 1988; Cembella *et al.*, 1990); more specifically DNA synthesis (Olson *et al.*, 1986). Specific enzymes, N-sulfotransesterases, found in several dinoflagellates, are now considered to be part of the PSP biosynthesis pathway (Oshima 1995), and significant changes in the composition of intracellular amino acids especially that of arginine and glutamine has been observed (Flynn *et al.*, 1995).

The biosynthetic source of saxitoxins in dinoflagellates is still subject to controversy, and two main schools of thought exist. One proposes that the organisms synthesise the toxins in a series of steps requiring multiple genes, and indeed early experiments (Burke *et al.*, 1960; Schantz 1963) showed that *Gonyaulax catenella* could produce toxin when cultured under axenic conditions, in the absence of symbiotic bacteria. Similarly, Sako *et al.* (1995) have reported stability, and heredity experiments with cultured dinoflagellates, show a 1:1 Mendelian behaviour pattern in toxin inheritance over progenies of many populations which has a composition very similar to those of wild strains.

The other theory proposes that the primary source of toxins is provided by endogenous (or possibly exogenous) bacteria that have a symbiotic-like existence with the dinoflagellates (Silva 1982; Kodama and Ogata 1988; Kodama *et al.*, 1996; Gallacher *et al.*, 1997). However, although bacterial toxigenesis was demonstrated, the

toxins levels measured were minimal compared to those produced by dinoflagellates (Kodama 1990). In addition, toxin composition and content was different (Doucette and Trick 1995) whilst Ogata *et al*, (1990) found that only two dinoflagellate species out of several collected from various locations produced PSP toxins. Tamplin (1990) suggests that low levels of toxin observed in laboratory cultures may indicate that the host environment has not been duplicated, or culture medium still requires refinement. Alternatively, some synergism may occur between a small number of symbionts and the host dinoflagellate that is lost when the bacteria are isolated in culture or perhaps a bacterial gene (Anderson 1994), or plasmid (Shimizu 1988) may be involved.

1.3. Accumulation of PSP toxin by shellfish

Suspension-feeding bivalve molluscs are susceptible to the accumulation of algal toxins present within the pelagic and benthic food webs. These filter feeders sequester the toxins in their tissues, thereby acting as a vector in the PSP food chain. The ingested cells are initially concentrated in the digestive gland (hepatopancreas and liver). At this stage toxin content and profile resemble that of the primary producer (Beitler and Liston 1990). The profiles of the toxins vary not only according to dinoflagellate species and location but also to seasonal changes (Cembella *et al.*, 1993). Superimposed on this are the changes due to processes in the accumulating organism, resulting in a compositional diagenesis during sequestration (Hall and Reichardt 1984).

Accumulation rates of PSPs from the food source varies between bivalve species. Mussels, e.g., *Mytilus edulis*, are known to accumulate toxicity very rapidly and may become toxic in less than one hour if exposed to a highly toxic strain of dinoflagellates (Bricelj *et al.*, 1990) whilst in other bivalve molluscs, such as scallops accumulation may take up to a week (Kodama and Ogata 1988). The monitoring of PSP in shellfish by the CSL, Aberdeen, is in fact based on toxin levels in mussel samples they receive for analysis.

There also appears to be a difference in the kinetics of sequestration and elimination of toxins between species. Mussels can excrete or degrade the poisons within 2-3 weeks of a bloom disappearing (Schantz 1984, Desbiens *et al.*, 1990),

whereas sea scallops and several clam species are capable of prolonged retention (Martin *et al* 1990, Cembella *et al.*, 1993). Phasic toxicity reported in the absence of blooms or during the winter is thought to be due either to the bioconversion of low to more potent STX derivatives (Cembella *et al.*, 1993) or to an exogenous source (Shumway and Cembella 1993) possibly cysts (Ingham *et al.*, 1968; Lassus *et al.*, 1989).

Compartmentalisation of the PSP toxins into various body tissues has been found to be species specific. Therriault and Levasseur (1992) suggest that shellfish may initially screen the toxic organism immediately following ingestion into the mantle cavity, on the labial palps or in the gut before digestion occurs. During the bloom, when toxicity levels are high, several clam species concentrate toxins rapidly into the viscera, mainly the mantle, kidney, pallial muscle, heart and pericardial gland, with lower amounts found in the gill and gonad. After a lag of about 4 weeks the siphon becomes the major repository organ, predominantly for STX. During bloom supression however, toxins transfer in high levels to the brown gland (which may function as part of the excretory system) (Schantz 1984; Beitler and Liston 1990; Martin *et al.*, 1990).

In North America and the UK only the large adductor muscle of scallops, which is usually free of accumulated toxins, is removed for consumption, while other tissues are normally discarded. However there has been a recent expansion in the sale of non-traditional scallop tissue (e.g. whole and "roe-on" scallops), and it is following this consumption of whole scallops that cases of PSP have been reported, with Japan ceasing to supply whole scallops to certain markets, especially France (Shumway and Cembella 1993). Levels of PSP toxicity in scallop roe (gonads) are usually below the regulatory limit, a typical value being 30-40 μg STXeq/100g. However, where extrememly high toxicity is detected in scallop digestive gland tissue, significant levels of toxicity are also found in the gonad, one sample recording1300 μg STXeq/100g (Cembella *et al.*, 1994).

1.4. The impact of PSP on commercial shellfish markets

There is great interest in PSP because of its economic impact on the multi-billion pound fish farming and shellfish industries. The expanding world-wide markets for such products have resulted in more widespread epidemics of human intoxications, rather than local outbreaks (Taylor 1990). The whole shellfish industry can be affected by such events, which reduce both consumer confidence and sales. The shellfish trade for Europe alone is in excess of 10^5 metric tonnes/annum. A PSP outbreak in the UK in 1990 resulted in 1000 boats being tied up and more than 3000 people put out of work for a period.

Statutory legislation in the UK now requires there to be monitoring and surveillance to ensure public health by preventing potential risks of fish and shellfish contamination by algal toxins. Monitoring of shellfish samples landed from potential PSP sites around the British Isles is currently under the directive of agencies of the Ministry of Agriculture, Fisheries and Food (MAFF). However, there are a number of shortcomings in both the scope of the current management regime in shellfish harvesting areas, and the quality control of the main screening method, the mouse bioassay.

All UK incidents of PSP have involved mussels. Between the years 1827 and 1975 ten outbreaks were identified, with 116 people affected and 14 deaths (Scoging 1991). The first well documented epidemic of PSP intoxication in Britain (Ingham *et al.*, 1968, McCollum *et al.*, 1968) occurred between Bridlington, N.E. England and Rosehearty, north of Aberdeen, Scotland (see Figures 1.2 and 1.3) when over 300 miles of coastline had contaminated mussel beds. Toxicity ranged from 2012 μg - 4160 μg STX equivalent/100g wet shellfish meat (10,062 to 20,800 Mouse Units (MU)/100g by the mouse bioassay). Seventy eight people were hospitalized. The causative agent was identified as excysted *G. tamarensis* (Adams *et al.*, 1968, Lewis *et al.*, 1995). Since 1968 PSP has been detected annually along the North East coast of Britain.

In contrast, the West coast of Britain was unaffected in 1968, and has tended to remain so, although *Gonyaulax spp.* have been detected around most of the UK coastline (Figure 1.1.b). However, in 1990, the first cases of PSP were recorded in West

Scotland, when a maximum concentration of 3,023 μg STX equivalent/100g wet shellfish meat (16,480 MU) was detected in mussels and scallops (van Egmond *et al.*, 1993). This is over 40 times greater than the critical safety limit for health consumption (Table 1.2). During the PSP season, normally between April and September, regular warnings are now posted by local authorities to alert the public about the danger of beach collection of shellfish (Figure 1.4).

Elevated concentrations of PSP continue to be detected at various sites, but not at levels found in the 1968 outbreak (Figure 1.2 and 1.3). The exception was again 1990, when the maximum concentration detected was found in a sample from Trow Rocks with a level of 4000 μg/100g shellfish flesh (MAFF 1994a; Joint *et al.*, 1997). In most cases, *A. tamarense* was reported to be the causitive organism (C. Murray *pers. comm.*; Lewis *et al.*, 1995). An example of the problems caused by PSP occurred in December 1991, when the European Commission placed a ban on all Scottish shellfish exports after traces of PSP were found in scallop gonads harvested from Orkney. This was thought to be from a frozen batch left over from an earlier PSP ban in the same year, but this was denied by the company involved. In 1994 the most toxic PSP sample detected from the N.E. coast registered 953.4 μg STX eq/100g tissue (M. Donald, MAFF, *pers comm.*). PSP has recently been reported in both *Alexandrium* and shellfish taken in Northern Ireland around Belfast Lough, but there are no reported incidences of human poisoning (McCaughey and Campbell 1992). Diarrhetic shellfish poison (DSP) still remains the predominant shellfish toxin in Ireland (Carmody *et al.*, 1996).

In 1992/1993 algal blooms caused a crisis in the New Zealand shellfish industry which subsequently sustained severe economic damage (O'Hara 1993). In North America, where PSP intoxication has been endemic for many years, STX levels in excess of 45,000 μg STX eq/100g have been recorded from Bay of Fundy scallops whilst the equivalent of 130,000 μg STX/100g, has been recorded from Japanese scallop tissue.

Sensitivity of humans to PSP intoxication varies considerably, and very few reliable records exist of the amounts of contaminated shellfish consumed. Extreme symptoms have been recorded with an intake estimated to be as low as 325 μg (2200

MU), but in other cases no symptoms of poisoning have been detected with an intake as high as 2720 μg (17,000 MU) (Quayle 1969). The lethal dose of STX was estimated to be 1 mg by Kao (1966), 1- 4 mg by Schantz (1984) and Anderson (1994), and 500 to 12400 μg (0.5 mg-12.4 mg) by Krogh (1987); the currently accepted lethal dose is taken to be 7-16 μg/kg body weight.

Most countries with shellfish growing areas have adopted a tolerance level of 80 μgSTX eq./100 g of shellfish tissue, as introduced in the US and Canada around 40 years ago (Table 1.3), and used in Britain for many years (MAFF 1994a). This level has also been adopted in Europe, with three exceptions: in the Netherlands and Italy tolerance levels of 40 μg/100g have been set (i.e. no detectable amounts by mouse bioassay), and in Austria, which has no shellfishing industry, a tolerance level of just 10μg/100g operates. It is noteworthy that since the introduction of the standard 80 μg safety cut off level, no death has occurred in the US following consumption of "controlled" seafood (Krogh 1987). The safety cut off level reflects the potential health hazard that might result from consuming contaminated tissue.

1.5. Clinical symptoms of PSP intoxication

The clinical symptoms of PSP intoxication (Table 1.2) involve mainly the neuromuscular, cardiovascular and respiratory systems. It is known that STX and related PSP toxins block nerve transmission by reducing the amplitude of the action potential, that motor and sensory nerves are equally affected and that synaptic transmission at the end plate is relatively unaffected (Kao and Nishima 1965; Evans 1969). Consistent symptoms have been reported in all documented cases. The first effects are parethesia and numbness around the lips, tongue and mouth, reflecting the fact that PSP toxins are readily absorbed through the buccal mucus membranes. A tingling sensation spreads to the fingertips and toes, frequently accompanied by headaches and dizziness. In moderate to severe cases parethesia spreads to the arms and legs, along with distinct motor weakness which Kao (1993) suggests reflects a progression from the blocking of the thin, unmyelinated sensory nerves to the inhibition of the larger diameter, myelinated motor neurons.

Sometimes vomitting, sweating, and convulsions are also evident (Bond and Medcof 1958), as are giddiness, incoherent speech, and sensations of "floating". These effects are possibly due to interference with afferent proprioceptive signals in the sensory system. Progressive intoxication is marked by ataxia, motor in-coordination and dysmetria. Respiratory difficulties begin with a tightness in the throat, and in severe cases there is paralysis of the intercostal muscle, resulting in respiratory failure. Death can occur within 2 to 24 hours, depending on how much poison is consumed. As little as 1 mg poison, which can be ingested from one highly toxic shellfish alone, has been known to be fatal in humans (Kao 1966; Schantz 1984).

The exact cause of respiratory depression is still unclear, but Murtha (1960) and Kao (1966) reported that both peripheral and central effects are involved. During the first British epidemic the severity of neuromuscular symptoms in some patients suggested cerebellar involvement which led McCollum *et al.* (1968) to suggest that STX can cross the blood-brain barrier. However Evans (1969) disputes the involvement of central depression, and postulates a peripheral paralysis of the respiratory muscles.

An anti-STX rabbit serum has been reported to neutralise STX (but no other PSP components) both *in vitro* and *in vivo* in mice (Davio 1985) but to date, there is no effective antidote for PSP intoxication. Therapy using 4-aminopyridine (4-AP), a known K^+ channel blocker, has restored the ability to breathe spontaneously in guinea pigs within minutes, following a lethal dose of STX (Chang *et al.*, 1996) or TTX (Lundh 1978; Kawai and Niwa 1980). The therapeutic effects of 4-AP reversed the blockade of diaphragmatic neurotransmission, vascular hypotension, myocardial anomalies, bradycardia and aberrant discharge patterns of medullary respiratory-related neurons to control levels. There is no indication however, of the success rate of 4-AP following administration of PSP rather than STX alone.

Most of the PSP toxins are positively charged (described below), due to the guanidinium moieties possessing alkaline pK_as, and are protonated with a net cationic charge at the human body pH of 7.4, yet they are easily absorbed through the gastrointestinal mucosa (van Egmond 1993). Elimination of the toxins from the body through urine excretion (Prinzmetal *et al.*, 1932; Stafford and Hines 1995), suggests a

half-time for elimination after intravenous injection of 100 mouse units (MU), equivalent to 20 μg STX, into a dog, is around 120 min. Thus in mild cases where toxin consumption is low, 12 h (six half-times) is probably a reasonable time period for a reduction of the toxin concentration to relatively harmless levels. Excretion probably takes place almost as fast as absorption, thereby preventing a high concentration of the toxin in the body fluids.

1.6. Stability of STX during cooking and processing treatments

Poisonous shellfish look and taste the same as normal ones, and so are frequently served and ingested unsuspectingly. Following two outbreaks of PSP intoxication in 1957 in New Brunswick, only 8 out of the 70 people affected had eaten raw bivalves, while the others had eaten cooked shellfish (Bond and Mecof 1958), highlighting the fact that the STX and related PSP toxins are not completely destroyed when heated by normal cooking procedures (Schantz 1984; Scoging 1991). Experiments following the 1968 UK PSP outbreak showed that the toxin retained 70% toxicity after 5 min of boiling and 60% after 20 min (McCollum *et al.*, 1968).

However in a series of tests simulating steaming, boiling and frying methods (Prakash *et al.*, 1971; Lawrence *et al.*, 1994) up to 70% of toxicity was destroyed either in cooked shellfish or lobster meat. This figure is raised slightly through transfer of some of the toxin to the stock solution which may be consumed with shellfish dishes (Quayle 1969). In controlled experiments where toxic butter clams were boiled under a range of low pH levels for varying times (2.5 - 10.0 min) there was no significant difference in toxicity detected in the mouse bioassay (Quayle and Bourn 1972).

Commercial processing treatments such as shucking and trimming of clams does not significantly alter their toxicity (Prakash *et al.*, 1971). Similarly, shucking or steaming toxic mussels and whelks does not alter the relative toxicity of their various organs. However, canning greatly reduces the toxicity of shellfish. Normal cooking and canning operations reduced toxicity in butter clams and oysters by 90% when analysed by the AOAC procedures (Quayle and Bourne 1972; Miyazawa *et al.*, 1995). Toxicity also deceases during storage. Thus if measured toxicity decreases to below 80 μg/100g,

tissue packs of shellfish can be released for marketing. However, the high storage costs and the threat of destruction of stock if toxicity does not decrease makes this a risky marketing strategy (Prakash *et al.*, 1971).

These factors have wider implications, due to the huge export market for shellfishery products, the various cuisine habits in different countries, and the increasing dependence of inhabitants of less industrialized countries on seafood products to supplement their diet. In these situations surveillance or monitoring programmes may be less efficient, public education may be more scant, any increased incidence of poisoning may go unnoticed, and medical help may be inadequate.

1.7. Tetrodotoxin (TTX)

Another guanindinium toxin of biological origin which has the same pharmacological action as STX is tetrodotoxin (TTX), which is associated mainly with the Japanese Fugu, or Puffer fish, occurring in the tissues of at least 40 species, mostly belonging to the family *Tetraodontidae* from which it takes its name (Fuhrman 1967). TTX is found in highest concentrations in the ovaries and liver of the Puffer fish, but also in the intestine and skin. There is a seasonal variation of toxicity, with the fish becoming more poisonous as they enter the spawning season (Kao 1966).

TTX has also been identified in eggs of various species of Western American newts (genus *Taricha*), in the skin of some Central American frogs, and a species of Pacific goby (Ritchie & Rogart 1977) as well as gastropods (Hwang *et al.*, 1994), octopus, salamanders (Yoshida 1994) and crabs (Tsai *et al.*, 1996). Yasumoto *et al.* (1986) inferred that TTX is produced by bacteria and transmitted to other animals through the food chain, and the synthesis of TTX by bacteria of *Vibrio* and *Pseudomonas* species is now established (Mosher and Fuhrman 1986; Yasumoto *et al.*, 1986; Hwang *et al.*, 1994).

Although the biological actions of TTX are very similar to STX a few differences in their actions have been documented. Small doses of STX can cause neuromuscular weakness without a concomitant reduction in blood pressure, whereas TTX always produces both effects. In anethestized animals, the hypotension provoked

by STX is generally less severe than TTX and there is a tendency for the compensatory release of catecholamines which may account for the apparent absence of hyoptension in human clinical cases of PSP intoxication (Nagasa *et al.*, 1971).

1.8. Detection and isolation of STX

Although the chemical structure of TTX was determined over 30 years ago (Mosher *et al.*, 1964) with a molecular formular of $C_{11}H_{17}N_3O_8$, the structure of STX ($C_{10}H_{17}N_7O_4$) was not elucidated until almost 40 years after its original identification when Schantz *et al.* (1975) established the structure of the molecule from a crystalline derivative using x-ray diffraction. Before that, the only analogue available had been a reduction compound of uncertain structure (Schantz *et al.*, 1975). With the improved extraction and purification techniques available over the past several years enormous progress has been made to further establish the identity of the full complement of natural STX analogues. To date at least 21 chemically-related derivatives have been identified and characterised (Wright 1995) and according to Hall *et al.* (1990) possibly 24 may exist if all combinations of possible variation are used.

Attempts to isolate and characterize the components of PSP toxin date back to 1885, when a mass intoxication from eating mussels occurred in Germany, and Brieger (1889, cited in Schantz 1960) claimed to have isolated what he called mytilotoxin. At around the same time Wolff (1886) found that the poison was localised in the mussel dark gland or hepatopancreas, whilst Salkowski (1885) described properties of the poison which enabled later investigators to make comparisons with toxin indentified from other sources. Following the outbreak of PSP poisoning in San Francisco in 1927, Sommer and Meyer (1937) showed that the toxin could be extracted with weak acid, and they later use cation exchangers to partially purify it (Sommer *et al.*, 1948; Schantz 1971). Work by Schantz in the 1940's found that the carboxylic acid exchange resins suitable for Pacific coast mussel and clam poison extraction were unsatisfactory for the purification of Atlantic coast scallops (Schantz 1960). He noted that the primary source on the Pacific coast was *G. catenella*, whereas on the Atlantic coast it was *G.*

tamerensis, and postulated that the scallop PSP toxin was actually chemically different, and that forms of PSP toxin existed other than STX.

Further progress in purification depended on locating rich sources of toxin, and the dark glands of California sea mussels (*Myltilus californianus*) and the siphons of Alaska butter clams (*Saxidomous giganteus*) were found to provide these. Butter clams often remain toxic all year (Schantz *et al.,* 1975) with approximately 70% of the toxin sequestered in the siphon. Therefore an almost constant source of concentrated toxic material was available for chemical analysis. Crude extracts of these tissues were subjected to a new purification technique involving filtration and extraction with water acidified to pH 3.0. Complete recovery of saxitoxin (STX) was thus obtained (Schantz 1971), and it was named after the the *Saxidomus* clam which provided the material.

1.9. Chemistry of STX

The structure of the STX molecule elucidated by Schantz *et al.* (1975) and its known derivatives is shown in Figure 1.5. STX is a water soluble, non-protein with a relative molecular mass of 299. It has a tetrahydropurine/perhydropurine skeleton with an additional five-membered 3 baron-ring fused at an unusual angular position between N-3 and C-4 (Shimizu 1988; Kao 1993). It possess two guanidinium groups (TTX possesses only one) fused together in a stable azaketal linkage (Baden and Trainer 1993). The 7,8,9, guanidinium moiety is essential for toxic activity (Ritchie and Rogart 1977). The dihydroxy, or hydrated ketone group on the five membered ring (C-12) is the other functional moiety essential for toxic activity. Water forms a reversible covalent bond, resulting in a gemdiol, $C(OH)_2$ (Hall *et al.*, 1990). Catalytic reduction of this group with hydrogen to a monohydroxy group eliminates activity, with the loss of toxicity being directly proportional to the uptake of hydrogen (Schantz 1963). Removal of the carbamyl group side-chain on the six-membered ring, leaving a hydroxyl group in its place, reduces toxicity to around 60% (Schantz 1984). Titrations of STX have revealed two proton dissociations at pK_a 8.22 and 11.28. The lower value, unusually low for a guanidinium group, is assigned to the guanidinium centred on C-8 and may be due to stereochemical strain of the five-membered ring (Shimizu 1988).

STX is very stable in acidic conditions of pH 5 or less. It can be kept in dilute HCl with no loss of potency for years, and as a hydrochloride salt is quite stable in boiling water (Schantz 1971). However, in concentrated acidic solutions, hydrolysis can occur. Under alkaline conditions, STX is extremely unstable, especially in the presence of oxygen, undergoing oxidative degradation (with the rupture of the C-12/C-4 bond) to yield aromatized aminopurine derivatives which are highly fluorescent (Shimizu 1988, Hall *et al.*, 1990). The presence of this active hydroxyl group allows the preparation of various STX derivatives for analytical purposes.

Advances in chemical separation techniques have yielded a number of chemically-related compounds with varying degrees of potency. Natural derivatives of the STX molecule have substitutions at amines R1-R4 (Figure 1.5). Substitution considerably modifies the individual potency of each toxin, *in vivo* and *in vitro*, and also alters the binding affinity (Baden and Trainer 1993). Variations in the three derivative groups occur mainly along the side chain on C-6 and positions N-1 and C-11 (Kao, 1993). The C-6 variations can occur as a carbamoyl function (–$CONH_2$, as first seen in STX), as a decarbamoyl group (–H, referring to the structure in STX as the basis), or, as a sulfocarbamoyl function (–$CONHOSO_3^-$). In each of these C-6 modified groups, further simultaneous variations can occur on N-1, where the –H can be replaced by an –OH, and on C-11, where the two protons can be replaced by –OSO_3^-.

The net charge of STX natural derivatives has a significant influence on their toxicity and pharmacology. Under acid isocratic conditions (0.1 M acetic acid) they elute from a polyacrylamide gel as three groups in descending order of net charge. The first to elute are the most strongly basic carbamate compounds, STX and neoSTX with a charge of +2, followed by the GTXs and B1 and B2 with +1, and finally the most neutral with no net charge, the C1-4 analogues (Hall *et al.*, 1990). In order of potency in mammals, the carbamate toxins (GTX I-IV, NEO, STX) are the most potent, the decarbamoyl (dc-) analogues are of intermediate toxicity and the *N*-sulfocarbomyl derivatives (B1, B2, C1-C4) have a much lower specific toxicity. (Cembella *et al.*, 1993) (Figure 1.6).

1.10. Structure of the principal PSP derivatives

NeoSTX (1-*N*-hydroxysaxitoxin) is now known to be a major component in most toxic shellfish and dinoflagellates. The structure of neoSTX was confirmed using nuclear magnetic resonance (^{15}NMR) (Hori and Shimizu 1983). Reduction of neoSTX results in the cleavage of the *N*-hyroxyl group to yield STX. Although almost equipotent to STX it is not as stable under acidic conditions, decomposing in HCl solutions (Shimuzi 1986).

GTX I and GTX II are also now known to be major components in many PSP samples and causative organisms. GTX I and its stereoisomer GTX IV, are probably the most unstable of the analogues (Shimizu 1988). Reduction of GTX I gives a mixture of neoSTX and GTX II, which can be further reduced to STX. GTX II was originally thought to be a free 11-hydroxyl derivative of STX but is now amended to 11-hydroxysaxitoxin sulphate.

GTX III is the 11-epimer of GTX II, generally forming a compound content ratio with GTX II, of 3:7, respectively. There is some speculation that GTX III is actually GTX II in some living organisms especially primary producers. GTX IV is the 11-epimer of GTX I and forms the same compound relationship as GTX II and III. Normally the 11-hydroxysulphate group are encountered as epimeric pairs: GTX I and GTX IV, GTX II and GTX III, C1 and C2. Thus extracts containing one generally contain an amount of the other, most likely due to the ease with which they spontaneously epimerize.

B1 (GTX V) in its natural form is almost non-toxic, but can be hydrolysed with weak acid to yield STX (Hall *et al.*, 1980; Harada *et al.*, 1982). B2 (GTX VI) is a carbamoyl-*N*-sulphate of STX, and exposure to dilute mineral acid cleaves the *N*-sulphate bond to liberate free neoSTX.

C1 and C2 are carbamoyl-*N*-sulfo derivatives of GTX II and GTX III respectively, easily hydrolysed under weak acid conditions into these GTX analogues. C1 is a compound that only becomes toxic during epimerisation to GTX II, whilst C2 (or GTX VIII) is considered naturally toxic (Wichmann *et al.*, 1981). C2 is also thought to easily epimerise to epigonyautoxin VIII, or C1 (Shimizu 1988). The other two C-

toxins, C3 and C4 both have a negative net charge and are not retained on a cation exchange column. Treatment with dilute acids releases free GTX I and IV from both C3 and C4.

dcSTX (decarbamoylneosaxitoxin/GTX VII) was first found in sea scallop samples. Its structure is very similar to decarbamoylsaxitoxin and is thought to be a product of biotransformation in shellfish.

1.11. The chemical stability of PSP toxins

The pH stability of the PSP toxins is an important consideration for analytical techniques. For example, at pH <2.0, the C-21 sulfo group will undergo indeterminate hydrolysis from the sulfamate compounds to yield the corresponding, but more potent, carbamate form. Thus, the low potency *N*-sulfocarbamoyl toxins C1-C4, hydrolyse to their GTX analogues. These N-sulfo compounds are not only extremely sensitive to acid lability, they are also poorly detected by the mouse bioassay (Boyer *et al.*, 1985). Some degradation of the low toxicity components B1 and B2 to the carbomates STX and neoSTX can also occur (Sullivan *et al.*, 1988). GTX 1 and IV, considered to be the most unstable of the PSP toxins may be lost during purification, but more importantly, may be altered, increasing the toxicity of the sample to more than originally observed (Shimizu 1988).

The N-sulfocarbamoyls, generally the major toxin component found in the dinoflagellates, constitute a substantial reservoir of cryptic or latent toxicity (Hall and Reichardt 1984). Therefore it is important to differentiate between the toxicity of a sample, observed when the sulfamates are present, and that which will result when they are converted to carbamates: the "potential" toxicity of the sample. From a public health standpoint, the "potential", i.e. that obtainable if transformations occur in a sample, is the relevant value to monitor.

These induced conversions are accelerated at high temperature. Quantitative hydrolysis of the sulfamate toxins can be attained by heating to 100° C in aqueous 0.1 M HCl for 5 minutes or less (Hall and Reichardt 1984). The use of hot 0.1 M HCl in the AOAC (1984) mouse bioassay therefore increases net toxicity, known as the "Proctor

enhancement"(Cembella *et al.*, 1993). Thus the mouse bioassay is an indicator of potential toxicity, not actual toxicity. Proctor (cited in Hall *et al.*, 1980, and Cembella *et al.*, 1993) observed an increase in toxicity in cell-free extracts of *Protogonyaulax* following brief heating at low pH.

In similar experiments, Hall *et al.* (1980) themselves reported that the toxicity of cell extracts increased to an approximately constant value after heating to 100°C in 0.1 M HCl for 2.5 to 25 min. Extracts containing the compounds B1, B2 C1 and C2 increased in toxicity by factors of approximately 10, 6, 20 and 5, respectively. Those containing primarily neoSTX and GTX 4 showed no significant change. However, following extraction under isocractic conditions using 0.1 M cold acetic acid Bricelj *et al.* (1990) and Cembella *et al.* (1993) reported complete recovery of compounds with no net change in toxicity values.

Nevertheless, according to Hall *et al.* (1990), the standard AOAC mouse bioassay procedure, which uses a 1:1 dilution factor of 0.1 M HCl to sample is insufficient (according to their earlier study) to produce complete sulfamate hydrolysis, although trace hydrolysis is difficult to avoid. Some conversion was observed at room temperature over long periods.

Problems of latent lethal toxicity from samples extracted under these AOAC conditions was commented on by Nagashima *et al.* (1990), who also suspected that the official AOAC method of using 0.1 M HCl may underestimate possible potency. Improved extraction methods for tissue containing predominantly N-sulfo-carbamoyl compounds were examined in a comparative study using various concentrations (0.01-5 N) of HCl, HNO_3, and H_2SO_4. It was concluded that extracts heated in 1.0 N HCl gave more reliable toxicity measures than the official AOAC method. Stability of some of the other toxins was also examined during heating with 1.0 N HCl. The findings indicated that STX and GTX II and III retained toxicity unchanged over a 15 min heating period, whereas GTX I and IV lost some toxicity when heated for only 5 min. Conversely, Jellet *et al.* (1995), found toxicities of pure-form PSP preparations were not affected by either the concentration (0.1 or 1.0 N) of HCl or the 5 min boiling procedure, the only exception being GTX II and III which increased in toxicity.

The variability of all these findings strongly implies that a greater effort is needed to optimise the primary extraction procedures, and that latent potency must not be overlooked by the chosen assay or analysis.

The stability of purified toxins particularly those used for reference standards is another important consideration. Alfonso *et al.* (1994) compared the stability of STX and neoSTX in acidic solutions and lyophilized (freeze dried) samples when stored at 37°C, 4°C. -20°C and -80°C respectively. Their results showed that below 4°C STX was stable in acid conditions for up to 2 years and that the lyophilized samples remained stable for around 6 months before deteriorating. NeoSTX however, had low stability even at -80°C, and at -20°C toxicity was almost totally non- detectable after one year of storage. The toxicity of the lyophilized samples decreased within 3 months of storage. However pure-form PSP extracts opened and then stored at 5° C in either ethanol or acetic acid showed little toxicity change when compared with a standard reference STX (Jellet *et al.,* 1995).

In a similar study using the same temperature range Louzao *et al.* (1994) examined the effect of lyophilization on the stability of the GTXs and the C-toxins. Lyophilization caused the C-toxins to become extremely unstable, with some transformation into the more toxic GTXs. GTX I, II, and III at all temperatures from -80°C to 4°C, whereas the lyophilization of GTX IV and VI (B2) did not modify the their stability at any temperature. The stability of toxins in frozen shellfish samples was questioned by Evans (1970) who observed a 93% loss of toxicity over a storage period of 6.5 days. Dilution during re-extraction may have caused some of the loss, but was not the full explanation.

Harada *et al.* (1984) found that the virtually non-toxic, N-sulfocarbamoyl derivative GTX V (B1) was converted to the potent carbamate STX under stomach gastric conditions, thus raising the possibility that similar transformations could be catalyzed by stomach acid following ingestion of contaminated shellfish.

These results raise the question of whether the AOAC mouse bioassay, which employs i.p. administration, is the proper means for toxic sample evaluation. When the toxin was given orally to mice there was a great reduction in toxicity (LD_{50} 263 μg

compared to LD_{50} 10 μg/kg i.p.) (Wiberg and Stephenson 1960). Incomplete or delayed absorption from the gut was suggested, but the alkaline pH of the intestinal tract and the presence of Na^+ ions in the intestinal juice may also tend to lower toxicity. Therefore, direct application of toxin to stomach gastric conditions could produce an inaccurate estimation of either sample toxicity or conversion of compounds.

1.12 Bioconversion of PSP toxins in bivalves

Differences exist in the relative abundance of the various PSP toxins not only in the causative dinoflagellate, but also in the various tissues of contaminated shellfish, together with significant changes in toxin composition. In two species of oyster, Kamiya and Hashimoto (1978) observed that the mantle and adductor muscle were only weakly toxic, but that GTX I and II, and STX, together with two new unidentified derivatives were detected in the vicera.

In the early studies of Burke *et al.* (1960) and Schantz (1963) it was concluded that no conversion of toxins derived from dinoflagellates occurred in shellfish extracts tested. However, studies by Shimuzi and Yoshioka (1981) and Sullivan *et al.* (1983) reported enzymatic transformations of PSP toxins from the weakly toxic B and C toxins to metabolites of the GTX's and STX in clam and scallop tissue homogenates. Similarly in a controlled feeding study, Bricelj *et al.* (1990) exposed soft shelled clams to *A. fundyense* and reported an increase in concentration of STX and a reduction of neoSTX and GTX II and III relative to the content of the original material.

Other comparisons between bloom organisms and contaminated shellfish have indicated rapid degradation of *N*-1 hydroxy toxins, and the conversion of the fairly unstable 11-β hydroxysulfates to their more stable 11-α epimers in shellfish tissue (Sullivan *et al.*, 1983; Hall *et al.*, 1990). A pronounced seasonal shift in PSP toxins has also been noted from the less potent *N*-sulfocarbamoyls toxin (C1/C2) which dominate in the primary organism, to higher toxicity carbamate derivatives such as GTXs, neoSTX and STX in the shellfish, especially the gonads (Cembella *et al.*, 1993,1994) which could account for some of the increase in toxicity observed over time.

Changes in PSP profiles between dinoflagellates where low toxicity derivatives or precursors were the predominant toxins to significant changes in profile composition in shellfish species (Noguchi *et al.*, 1990; Asakawa *et al.*, 1995; Bricelj and Cembella 1995) have similarly confirmed that PSP components exist in bivalves in the form of the chemically more stable α epimers of the toxins. Three species of clam studied contain a hydrolytic enzyme catalysing decarbamoylation of the STX to produce completely different profiles from that of the causative dinoflagellate primary source (Oshima *et al.*, 1990; Oshima 1995a).

Thus, differences in PSP toxin composition between bivalves and ingested dinoflagellate cells can arise through several mechanisms. Selective retention, binding or elimination of specific toxins may occur, coupled with biotransformation among toxic compounds within selective tissues. Conversion seems to be mediated by a variety of biochemical (e.g. enzymatic) and physico-chemical (e.g. pH, temperature) mechanisms. The modification of chemical properties of the toxins result in epimerisation, the conversion of *N*-sulfocarbamoyl moieties to carbamates, or through a different position, the removal of the sulfamate or carbamate side chains yielding the decarbamoyl derivatives; and reduction which results in the elimination of *O*-sulfate. Perhaps the clearest evidence for bioconversion is the *de novo* appearance of a toxin component in shellfish, not present in the causative dinoflagellate.

1.13. The structure of sodium channels

Ion channels in plasma membranes are frequently the primary targets of marine and other natural toxins. These channels are important regulators of the cell's physiology through the control of intracellular pH, ion concentration, and membrane potential, and many of the patho-physiological effects of toxins arise from actions on ion channels. The target of STX is the sodium (Na^+) channel of nerve and muscle membranes, and many excellent reviews have described the structure, function, molecular properties and expression of these Na^+ channels (e.g. Catterall 1984, 1988, 1993; Kandel *et al.*, 1991; Hille 1992; Kallen *et al.*, 1993)

The first of a family of isoforms of the principal subunit of the Na^+ channel was cloned by Noda *et al.* (1984). The Na^+ channel is a transmembrane protein comprising of up to three (α and β) subunits that lies in the lipid bilayer of the membrane, and is anchored to other membrane proteins or to elements of the intracellular cytoskeleton by hydrophobic, electrostatic and covalent bonding. The macromolecule is large (*ca.* 260,000-450,000 D), consisting of 1,800 - 4,000 amino acids arranged in one or several polypeptide chains with some hundreds of sugar residues covalently linked as oligosaccharide chains to amino acids on the outer surface. It is heavily acylated with fatty acids (Agnew *et al.*, 1986) and carries a net charge or dipole moment (Armstrong and Bezanilla 1974). Each α subunit of the Na^+ channel contains associated regulatory subunits, i.e., β1 and β2 units in a 1:1:1 stoichiometry. The β2 subunit is covalently attached to the α subunit by disulfide bonds while the β1 subunit is associated non-covalently.

Cloned cDNAs encoding α subunits of rat brain Na^+ channels injected into *Xenopus* oocytes have established that the α subunit is the principal unit of the Na^+ channel, able to carry out basic voltage-gated channel functions by itself (Goldin *et al.*, 1986). The α subunit of the voltage gated channel (Figure 1.7) is organized into four highly homologous domains or repeats (I-IV). Each repeat is composed of six transmembrane segments or subdomains (S1-S6). On the basis of biophysical and chemical data the extracellular segment between S5 and S6 of each repeat is thought to fold back into the membrane to form part of the ion-conducting pathway (Guy and Conti 1990). In addition the loop between S5 and S6 is postulated to form a hairpin, designated the SS1 and SS2 segments (Imoto 1993) and is considered to be the binding site for STX (see **section 1.14**).

1.14. Voltage-dependent activation of the Na^+ channel

The electrical excitability of nerve and muscle (which is specifically blocked by STX and TTX) is due to the presence of voltage-sensitive ion channels in the plasma membrane of these cells. Hodgkin and Huxley (1952) first showed by voltage clamp methods that the action potential in squid axon results from time- and voltage-dependent

increases in axonal membrane permeability to Na^+ and K^+. Thus nerve excitation takes place as a result of opening and closing of Na^+ and K^+ channels in the membrane.

Activation of the voltage-gated Na^+ channel is assumed to result from a voltage-driven conformational change that opens the transmembrane pore. The pore is much wider than an ion over most of its length and may narrow to atomic dimensions in a discrete part to form what is called the selectivity filter, where ionic selectivity is established (Hille 1992). Depolarisation of the membrane exerts an electrical force on voltage sensors that contain the gating charges of the channel located within the transmembrane electrical field. These gating charges are thought to be charged amino acid residues located in the membrane-associated segments of the protein.

Mutation and electrophysiological studies suggest that S4 (Figure 1.7) may serve as the voltage sensor because neutralisation of charged residues in this segment of the domains (I-IV) has major effects on the voltage-dependence of activation (Auld *et al.*, 1990; Chabala and Anderson 1992; Kra-Oz *et al.*, 1992) The S4 segments are proposed to form a sliding-helix, with arginine residues forming a spiral of positive charge around the core of the helix which form ion pairs with negative charged amino acid residues from the surrounding transmembane α helices of S1, S2 and S3 segments. (Catterall 1988). The force of this electric field sets the resting membrane potential. Contradictory to this view, Sato and Matsumoto (1992, 1995) and Noda (1993) propose that the four linkers of S5-6 between segments S5-S6 regions act as the voltage sensor and these slide through a guiding pore formed by segments S2 and S4 to the cytoplasmic side.

Depolarisation reduces the force and therefore the charge, and the S4 helix is thought to undergo a spiral motion resulting in a gating charge movement which coincides with the flow of ionic current. Since the toxins STX and TTX only block when applied to the extracellular surface, the inactivation gate is assumed to be situated at the inner end of the pore. A three-residue hydrophobic motif, referred to as the IFM (Ile-Phe-Met) particle that lies within one of the intracellular loops between domain III and IV (Figure 1.7) and may function as an inactivation gate that blocks ion conduction (Gawley *et al.*, 1995).

It is thought that the Na^+ channel can exist in three functionally distinct (and presumably allosterically distinct) states: open (O), closed/resting (C/R), and inactivated (I). Only the open state is conducting, and the closed conformation is the resting state. After activation by depolarization (R $\rightarrow$ O), the channel enters an inactivated (non-conducting) state before returning to the closed, or resting state. In the resting state, most Na^+ channels are closed while some K^+ channels are open, bringing the membrane potential close to the equilibrium potential for K^+ as defined by the Nernst equation (around -80 mV, inside). Following a depolarising stimulus, the Na^+ channels open quickly allowing a rapid movement (10^8 ion/sec) of Na^+ ions to flow down their electrochemical gradient into the cell. Voltage clamp studies have confirmed that these permeability increases are mediated by separate voltage gated Na^+ and K^+ channels (Catterall *et al.*, 1982).

Thus activation can be viewed as the primary voltage-dependent event in Na^+ channel function (which controls the rate and voltage-dependence of Na^+ permeability increase following depolarisation), and that inactivation, (which controls the rate and voltage -dependence of the subsequent return of the Na^+ permeability to the resting level during a maintained depolarisation) derives its apparent voltage-dependence from coupling to activation, and is not a simple reversal of events but involves a third transition of the S4 voltage sensor (Keynes 1992).

1.15. Interaction of STX and other toxins with Na^+ channels

Marine toxins act on ion channels by four basic biochemical mechanisms: ion flux enhancement through the endogenous channels in membranes, ion flux inhibition through the same membrane channels, enzymatic inhibition and CNS receptor antagonism (Baden *et al.*, 1995). Some of the toxins known to affect voltage-gated Na^+ channels include both enhancers (activators or modulators) and inhibitors (Table 1.4), some of which bind at the same site and may therefore act competitively with one another (Hille 1992). Others, normally those binding to different receptors sites, may act synergistically or cooperatively (Catterall 1975; Jacques *et al.*, 1980; Baden and Trainer 1993).

Toxins that act by modifying Na^+ channel activation and inactivation mechanisms include sea anemone toxins, such as ATx-II, from *Anemonia sulcata*, which contain proteins that enhance persistent activation of the channel causing hyper-excitability (Norton 1991). Toxins from New World scorpions can slow the inactivation process by prolonging the channel opening time (the α-toxins), or cause Na^+ channels to open at membrane potentials at which they would normally be closed (the β-toxins) (Harvey *et al.*, 1993). Venom from two species of funnel web-spider have a very similar effect to both the ATx-II and the α-toxins (Nicholson *et al.*, 1996). Brevetoxin (PbTx) from the dinoflagellate *Gymnodinium breve* (= *Pytochodiscus brevis)*, causes a prolonged opening and a shift of activation potential to more negative values (Baden 1989; Gawley *et al.*, 1995). Ciguatoxin, another dinoflagellate (*Gambierdiscus toxicus*) toxin opens Na^+ channels at resting potentials, which are then unable to inactivate during subsequent depolarisation (Baden and Trainer 1993). Dibromosceptrin (DBS) an alkaloid toxin from tropical marine sponges of the genus *Agelas*, decreases Na^+ conductance, but appears to act more as a modulator than a blocker of the channel (Rivera Rentas *et al.*, 1995; Rosa *et al.*, 1995).

Of particular interest in this study (**sections 2.4.7, 6.3.4, & 7.3.8**) is the Na^+ channel modifying toxin, veratridine (VER), a colourless, odourless, lipid-soluble, alkaloid from the lily plant family, *Veratrum*. It is a reversible activator or agonist, that binds to Na^+ channels in their open conformational state and causes depolarisation of the cell membrane, during which a prolonged influx of Na^+ ions occurs, disrupting normal conductance (Catterall *et al.*, 1986; Strichartz *et al.*, 1987; Corbett and Krueger 1989). This modified state lasts on average for a second until the toxin dissociates again. Unbinding does not result in immediate inactivation of the channel protein. Voltage clamp studies have shown the Na^+ current (I_{Na}) persists for several seconds after the voltage test step has been returned back to resting potential (Barnes and Hille 1988).

Fewer toxins actually inhibit the Na^+ channel (Table 1.4). Of these the μ-conotoxins from marine snails, *Conus* sp. block the Na^+ channels of skeletal muscle but have little effect of action potential conduction of nerve fibres (Cruz *et al.*, 1985; Narahashi *et al.*, 1994). Only STX and TTX are known to inhibit Na^+ ion influx across

both nerve and muscle membranes at concentrations as low as 5 ng/ml (Noda *et al.*, 1986; Strichartz 1988). It is of interest that the single-celled dinoflagellates (marine eukaryotes) and cyanobacteria (freshwater prokaryotes) which synthesise the STXs do not possess Na^+ channels although the adaptive significance of this has not yet been explored (Hille 1992).

STX and TTX block nerve conduction without any change in resting membrane potential. They selectively block Na^+ channels and have no effect on voltage-gated K^+ channels, although this block is antagonized by Na^+, Ca^{2+} and H^+ (Stritchartz *et al.*, 1986). Their action is rapidly reversible, although nerves recover faster from a STX block than from a TTX block (Narahashi 1989). The rapid kinetics of activation and inactivation, and the movement of charge associated with gating currents are unaffected by STX or TXX (Armstrong and Benzanilla 1974). Thus, these toxins have become extremely useful tools for studying the Na^+ channel density in membranes (Ritchie and Rogart 1977), the kinetics of Na^+, and also K^+ channels (Ulbricht *et al.*, 1986; Narahashi 1988), the mechanism of transmitter release from nerve terminals, and in the isolation of Na^+ channels from membranes.

Both toxins exhibit a high affinity with values of $K_{0.5}$ from 1 to 10 nM on axons and skeletal muscle of mammals, amphibians and fish, to values of 1.0 to 6.0 μM for Purkinje fibres and ventricular fibres of mammalian heart, which are less sensitive (Evans 1972; Rogart 1986; Strichartz 1988; Narahashi 1989; Schwartz *et al.*, 1990). Similar values are reported for cells of smooth muscle and chronically-denervated muscle. Certain CNS and peripheral ganglia are not very sensitive in adult mammals. In addition, some fraction of Na^+ channels in embryonic neurones and skeletal muscles are resistant to both types of toxins. (Ritchie and Rogart 1977; Yoshida *et al.*, 1978; Narahashi 1989, Hille 1992; Ogata and Tatebayashi 1992).

Interestingly, the nerves from puffer fish and salamanders which synthesise TTX, and certain species of shellfish and crabs and other organisms which accumulate STX in their tissues, are highly insensitive to these toxins (Mosher and Fuhrman 1984; Louzao *et al.*, 1992; Yoshida 1994) which may be due to the presence of STX-resistant Na^+ channels which afford protection.

Whilst there is general agreement that both STX and TTX bind at site 1 of the Na^+ channel α subunit (**section 1.12**), a consensus still has to be reached as to the exact binding site and binding mechanism of the toxin molecule to the receptor site. Of the earlier hypotheses Narahashi (1974) and Ritchie and Rogart (1977) suggested that a binding configuration exists between STX and the α subunit such that the cationic charge of the toxin molecule impedes access of Na^+ ions through the pore. Hille (1975) suggested that the guanidium group of STX or TTX penetrates the pore and thus blocks it (the plug-in-the-channel model), but this hyphothesis seems unlikely (Lönnendonker *et al.*, 1990). Kao (1983) postulated that both toxins bind to an extracellular receptor site, with the +vely charged planar guanidinium group projecting over the opening of the Na^+ channel, producing the block.

Strichartz *et al.* (1986) hypothesized that toxin bonding occurred through a combination of ion-pairing between the guanidinium moities and surface negative charges which are present in higher density around the opening of the pore, and hydrogen bonding between various hydroxyl groups and deprotonated carboxylate functions in the Na^+ channel protein. Kao and Hu (1989) and Yang and Kao (1992) concluded from biochemical experiments with neoSTX that the binding site for STX was likely to be in a fold or a crevice of the channel pore rather than on the planar portion of the protein.

As molecular and biochemical techniques improve, methods to locate the binding domain have advanced. A site-directed mutation study of cloned cDNA for Na^+ channel proteins expressed in *Xenopus* oocytes involving neutralization of a glutamine residue located just outside transmembrane segment S6 (in the SS2 loop) of domain 1 causes a reduction in net negative charge in this region and a complete loss of both STX and TTX inhibition (Noda *et al.*, 1989). In addition to a glutamine, a nearby tyrosine residue is also required for high affinity of TTX binding in skeletal muscle and in cardiac (h1) Na^+ channels. These data suggest that the amino acid residues in analogous positions (S6) in each domain form a single binding site for TTX or STX in or near the extracellular pore of the channel.

Other mutation studies (Lipkind and Fozzard 1994) have also supported a similar binding site model for STX and TTX, but that differences in binding exists between TTX, which interacts directly with the SS1 and SS2 segments of repeat I and II, and that of STX, which additionally interacts with the segments of repeats III and IV through its second guanidinium group. Further work using a bioactive TTX derivative, to photolabel the Na^+ channel from eel electroplax also showed the TTX binding site is formed by close apposition of two discontinuous regions at domains III and IV (Nakayama *et al.* 1993). Thus, it now likely that the binding site for STX and TTX is located on or near the SS2 segment, especially as mutations of specific amino acids at this position have led to a reduction in the negative charge and this has significantly reduced toxin-binding sensitivity.

STX and TTX binding was previously thought to be voltage-independent, with molecules being able to bind equally well to open, inactive or closed Na^+ channels in a 1:1 stoichiometry (Hille 1968, 1992; Ritchie and Rogart 1977). There is now evidence from electrophysiological experiments for complex voltage and use-dependence of STX and TTX block of the Na^+ channel. Use-dependent block is highly dependent on membrane potential (being augmented by hyperpolarising the membrane potential) as well as the stimulus frequency (Salgado *et al.*, 1986; Narahashi 1990; Patton and Goldin 1991; Conti *et al.*, 1996). Voltage-dependent block is affected by changes in membrane potential and Ca^{2+} concentration (French *et al.*, 1984; Krueger *et al.*, 1986; Narahashi 1988), but the mean block time for both STX and TTX subsequently becomes independent of toxin concentration (Moczydlowski *et al.*, 1984). Hyperpolarisation increases Ca^{2+} binding to the STX site, thus preventing the binding of STX due to its cationic nature (French *et al.*, 1984; Salgado *et al.*, 1986; Narahashi 1988) and implying some form of a conformational change (Moczydlowski *et al.*, 1984; Krueger *et al.*, 1986; Patton and Goldin 1991).

Aims of this study

The primary aim of this thesis is to study the physiological effects of PSP toxins, especially STX, upon various types of nervous tissue, which are their primary targets.

Some of the preparations are considered as possible novel assay methods for rapid or sensitive biological screening for STX (reviewed in **Chapter 2**): the lobster nerve muscle preparation (**Chapter 3**), the chick biventor nerve muscle preparation (**Chapter 4**) and the frog sciatic nerve (**Chapter 5**), as well as for investigating the effects of "whole" tissue extracts from shellfish on the electrophysiological behaviour of the nervous tissue. Electrophysiological investigatations of the Na^+ channel properties and their modification by STX in the Neuro 2a tissue culture neuroblastoma cell line used in cell bioassays are also performed (**Chapter 6**), and are compared with the properties of a number of other cultured cell lines and dissociated neurones (**Chapter 7**). From these studies, an assessment is given of the suitability of various PSP bioassay procedures, and a contribution is made to our understanding of the underlying physiological mechanisms (summarised in **Chapter 8**).

Table 1.1. Algal and cyanobacterial toxins known to cause severe hazards to human health and economic damage.

Form of Poisoning	Algal species (examples)
Paralytic Shellfish Poisoning (PSP)	Dinoflagellates : *Alexandrium catenella, A. acatenella, A. minutum, A. tamarense, A. cohorticula, A. fundyense, Gymnodinium catenatum, Pyrodinium bahamense, P. concavum*
Diarrhetic Shellfish Poisoning (DSP)	Dinoflagellates : *Dinophysis acuta, D. acuminata, D. fortii, D. norvegica, D. rotundata, Prorocentrum lima*
Ciguatera Poisoning	Dinoflagellates : *Gambierdiscus toxicus, Prorocentrum spp., Coolia monotis ?*
Neurotoxic Poisoning (NSP)	Dinoflagellate : *Gymnodinium breve, Ptychodiscus brevis*
Amnesic Poisoning (ASP)	Diatoms : *Nitzschia pungens, N. pseudodelicatissima, N. pseudoseriata*
Maitotoxin Poisoning (MTX) Gambiertoxin	Dinoflagellate : *Gambierdiscus toxicus, Porocentrum lima*
Cyanobacterial Poisoning	Cyanobacteria : *Anabaena flos-aquae, Microcystis aeruginosa, Nodularia spumigena*

Table 1.2. The site of action, clinical symptoms and physiological effects of the predominant phyco- and cyanobacterial- toxins known to cause severe illness in humans.

Algal Toxin	Site of Action	Physiological Effect	Symptoms
Paralytic Shellfish Poisons Saxitoxin + 21 known analogues Tetrodotoxin	Site 1 on voltage dependent sodium channels	Inhibit inward flow of sodium ions, block action potential	Tingling, numbness, ataxia, giddiness, staggering, dry throat, fever, rash, incoherence, dysphasia, aphasia, respiratory failure/death
Diarrhetic Shellfish Poisons Okadaic acid Dinophysis toxin Yessotoxin Pectenotoxins	Catalytic subunit of phosphorylase phosphates	Inhibition of phosphorylase phosphatases 1 and 2a	Diarrhoea, nausea, vomiting, abdominal pain
Ciguatera Poisons Ciguatera	Site 5 on voltage dependent sodium channels	Repetitive firing, shift of voltage dependence of activation, increase Na^+ permeability	Muscle aches, sweating, tingling, numbness of lips/tounge, weakness, arthralgias, anxiety, chills, dilated pupils, extended debilitation
Neurotoxic Poisons Brevetoxin	Site 5 on voltage dependent sodium channels	Repetitive firing, large depolarisation, shift in voltage dependence of activation, increase in transmitter release	Sensory, motor or cerebellar neurological symptoms, hyper-excitability of NS, numbness of lips/tongue
Amnesic Poisons Domoic acid	Kainate receptors in CNS	Receptor induced depolarisation and excitation	Gastro-intestinal: abdominal-cramps, vomiting, neurological: memory loss, disorientation
Maitotoxin	Calcium channels	Calcium ion influx, stimulates phospho-inositide breakdown. Sodium influx in excitable cells	Contraction of smooth muscle,
Cyanobacterial Poisons Anatoxin-a Anatoxin-a(s) Saxitoxin	Acetylcholine receptors	Mimics ACh, no degradation, repetitive muscle contraction Continuous availability of ACh, repetitive muscle contaction	Overstimulates muscles, twitching, cramps, fatigue, paralysis, convulsions, suffocation/death

Table 1.3. Levels of shellfish contamination by the major dinoflagellate toxins that are considered dangerous or unfit for human consumption.

Poison	µg/ gm	µg/ 100gm	LD_{50} µg/kg (i.p.)	LD_{99} µg/kg (i.p.)
PSP	0.8	80	10	7-16
DSP*				
okadaic acid	2.0	200	192	200
dinophysistoxin-1	1.8	180	160	
dinophysistoxin-3				160
ASP	20	2000		
Maitotoxin			0.17	
Ciguatera*			0.45	
Palytoxin			0.025	

* Rarely fatal intoxication occurs

Table 1.4. Neurotoxin receptor sites associated with voltage-gated Na^+ channels and their physiological effects.

Receptor site	Toxin	Physiological Effect
1	Saxitoxin Tetrodotoxin μ Conotoxins	Inhibit transport
2	Veratridine Batrachotoxin Aconitoxin Grayanotoxin	Cause prolonged activation
3	α-Scorpion toxins Sea anemone toxins	Delayed inactivation Enhance persistant activation
4	β-Scorpion toxins	Repetitive firing Shift activation
5	Brevetoxin Ciguatoxins	Repetitive firing Enhance persistant activation Change in voltage dependence of activation
6	Pyrethroids Pumiliotoxins Goniopora coral toxins	Prolonged activation

Plate 1.1.

The dinoflagellate *Alexandrium tamarense*, causative organism of paralytic shellfish poison (PSP), from British coastal waters (SEM courtesy of Jane Lewis). Scale bar = 10 μm.

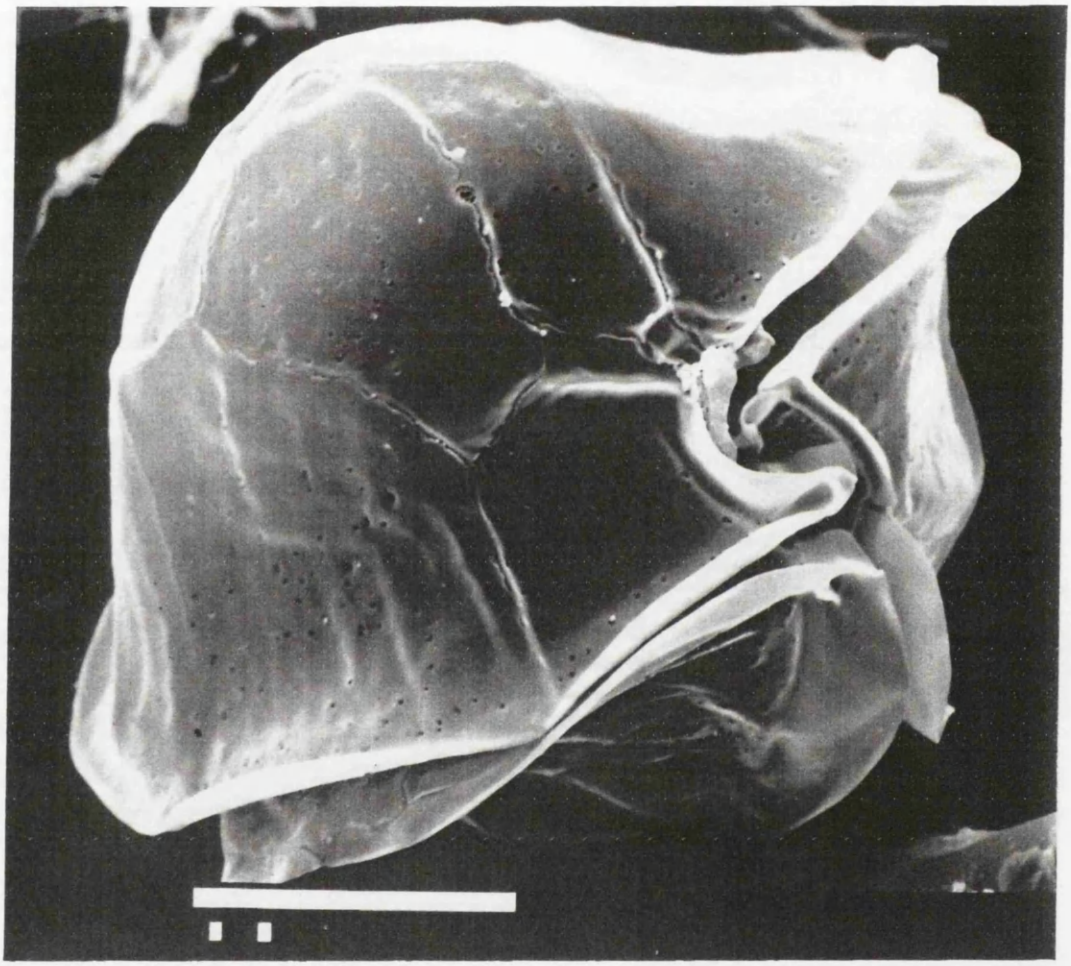
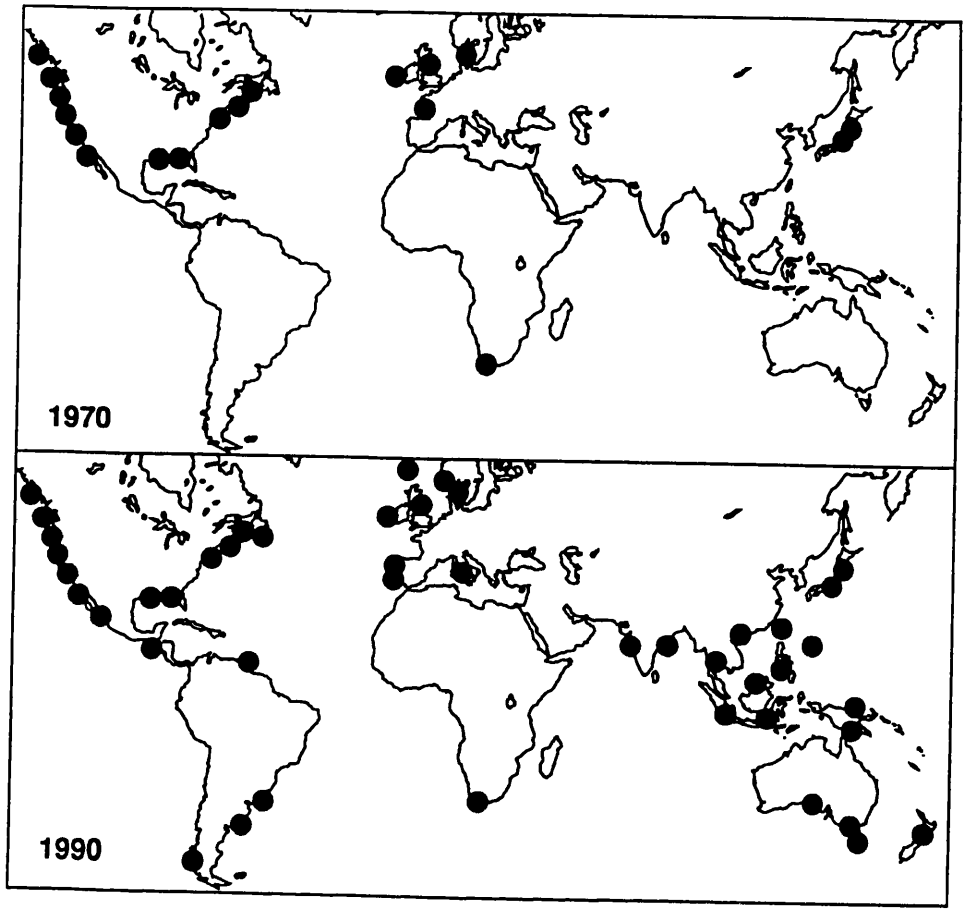


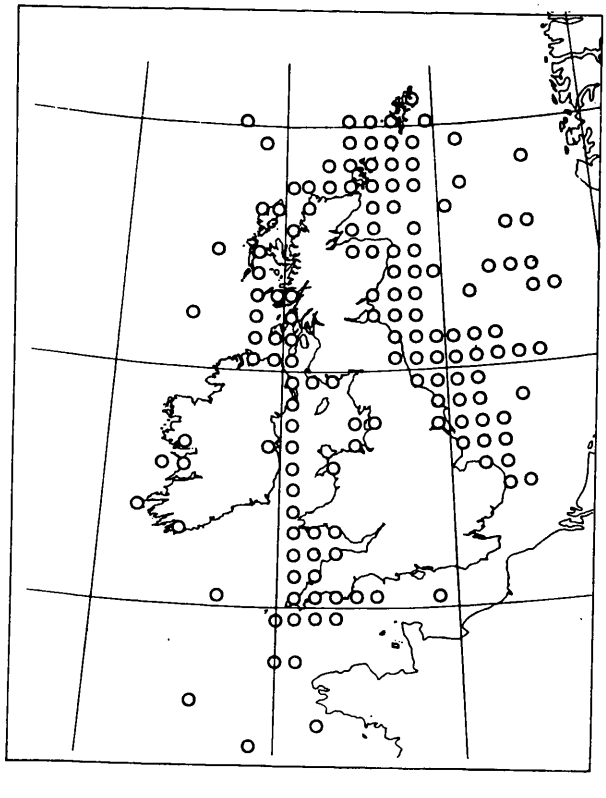
Figure 1.1.

a) Changes in the known global distribution of paralytic shellfish poisoning (PSP), indicated by the black dots, between 1970 and 1990.

b) Recorded distribution of the dinoflagellate *Gonyaulax* (= *Alexandrium*) sp. around British coastal waters (indicated as an open circle).



a)



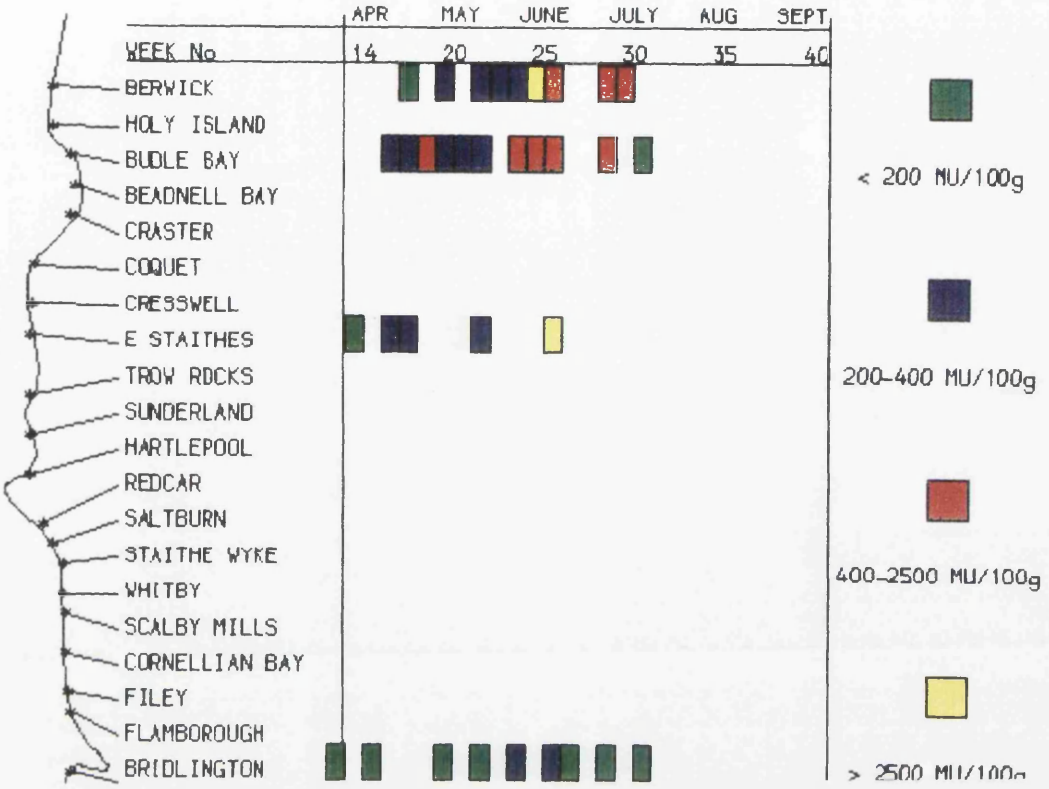
b)

Figure 1.2.

Concentration of PSP in mussel extracts from samples collected from MAFF (CSL) monitoring sites off the NE coast of Britain from the beginning of April through to August, for the years 1970 (top) and 1980 (lower graph).

The measure of PSP toxicity is given in mouse units (MU)/100g of wet shellfish tissue, described in detail in **section 2.4.1**.

PSP Survey N.E. England 1970 (Mussel)



PSP Survey N.E. England 1980 (Mussel)

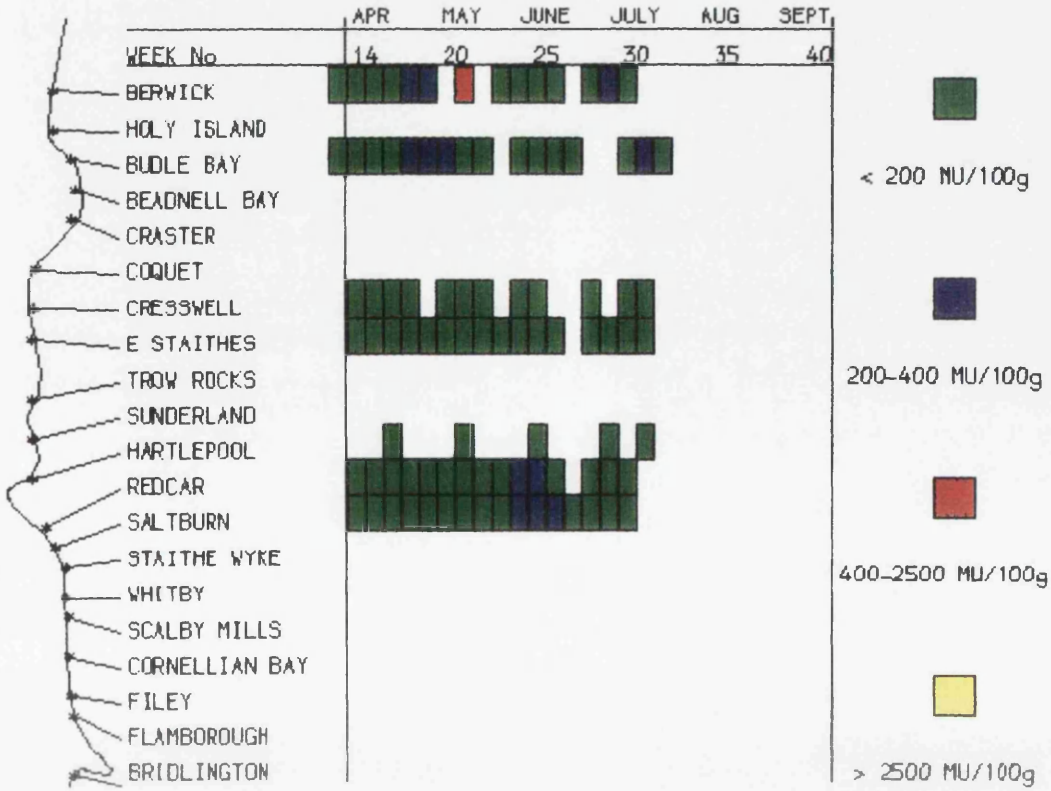
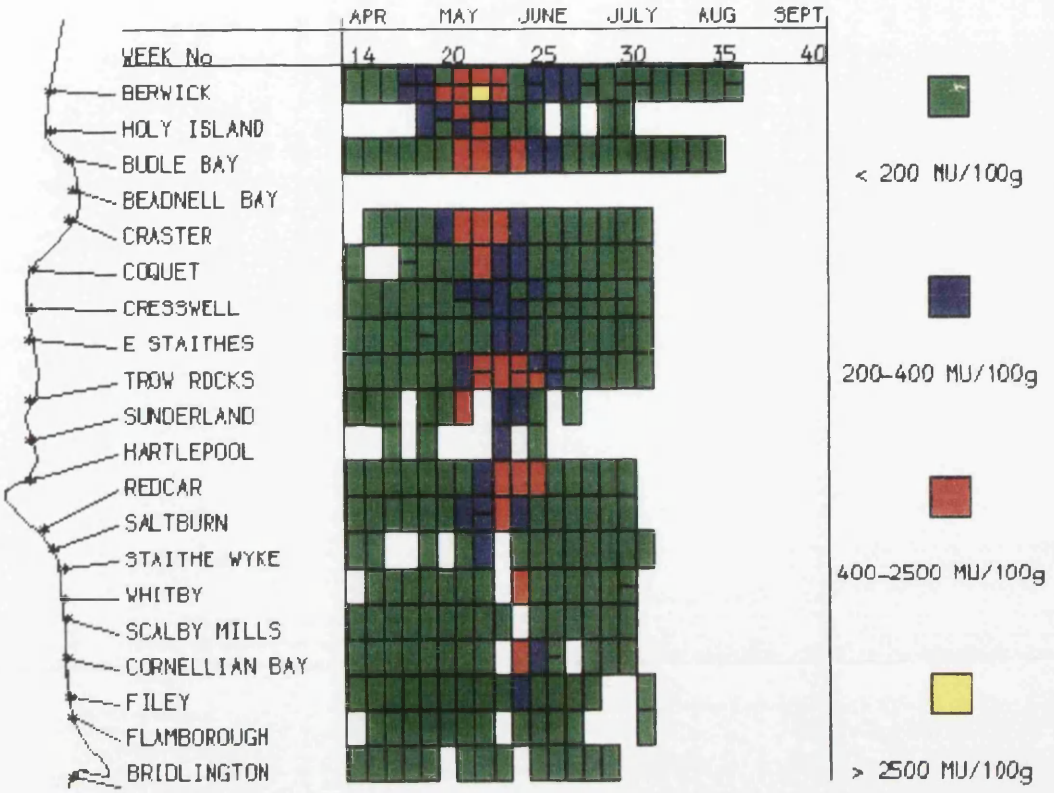


Figure 1.3.

Concentration of PSP in mussel extracts from samples collected from MAFF (CSL) monitoring sites off the NE coast of Britain from the beginning of April through to August, for the years 1991 (top) and 1992 (lower graph).

The measure of PSP toxicity is given in mouse units (MU)/100g of wet shellfish tissue, described in detail in **section 2.4.1**.

PSP Survey N.E. England 1991 (Mussel)



PSP Survey N.E. England (Mussel)

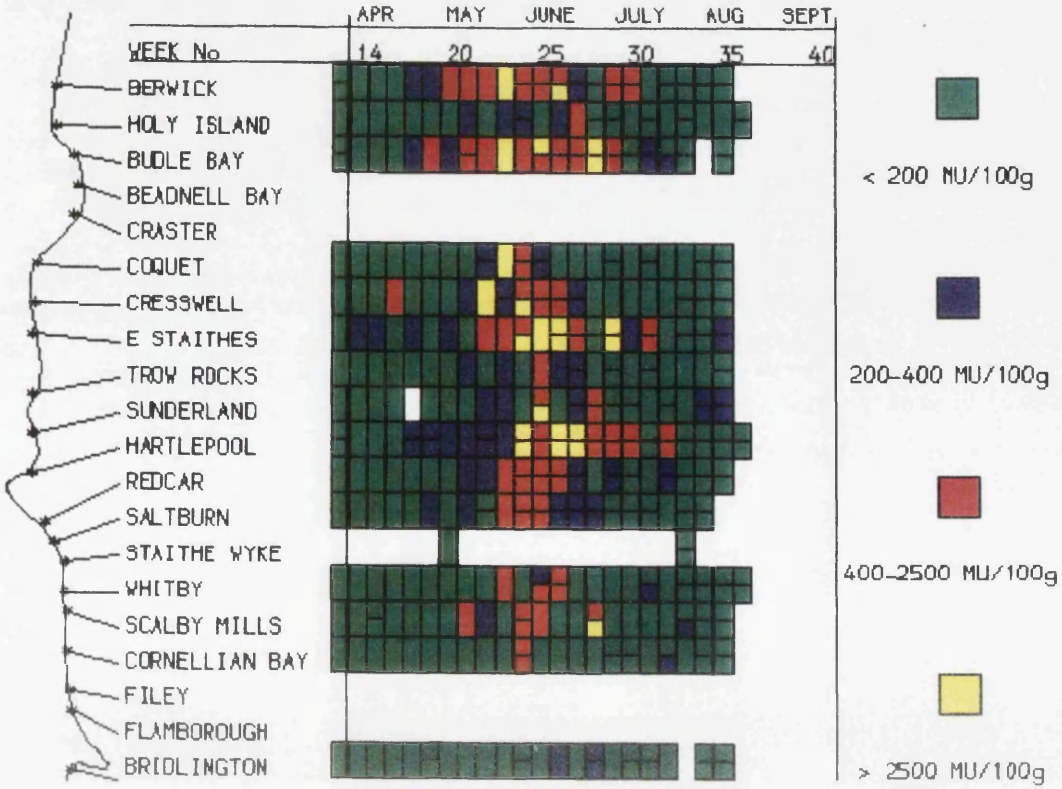


Figure 1.4.

Food health warning notice for PSP posted following collection of contaminated shellfish samples from within that area.

SKYE & LOCHALSH DISTRICT COUNCIL

FOOD SAFETY ACT 1990

FOOD HAZARD WARNING

PARALYTIC SHELLFISH POISONING

THE AREA EXTENDING ALONG THE COASTLINE FROM MURCHISONS MONUMENT BY BALMACARA VIA KYLE OF LOCHALSH TO RUBHA NA GUAILNE (WEST OF APPLECROSS BAY) (INCLUDING LOCH KISHORN TO LOCH CARRON):

HAS BEEN IDENTIFIED AS BEING AFFECTED BY MARINE ALGAL TOXINS

DO NOT COLLECT OR EAT BIVALVE MOLLUSCS SUCH AS MUSSELS, COCKLES, SCALLOPS, QUEENS, OYSTER AND RAZOR SHELLS FROM THIS AREA UNTIL FURTHER NOTICE IS POSTED

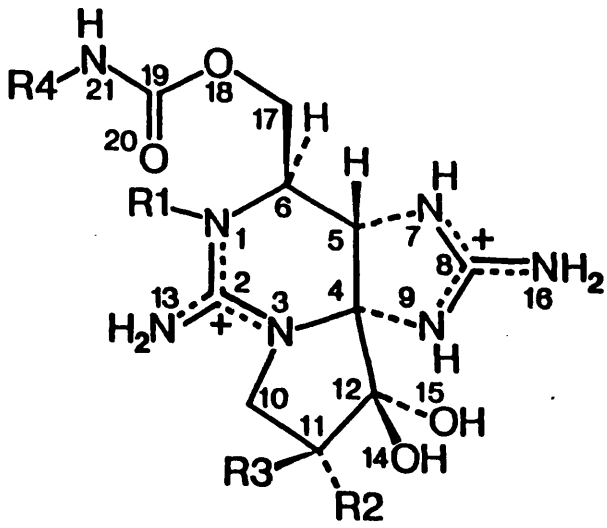
ENVIRONMENTAL HEALTH DEPARTMENT
TIGH NA SGIRE
PARK LANE
PORTREE
ISLE OF SKYE TEL: 0478 - 2341

DATED: 30th MAY, 1991.

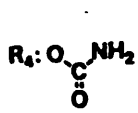
OTHER AREAS OF SKYE & RAASAY ARE AFFECTED. PLEASE CHECK NOTICES POSTED IN THESE AREAS.

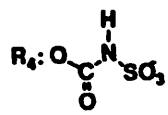
Figure 1.5.

The structure of saxitoxin and its known analogues that are collectively known as PSP (adapted from Oshima 1993).



R1	R2	R3	Carbamate Toxins	N-Sulfocarbmoyl Toxins	Decarbamoyl Toxins	Deoxydecabamoyl Toxins
H	H	H	STX	B1	dc-STX	do-STX
OH	H	H	NEO	B2	dc-NEO	--
OH	H	OSO_3^-	GTX 1	C3	dc-GTX 1	--
H	H	OSO_3^-	GTX 2	C1	dc-GTX 2	do-GTX 2
H	OSO_3^-	H	GTX 3	C2	dc-GTX 3	do-GTX 3
OH	OSO_3^-	H	GTX 4	C4	dc-GTX 4	--





R_4 : OH

R_4 : H

STX: saxitoxin
NEO: neosaxitoxin
GTX: gonyautoxin

dc: decarbamoyl
do: deoxydecarbamoyl

Figure 1.6.

Toxicity values of STX and its derivatives based on mouse unit (MU) values calculated from Fix Wichmann *et al.* (1981), Koehn *et al.* (1982) and Sullivan *et al.* (1983).

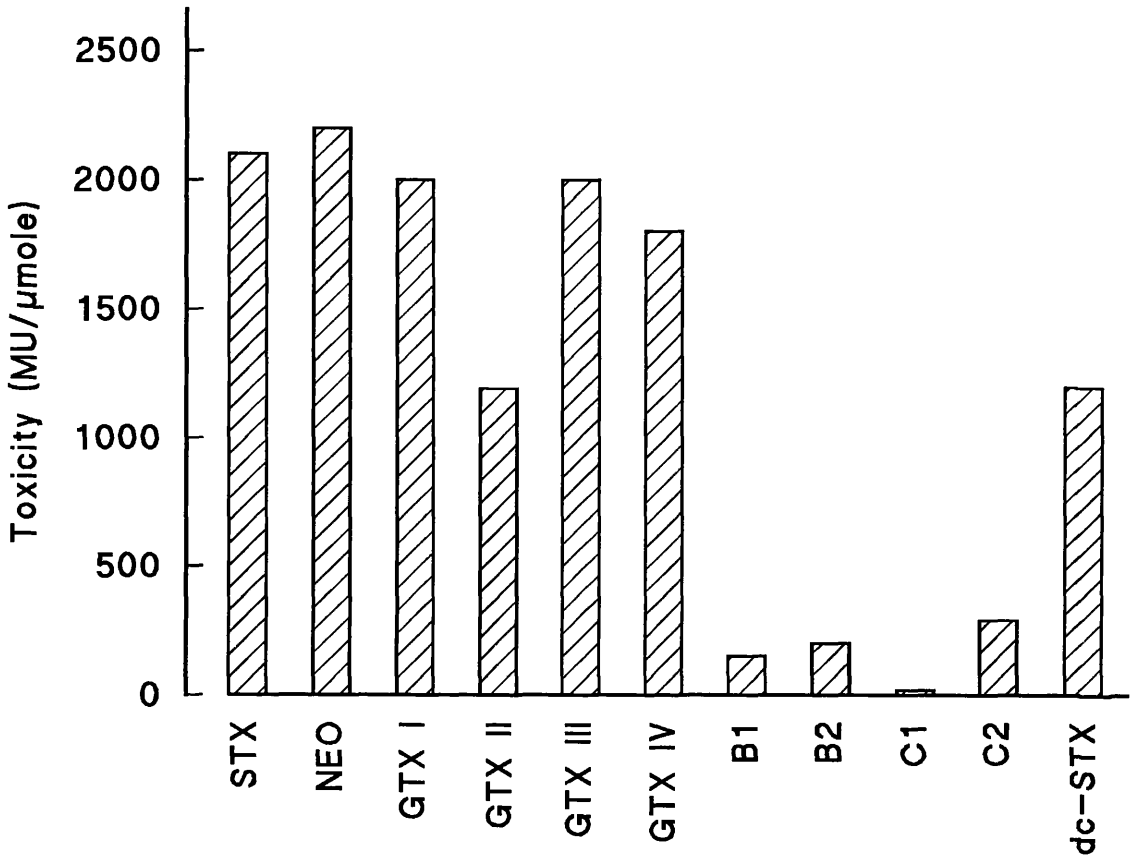
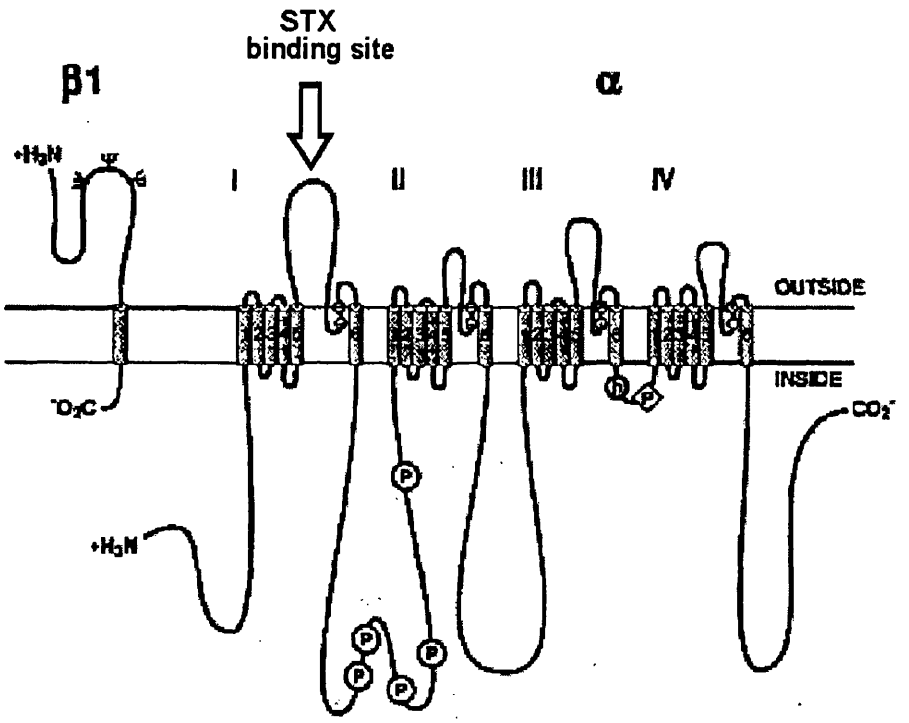

Toxicity (MU/μmole)
2500
2000
1500
1000
500
0
STX
NEO
GTX I
GTX II
GTX III
GTX IV
B1
B2
C1
C2
dc-STX

Figure 1.7.

Structural model of the Na channel showing the common elements of the α- and β- subunits. The α-subunit is comprised of four highly conserved homologous domains (or repeats) and six subdomains with intracellular and extracellular loops. The extracellular loop between S5 and S6 is thought to fold back to form part of the ion conducting pathway. In addition, the loop between S5 and S6 is considered to be the binding site for STX and TTX (described in **sections 1.13 & 1.15**)


STX
binding site
β1
α
+H3N
I
II
III
IV
OUTSIDE
INSIDE
-O2C
CO2-
+H3N
P

Chapter 2

Analysis Techniques and Assays for the Detection of PSP Toxins in Shellfish

2.1. INTRODUCTION

Detection of seafood contamination with PSP toxins and reliable testing protocols are foremost requirements to avoid risks to public health. Increasing international trade demands the ability of both established and developing nations to comply with the AOAC approved requirements to provide adequate safety measures. However, there are differences between countries as to the preferred method of sampling and analysis. Furthermore there is not total agreement between all countries concerning tolerance levels of the various toxins in seafoods (**section 1.4**). Pressure is constantly increasing to develop sensitive, rapid, reliable and inexpensive chemical, biochemical or biological screening techniques that could ideally be used as kits on board ship, at the dockside, or in local laboratories by field technicians.

The purpose of this chapter is to present an overview of the more commonly used analytical and assay techniques for the detection of PSP toxins in shellfish destined for the consumer market, as well to outline some novel methods still to be fully evaluated. Several excellent reviews have already been published e.g., Hungerford and Wekell (1993); Sullivan (1993) and van Egmond *et al.* (1993) where greater detail of the techniques described here is given.

Various assay and analysis techniques that encompass biological, biochemical and chemical methods have, and are being, developed to determine PSP in shellfish. Biological assays remain the mainstay of most monitoring programmes in Europe. Differences in toxin structure and potency, and complications introduced by complex organic matrices, clearly pose a challenge to the development of *in vitro* biochemical and cellular diagnostic tests. Such methods should be capable of simultaneously detecting the presence of multiple toxin derivatives and estimating total toxicity, referable to that derived from whole animal bioassays (Cembella *et al.*, 1995).

Currently, the most widely applied test method is the classic mouse bioassay, with animal death as the toxicity criterion (AOAC 1984). There are numerous inherent and operational deficiencies in the accurate quantification of toxins with such an assay, and increasingly there are mounting ethical and moral pressures to develop alternative methods. In Scandinavia, particularly in Norway, the use of animals for routine assays has been strongly discouraged since the early 1980s. Chemical methods such as the fluorimetric method developed by Bates and Rapoport (1975) or HPLC

(Sullivan and Iwaoka 1983) are commonly used with reputedly satisfactory results (Tangen 1992).

Immunoassays, enzymatic tests and cell culture bioassays are also less controversial alternatives to whole animal assays. These *in vitro* methods can be categorised into two general sub-types: structural and functional. Structural or biochemical assays depend on the conformational interaction of the toxin with an assay recognition factor, e.g., epitopic binding sites in immunoassays. Functional assays are based on the mechanistic behaviour of the toxins at cellular level, i.e., binding to ion channels or neuroreceptors. Many can now be performed using microtitre plates or automated plate readers, permitting rapid parallel screening of large numbers of samples. These tests have advanced beyond the developmental stage, and they compare well with the mouse bioassay for measurement of total toxicity (Jellet *et al.*, 1992, 1995; Usleber *et al.*, 1997).

The more sophisticated chemical analytical techniques, capable of determining individual toxins, e.g., HPLC and chromatographic methods, are frequently used at the expense of time, cost, and ease of use, requiring sample clean up and complex operational procedures performed in laboratories. The development of many chemical methods is also presently handicapped by the lack of commercially available standards and reference materials, and further complicated by the large number of closely-related compounds involved (21 derivatives to date, see **sections 1.8** and **1.10**). In fact, all tests and assays, whether routine or under development, require calibration against known toxin standards, and a global lack of standards is probably the greatest hindrance. The lack of liaison between agencies and/or laboratories in obtaining information and thus correlating assays under development is also a considerable obstruction to establishing reliable testing methods (van Egmond *et al.*, 1993).

A useful distinction between assays and analyses for the quantitative determinations for PSP toxins has been made by Sullivan *et al.* (1988). An assay (e.g. mouse) provides a single response that is the net effect of all active substances present in the test material. It therefore only tests overall toxicity or biological activity, and cannot identify which individual toxins are present. Analyses however (e.g. HPLC), resolve the compounds of interest so that individual components can be separately quantified.

2.2. CHEMICAL DETECTION METHODS

The following chemical detection methods have been developed for the quantification of the individual components of a PSP sample.

2.2.1. Fluorimetric techniques

A simple, but sensitive (1μg STX/100g), peroxide oxidation determination using fluorimetric techniques without chromatographic resolution was developed by Bates and Rapoport (1975), and Bates *et al.* (1978). However, the technique suffered from difficulties in the ion-exchange clean-up step with erratic recovery of STX from the columns. Additionally the toxins did not fluoresce equally, especially neoSTX and the carbamate toxins which exhibit poor fluorescence. Consequently this fluorimetric method generally produces only an approximation of toxin content or total toxicity, but at 80μg/100g wet tissue (the AOAC safety cut off level) it is able to discriminate between acceptable and unacceptable samples, with good correlation to the mouse bioassay. Hungerford *et al.* (1991) have automated a fluorescence method using flow injection analysis which corrects for background fluorescence, and which could provide a useful tool for the study of the PSP toxins

2.2.2. Column chromatographic techniques

The pioneering work of Schantz and co-workers using ion-exchange resins to separate STX from crude shellfish extracts (Schantz 1960) established the basis for most of the column chromatography procedures utilized since then. Since all the PSP toxins have a different net charge, ranging from +2 (e.g. STX) to zero (C1-C4), this property can be employed to retain and separate the compounds on ion-exchange resins. By employing a weak acetic acid gradient the following toxins can be partially separated, and eluted in the order B2, B1, GTX I/IV, GTX II/III, neoSTX and STX. C1-C4 are not retained because of their zero charge. In addition to cation-exchange resins, porous polymers, such as Bio-Gel P2, have been used to separate the various toxins (Hall *et al.*, 1990). The toxins elute in roughly the reverse order to ion-exchange resins, with the C-toxins eluting last.

2.2.3. Thin layer chromatography

The early thin layer chromatography (TLC) techniques described by Mold *et al.* (1957), who separated STX from extracts using paper chromatography, and later Proctor *et al.* (1975) who used silica gel, lacked specificity and sensitivity. In a TLC technique developed by Buckley *et al.* (1976), similar to the fluorimetric technique of Bates and Rapoport (1975), the toxins are separated on silica gel TLC plates, sometimes referred to as slab electrophoresis (van Egmond *et al.*, 1993), and exposed to peroxide, forming fluorescent spots which can be visualised under UV light. This method remains one of the best for general resolution of the saxitoxins and has been used extensively in PSP toxin studies. Hall *et al.* (1980) have used TLC together with column chromatography to resolve various dinoflagellate toxins, and an affinity chromatography methodology (Shiomi *et al.*, 1993) can analyse samples with low concentrations of TTX, although it cannot bind STX.

2.2.4. Electrophoretic techniques

Being charge-bearing molecules, the PSP toxins are also amenable to separation by electrophoresis. The toxins are separated by charge along an electrochemical gradient, with STX and neoSTX (+2) migrating furthest toward the -ve electrode. A sensitive capillary electrophoresis (CE) technique involving the use of laser based fluorescence to detect fluorescent PSP derivatives was developed by Wright *et al.* (1989), but despite its sensitivity and separation powers it has not been widely developed.

Modifications of CE involving high sensitivity UV detectors or ion spray mass spectrometry (MS) have provided clear identification of PSP toxins, but the method has low sensitivity (Thibault *et al.*, 1991). Further development by Locke and Thibault (1994), using a combination of CE and isotachophoresis (concentrating molecules into a narrow band), has increased the sensitivity to within the limits of regulatory detection. A further step is the application of a sample stacking procedure prior to CE separation which is fully compatible with MS and has provided quantitative identification in the micromolar range of PSP toxins in crude digestive material (Buzy *et al.*, 1994)

2.2.5. High performance liquid chromatography

Following the advent of high performance liquid chromatography (HPLC) in the early 1970s, it has become the most widely used analytical method for the individual quantitative determination of PSP compounds. The method involves the chemical conversion of PSP toxins to fluorescent derivatives, commonly using a silica C-18 ion exchange column (Hungerford 1993), either after chromatographic separation (post-column derivatization - POCD), or before chromatography (pre-column derivatization - PRECD), based on ion pairing of the highly charged analytes.

The original POCD method (Sullivan and Iwaoka 1983) was sensitive in the nanogram range (0.5 ng for STX to 25 ng for neoSTX) but suffered numerous instabilities, now reportedly overcome (Sullivan and Wekell 1984). A POCD method for resolving almost all of the 21 known derivatives, with detection limits ranging from 20-110 fmol, has been developed (Oshima 1995b) but the system is rather complex and expensive for routine screening. A further development of a solid phase oxidant POCD (Lawrence and Wong 1996) which is sensitive to 0.10 ng STX and to sub-nanogram, or above, concentrations of non-hydroxylated or hydroxylated toxins, respectively, has quantified PSP extracts with a 10% error rate (which is half the error rate of the mouse bioassay).

PRECD-HPLC is also sensitive in the nanogram (3-12 ng/g) range depending on the individual PSP compounds. Pre-column separation and oxidation step modifications have improved toxin quantification, especially for the epimer pairs and the C-toxins, and some can allow automated analysis (Janecek *et al.,* 1993; Lawrence *et al.*, 1995, 1996).

Despite the advantages of individual toxin quantification using HPLC, and the small sample size (< 1g tissue) required, the method is still slow compared to the mouse bioassay, and Waldock *et al.* (1991) questioned the robustness of the system when HPLC failed to support a survey following a large toxic bloom off the NE coast of Britain in 1990. The other main drawback with HPLC methods is the difficulty in correlating the data obtained with toxic effects in humans and with the results of the mouse bioassay (MAFF 1994b). However, a fast (12.5 min), low-temperature PRECD method has been developed by Flynn and Flynn (1996) that could prove to be very useful for the rapid screening of many samples, especially when the extra expense in

time and instrumentation does not justify the use of a POCD analysis system. A novel analytical method with diagnostic potential can measure STX isolated from rat urine and incorporates an ion-exchange procedure and a PRECD coupled with fluorescence detection (Stafford and Hines 1995).

2.2.6. Inter-laboratory comparison of HPLC and ELISA methods

An inter-laboratory comparative study of STX determination methods by 18 laboratories, organised within the European Commission's Measurements and Testing Programme (van Egmond *et al.*, 1994), used either HPLC (4 methods) or an ELISA technique (see **2.3.1.**). Three of the HPLC methods (Lawrence *et al.*, 1991, PRECD, Thielert *et al.*, 1991 and Oshima *et al.*, 1989, both POCD) were able to quantify STX in the PSP-positive samples provided. However, the Sullivan and Wekkell (1987) POCD method gave a significant over-estimation, blamed on inadequate separation of STX from matrix compounds and the appearance of interfering peaks on the chromatogram. The ELISA method of Usleber *et al.*, (1991) also grossly over-estimated STX, due to cross-reaction contamination by other PSP matrix components with the antibodies.

The study highlighted the need for further improvements in HPLC analytical methods. Several laboratories commented that the STX standard provided was only around 60% of the quoted concentration when compared to STX from commercial sources. In the absence of interfering substances in the STX extract, a coefficient of variation of 33% at a concentration of 0.5 μg/ml was determined. This is still larger than that of *ca.* 20% for the mouse bioassay. Thus, inter-laboratory reliability using these techniques still requires many improvements in accuracy and standardisation before such methods can replace the AOAC mouse assay.

2.3. BIOCHEMICAL METHODS

2.3.1. Immunoassays

These assays depend on the production of antibodies against the toxin of interest. The toxin is conjugated to some larger molecule, usually a protein. Both monoclonal antibodies (usually produced by injecting mice with the toxin conjugate),

more suited for a single epitopic site, or polyclonal antibodies (usually obtained from rabbits) with a high affinity for multiple, epitopic sites, i.e., broad spectrum assays, are acquired from the antiserum and purified. Enzyme-linked immunosorbent (ELISA) assays are usually carried out in multiwell microtitre plates that can be read automatically and rapidly, allowing large numbers of samples to be screened.

The application of immunological techniques for the determination of PSP toxins was initially attempted by Johnson and Mulberry (1966), by coupling STX to bovine serum albumin (BSA) with polyclonal antibody preparations obtained from rabbit antiserum using a formaldehyde treatment. The assay was reasonably specific for the PSP toxins, but it lacked the necessary sensitivity required for routine use. Since then, a wide array of immunological assay techniques has been developed, including direct- and indirect-coupling, competitive interaction, and "sandwich" assays (Cembella *et al.*, 1995), which commonly make use of markers such as radio-label (RIA), a coupled enzyme reaction (EIA), or a fluorescent marker (FIA).

RIA methods developed by Carlson *et al.* (1984) and Davio *et al.* (1985), and ELISA assays (Chu and Fan 1985; Davio *et al.*, 1985), employed various cross-linking agents and STX derivativization reactions to produce antibodies. Where cross reactivity with other PSP toxins was evaluated, it was found that the critical deficiency in most immunoassays was the weak cross reactivity with the neoSTX sub group. The ELISA method was found to be the more sensitive assay, able to detect STX at concentrations as low as 0.5 ng. ml^{-1}. Another handicap of the RIA method is the short shelf life of the radiolabelled material.

An absorption-inhibition ELISA technique, based on a polyclonal anti-STX antibody developed by Cembella *et al.* (1990), was evaluated against a number of standards, including neoSTX, GTX II and III and the *N*-21 sulfocarbamoyl, C1 and C2 compounds. No cross reaction was evident with C1 and C2, but there was high affinity for STX, with cross-reaction to various degrees with neoSTX and GTX II and III. Similarly, a direct EIA method, prepared from a polyclonal anti-STX antibody (Usleber *et al.*, 1991), showed high sensitivity of 3-4 ng/g tissue, but cross reactivity was again weak.

The production of a chromogenic dipstick ELISA kit for STX, neoSTX and several other derivatives, but still not the C compounds, was reported to be available by Wekell (1991). Good correlations with the mouse (90%) and LC (88%) methods

were obtained. This "stick test" might provide a convenient field sampling immunoassay kit. A similar simple and rapid, qualitative type screening assay has been developed from a competitive enzyme-linked immunofiltration assay (ELIFA) which could be performed outside of a well equipped laboratory (Usleber *et al.*, 1995). A novel monoclonal ELISA developed for saxitoxin-induced-protein (SIP) detection in shore crabs extracts (Smith *et al.*, 1989, Smith and Kitts 1994) could provide the basis for rapid screening of shellfish for PSP contamination.

Whilst high specificity for STX and neoSTX improve as immunoassays continue to develop (Burk *et al.*, 1995; Chu *et al.*, 1996), the weakness in cross-reaction to the STX sub groups remains inherent. An evaluation of an ELISA kit with the mouse bioassay (Kasuga *et al.*, 1996) again showed unpredictable cross-reactivity to the GTXs and an underestimation to mouse bioassay toxicity values.

A comparison of a competitive direct ELISA and the mouse bioassay (MBA), of shellfish samples gathered during MAFF (CSL, Torry) routine surveillance found no false positive results out of a total of 45 positive MBA samples and only one false positive from 15 negative MBA samples, obtained with the ELISA method. At the AOAC regulatory level (80 μg/100g tissue), 39 of the samples were correctly determined by ELISA to be above or below this level, but toxicity was overestimated in two samples and underestimated in four samples (Usleber *et al.*, 1997).

2.4. BIOLOGICAL ASSAYS

The biologically-based assays can be described under the broad categories of classical (e.g., the mouse bioassay), more novel approaches (e.g. fly, lobster, frog bladder) and the cell-based assays (which can be subdivided into binding methods, cytotoxicity assays and methods that target the Na^+ channel more directly).

2.4.1. Mouse bioassay

The original bioassay for PSP, and still the major assay in use, is based on intraperitoneal injection (i.p.) into laboratory mice. It was described in its original form by Sommer and Meyer (1937) and was later standardised by Medcoff *et al.* (1947). This involves i.p. injection of an acidified test solution, typically 1 ml, into a mouse weighing 19-21 g, and observing the time from injection to death. From the

death time and mouse weight, the number of mouse units are determined and converted to toxin specific units equivalent to μg STX/100 g wet/fresh shellfish tissue, based upon the toxicity response calibrated with reference to a toxin standard table. One mouse unit (MU) is defined as the amount of toxin that will kill a 20 g mouse in 15 min (AOAC 1984, Sommer and Meyer 1937). This has been reported to be equivalent to *ca.* 200 ng STX (Tamplin 1990) and 220 ng TTX (Yasumoto *et al.*, 1995). The generally-accepted rule is that 1 MU is equivalent to 0.2 μg STX eq/100g tissue, but variations between 1.8 and 2.3 μg are recorded, depending on the conversion factor method used. The sensitivity of the mouse population used is calibrated using a reference standard STX solution of 1μg/ml.

In practice, the concentration of both the STX standard and test solution is adjusted, using 3-5 mice per dilution, to result in a median death time of 5-7 min. Once the correct dilution has been established, and a conversion factor (CF) value calculated, expressing μg poison (e.g. PSP in sample) equivalent to 1 MU, another group of 3-5 mice normally provide a result that differs by less then 20%. Variation greater than this represents a significant change in response of the mice to the toxic sample or technique in the assay (AOAC 1990). The adoption of a CF value is therefore crucial for inter-laboratory reliability as there are still inherent weaknesses in the assay that have to be considered. The regulatory AOAC safety limit of 80 μg STX eq/100 g tissue (400 MU) is only twice the nominal toxicity detection limit (*ca.* 40 μg STX eq.) providing only a narrow security margin for technical errors.

Female mice are slightly, but significantly, more sensitive then males (8:10 μg STX/kg LD_{50}, respectively). The lowest detectable level of PSP is about 40 μg STX/100g, although the exact level depends on the mouse strain employed. The higher the concentration of NaCl (more so than $NaSO_4$, $-PO_4$, or $-NO_3$) in the sample, the "salt-effect", the less potent the sample will be, thus extending death time (Wiberg and Stephenson 1960). Excess zinc in the shellfish tissue (McMulloch *et al.*, 1989) will also interfere with assay values. Inter-laboratory variation can be extremely large when toxicity levels in samples are high (Park *et al.*, 1986). The presence of unknown or poorly-defined toxic components in the extract matrix can be screened inadvertently (Wright 1995). This is particularly a problem for samples which contain multiple toxin analogues (**section 1.11**) or the co-occurrence of different phycotoxins,

which can lead to synergistic or antagonistic biological responses, e.g., the simultaneous presence of a Na^+ channel activator and a blocker.

The mouse bioassay is performed at pH 2.0 - 4.0 (never > pH 4.5). This appears to be for several reasons. Firstly, the STXs are more stable under acidic conditions. These low pH values are used to mimic the gastric environment in the stomach, although the route of administration is normally by i.p. injection, not orally. This is reflected in the variance of the LD_{50} dose between the two routes, i.e., 10 µg/100g i.p. versus 263µg/100g orally. Secondly, and perhaps most importantly, the LD_{50} increases if the pH of the sample is elevated, with mice tolerating increasing amounts of toxin, thus increasing the time to death above pH 4.5. This is because samples become less active at pH 6 or above (Park *et al.*, 1986). Below pH 4.5 (i.e., 3.0- 4.5) as prescribed by the AOAC method, median death time is essentially unaltered (Wiberg and Stephenson 1960) giving good correlation with CF values. However, at pH 2- 3 the less toxic sulfocarbamoyl compounds (-$CONHOSO_3$-) are easily converted to the carbamoyl analogues (-$CONH_2$). Thus if a sample contains a high percentage of the sulfocarbamoyl compounds an increase in toxicity of around 40-fold can occur (Kao 1993).

When well administered and properly calibrated the toxicity determination using the mouse bioassay is directly relevant to human toxicity effects, although accurate extrapolation of results is difficult as they are derived from an alternate route of administration (i.p. injection) to human oral potency (Fernandez and Cembella, 1995).

2.4.2. Fly bioassay

Use of the common house fly (*Musca domestica*) as an alternative to the mouse bioassay has been advocated (Ross *et al.*, 1985). Anaesthetised flies are injected with an AOAC prepared sample. They are then scored for motor ability (moving or not moving) after 10 min. The results showed that there was a linear relationship between the probit of the fraction of the animals affected and the logarithm of the dose. The 50% effective dose (ED_{50}) is established for unknowns and the concentration of toxin estimated from dose response standard curves. Again, this assay measures total toxicity but claims to be more sensitive, cheaper and does not exhibit a "salt-effect" compared to the mouse assay, this last point resulting in the

actual concentration in any given sample being higher in the fly compared to the mouse. Disadvantages include microinjection of flies, which is considered awkward by some operators, the fact that the assay still requires a trial and error dilution step, and the need for each analyst to produce their own calibration curve (Hurst *et al.*, 1985).

2.4.3. Lobster nerve-muscle assay

This bioassay makes use of the inhibitory action of PSP on Na^+-dependent excitable membranes, and utilizes a nerve-muscle preparation from the walking leg of the lobster (*Panulirus japonicus*) (Daigo *et al.*, 1989). It is based on a dose-dependent reduction in the amplitude of nerve action potentials, reflected in an equivalent reduction of excitatory post synaptic potentials (epsp's) recorded from the stretcher muscle, following electrical stimulation of its associated motor nerve.

It is a highly sensitive assay, with a detection limit for STX around 10^{-14} mol (40% blockage). It is even more sensitive to GTX-rich and PTX-rich samples, with complete epsp blockage at 10^{-16} mol. Oyster toxin and *Gymnodinium* extracts strongly blocked at 10^{-17} mol and 10^{-15} mol respectively. However, with unknowns it will only detect total toxicity as do the mouse and fly bioassays. A useful feature is that following thorough washing of the preparation, a complete recovery of epsp's is observed. Thus the effect of 5-10 toxins could be examined on the same preparation. Disadvantages include the fact that washing time between toxins is at least 2-3 h (Prof. Kawai, *pers. comm.*), operators require some skills in dissection and electrophysiology, and the extreme sensitivity of the system may not be suitable for the high toxin content of many PSP samples.

2.4.4. Frog bladder membrane assay

A simple, rapid, tissue biosensor able to detect very low levels of TTX and STX has been developed using a section of frog bladder (Cheun *et al.*, 1996). The tissue biosensor consists of a Na^+ electrode intergrated within a flow cell. The tip of the electrode is covered with frog bladder membrane, sandwiched between two cellulose acetate membranes, and is positioned in a flow cell in which a NaCl control solution is circulating. Toxicity is measured as the inhibition ratio of the sensor peak output after the addition of TTX/STX into the flow system. Each section of frog

bladder can determine TTX concentrations for up to 250 h continuously, depending on the external Na^+ concentration.

The assay is reportedly able to detect a lower limit of 86 fg of TTX, with a linear relationship between Na^+ concentration and TTX quantity of 86 fg - 600 fg. Such an extremely sensitive system would not be suitable for routine screening, especially as the linear relationship between sensor output and TTX only exists up to 600 fg, suggesting that toxin saturation of the system occurs above this concentration. STX was reported to dissociate from the membrane Na^+ channels more slowly than TTX, which is the opposite to the dissociation times observed in other studies (e.g., Narahashi 1988). Further work is required to explain this difference.

2.4.5. Solid-phase radioreceptor assay

Vieytes *et al.* (1993) have described a direct solid-phase radioreceptor binding assay (RRA) for PSP toxins which uses rat brain membrane preparations. PSP toxins were effectively quantified by their specific ability to competitively displace [^{3}H] STX from its receptor on rat brain membranes bound to a solid-phase RRA. PSP mussel fractions tested were found to contain STX and dcSTX, GTXs, and C1 and C2 which correspond to results obtained by HPLC. The IC_{50} values for the mussel extracts ranged from 0.03 to 0.30 ng/ml which is far more sensitive than the mouse bioassay (Table 2.1). Variability of the RRA is 10-15%. Each toxin could, in principle, be quantified with this assay, because of the competitive binding nature of all the PSP toxins, but at the moment further development is limited by the lack of availability of toxin standards.

2.4.6. Competitive binding assays

A competitive displacement assay (CDA), also referred to as a competitive binding assay, is based on the ability of the non-radioactive form of the toxins (TTX or STX) to competitively displace a radiolabelled form of the toxin (e.g., [^{3}H] STX) from its receptor in rat brain membranes when incubated together (Davio and Fontelo 1984). The displacement is quantified by comparison with a specific binding curve previously determined by incubating known concentrations of [^{3}H] STX in batches of rat brain membranes and parallel samples of [^{3}H] STX prepared containing an additional 5 μM unlabelled STX. Following incubation and filtration, the fractions are

read on a scintillation counter. The specifically-bound [^{3}H] STX is then quantified by subtracting non-specifically bound [^{3}H] STX (in additional 5 μM STX) from the total bound [^{3}H] STX (with no 5 μM STX added) to produce a binding curve.

The CDA has a sensitivity of 0.15 ng STX/ml (= 0.3 μg STX/100g) making it around 1000 times more sensitive under ideal conditions than the mouse bioassay (Table 2.1). Similarly for TTX the lower detection limit is 0.8 ng/ml (=1.6 μg STX/100g). A rat brain synaptosome CDA assay developed by Doucette *et al.* (1995) has a completed assay time of *ca.* 4 h with a detection limit of *ca.* 4 ng STX/ml in the original sample extract. Although the detection limit is slightly higher than that of Davio and Fontelo (1984), it is still far more sensitive than the mouse bioassay, and has the advantage that it is automated, using 96 microwell plates and scintillation counting technology.

Owing to the extreme sensitivity and excellent correlation achieved, Sullivan *et al.* (1988) suggest the CDA as a promising method for determining total toxicity in shellfish samples. Hall (1985) applied a similar CDA method on spiked-shellfish samples, and found it to be at least 10 times more sensitive than the mouse bioassay. It is thought that a good correlation response between mouse intraperitoneal potency (MIP) and human oral potency (HOP) will be found. It is already documented (Hall and Reichardt 1984) that extracts administered intraperitoneally to mice are 40 times more toxic than when administered orally, and a great deal of uncertainty exists to date regarding the non-uniform relationship between MIP and HOP. Hall (1985) proposes the CDA method to be a reliable predictor of mammalian toxicity since the population of Na^+ channels in rat brain should have similar properties and affinities to those in the human population at risk. Although at present animals are required for isolation of target membranes, it is possible that a suitable cell line could be identified and developed for use instead.

2.4.7. Cell-based assays

A number of cell based assays have recently been introduced for the detection of PSP in shellfish samples. According to Hungerford (1993) it is necessary for these assays to satisfy the following basic criteria:

- they should respond within the dose range of the suspected or known *in vivo* effect.

- they should exhibit low inherent variability, and ideally give a linear dose response curve.
- they should be rapid and relatively simple.

A tissue culture assay using the mouse neuroblastoma cell line, Neuro-2a, was developed by Kogure *et al.* (1988) for the detection of TTX, STX and related toxins. The cells are treated with pre-calibrated concentrations of veratridine (VER) (known to depolarise the cell membrane) and ouabain (OUB) (a specific Na^+-K^+ ATP -ase inhibitor). The combination of these two agents causes a prolonged, increased influx of Na^+ (and Ca^{2+}) ions into the cell, whereupon, the cells swell and lyse, or lose their morphological rigidity.

The assay is based on the potent Na^+ channel blocking actions of TTX and STX, which antagonise the actions of VER and OUB. Triplicate wells are prepared for each sample and are incubated for 6-48 h. The viability of the cells is estimated by the operator using an inverted microscope to count the number of live cells amongst 200 randomly chosen cells per well. Dose response curves for cells incubated for 6 and 18 h, are constructed from % viable cells v. concentration of TTX. A good correlation exists up to a concentration of 50 nM TTX. Below 10 nM, TTX did not prevent cells from rounding up, whilst a concentration of 100 nM TTX provided over 90% protection. The minimum reported detectable level of TTX was 3 nM (ca. $1x10^{-3}$ MU).

Although this assay is sensitive (Table 2.1), relatively inexpensive compared to many of the chemical based methods, and simple to operate with the potential to process several samples at one time, it cannot distinguish between toxins, requires a long incubation period and is liable to operator subjectivity at the counting stage.

A quantitative tissue culture assay for Na^+ channel blocking toxins in bacterial culture supernates, based on the Kogure method, has been developed for use with a microtitre plate reader (Gallacher and Birkbeck 1992). Cells are incubated for 24 h in multiwell plates in a combination of VER and OUB, plus a test sample in quadruplicate sets. Viable cells are detected by their ability to take up the vital stain, Neutral Red, after 30 min of incubation. The percentage protection by TTX was calculated from the absorbance at 540 nm of viable cells in test samples/solutions as a fraction of control cells minus cells in VER and OUB alone. TTX was used as a standard and a sigmoidal dose response curve was obtained; 50% protection required

a 50 nM concentration of TTX and protection was measurable over the range 10 - 500 nM TTX (3.2 - 160 ng/ml).

Gallacher and Birkbeck (1992) postulate that since the sensitivity of the assay permits direct assay of culture supernates, it will allow quantification of Na^+ channel blocking toxin concentrations without the prior need for partial purification. The assay has greater convenience in terms of the number of samples that can be tested in one reading, the speed at which tests are read, and the removal of operator subjectiveness in counting cells. The main disadvantage is still the long incubation period of samples. Stability of the Neuro-2a cell line (e.g., cell culture contaminations and loss of Na^+ channel expression) has caused technical difficulties, with indeterminate and inconsistent pharmacological reactions to known toxic PSP samples. These problems were further highlighted in the 1994/5 MAFF Ring Trial by S. Gallacher (MAFF Report FD94/161) who reported poor correlation between the mouse bioassay and tissue culture results. This included detection by the cell bioassay of Na^+ channel blocking activity in three shellfish samples determined as containing 0μg STX eq. by the mouse bioassay.

In order to improve assay time and sensitivity several chemical reagent modifications have been investigated with varying degrees of success (Munro and Birkbeck, MAFF report 94/161). These have included the use of the fluorescent dye, sodium-binding benzofuran isophalate acetoxymethyl ester (SBFI-AM), as a measure of intracellular sodium. SBFI-AM is considered more sensitive than neutral red in STX detection, and should eliminate the need for a washing step in the processing. This would reduce assay time and the need for cells to adhere to the substrate. Another fluorescent cell-impermeant dye form, sodium-green-tetraacetate has also been investigated. Both dyes gave only limited success as the esters appear to have been internally compartmentalized, with little evidence of cleavage within the cells. Only extracellular calibration curves could be obtained.

Another agent, Calcein-AM, which is an indicator of esterase activity in live cells, measures viability by a fluorescence generated by the hydrolysis of Calcein-AM to Calcein, which is retained within the cell as a green fluorescence and can be read by a plate reader at 530 nm. It has also been tried, with limited success.

Two potentially promising tissue culture assays are those developed by Jellet *et al.* (1992) and Manger *et al.* (1993, 1994, 1995a,b), the first being an automated

endpoint determination of absorbance of crystal violet-stained cells, the second being based on cell dyhydrogenase activity, which is detected using a simple colorimetric method (MTT) to assess cytotoxicity.

Jellet *et al.* (1992) have exploited the fact that cells affected by VER and OUB lose adhesion to the plastic walls of the standard 96 well plates in which they are cultured. It is this phenomenon which permits automation of the determination of STX in test samples. During the rinsing, fixing and staining stages, cells affected by OUB and VER are washed away. The absorbance at 595 nm of the remaining cells (protected by STX) is measured automatically with a plate reader. The absorbance (purple colouring) was found to be proportional with the concentration of STX over a range of concentrations of 0 - 0.6 ng STX/10µl (= 0 - 60 ng STX eq. ml^{-1}). The lower detection limit was reported as 10 ng STX eq. ml^{-1} (= 2.0 µg STX eq./100g tissue). A hyperbolic relationship between STX concentration and percentage of cells protected (absorbance) was reported, similar to that found by Kogure *et al.* (1988).

The bioassay was compared with the standard mouse bioassay using 10 extracts from PSP toxic dinoflagellates and 47 shellfish extracts (standard AOAC method). The cell assay gave almost identical results, and was moreover far more sensitive (Table 2.1). Reliability variation was ± 20%, which matched that of the mouse bioassay. Results of HPLC analyses of 12 of the extracts were less consistent compared to both bioassays. A disadvantage of the assay is again the long incubation period required. Jellet *et al.* (1992) state that it is imperative for the cells to be subcultured regularly to maintain active growth characteristics, otherwise a degree of sensitivity to OUB and VER is lost. Failure to use actively growing cells for the bioassay will also result in an increase in the detection limit and a decrease in the accuracy of the results.

Unknown materials co-extracted with the STX in the shellfish samples, but not the dinoflagellate extracts, caused some lytic effects on the Neuro-2a cells and affected toxicity values. Attempts to identify whether geographic location or shellfish species was the common denominator proved inconclusive. However, filtration with a 10 kD cut off filter failed to remove the causative material, and therefore is was assumed that the compound had a mass of less than 10 kD (Jellet *et al.*, 1992).

Co-extracted materials also reduced toxicity values when known amounts of STX were added to non-toxic extracts. This observation is important given that

bioassay calibrations are performed using pure standards. Sato *et al.* (1988) suggest that co-extracted proteins may adsorb some of the added toxin. Manger *et al.* (1995a) observed non-specific toxic effects with crab viscera extracts, but the problem was overcome by dilution. A similar problem with unidentified compounds eluting off during thin layer chromatography (TLC) analysis, at the same time as STX, neoSTX and GTX I-IV, was observed by Park *et al.* (1986) in a study of variability in mouse bioassay results. Zinc found in some mussel extracts has been shown to have a toxic effect on mice (Sullivan 1993). Thus these co-extracted materials can influence values both in cultured cells and live mice, and therefore some partial cleaning-up of extracts prior to assaying would be desirable. This problem is not peculiar to PSP extracts, since Onoue *et al.* (1984) discovered that some amines (e.g., ethanolamine, lysine) and even ammonium ions can exert a major interference on the detection of TTX from crude toxin preparations.

Interestingly, a further study (Jellet *et al.*, 1995) using pure forms of PSP toxins: STX, neoSTX, GTX II/III, GTX II, dcSTX found that boiling in 0.1 or 1.0 N HCl did not affect toxicities, except that of GTX II/III which increased. Also significant were the findings that matrix conditions (acetic acid v ethanol), filtration (filtered 0.22 μm m or unfiltered) and time in storage at 5°C had almost no affect on EC_{50} values.

Despite the handicaps outlined above, the Jellet *et al.* (1992) assay has been shown to be sensitive, reliable, flexible, has eliminated individual cell counting and has low maintenance requirements, although Manger *et al.*, (1995b) comment that mechanical removal of cells during processing in the Jellet *et al.* assay is still time-consuming and may be subject to operator variability.

The bioassay developed by Manger *et al.* (1993, 1994, 1995a,b.), again using Neuro-2a cells, also utilizes Na^+ influx enhancement, initiated by OUB and VER. The assay is very versatile, being able to detect both Na^+ channel blocking agents such as STX and Na^+ channel enhancers such as brevetoxins and ciguatoxins.

Modification of the Kogure method was undertaken by incorporating a sensitive endpoint determination colorimetric test of cellular metabolism originally described by Mossman (1983), and this has successfully streamlined the assay. Cells are incubated for 24-48 h in VER and OUB, and a sample, in replicates of 3-5 per sample. The medium is replaced with an MTT solution, thus removing the previous

wash step, and incubated for 15 min. Active cells (those protected by STX) reduce the tetrazolium compound MTT, to a blue-coloured formazan product. The absorbance of the MTT into the cells is read at 570 nm with reference at 630 nm on an automated multiwell scanning spectrophotometer (ELISA plate reader). A linear relationship between cell density and MTT metabolism was observed.

At 15 min the minimum detectable level of STX was 0.1 ng/10 µl (2 µg STX eq./100g). With increased development time (*ca.* 45 min), sensitivity could be enhanced to 0.02 ng/10 µl (0.4 µg STX eq./100g) but the response tended to plateau at higher concentrations. Dose-dependent inhibition of OUB/VER-induced cytotoxicity was examined with standards of STX-1; NEO-1; GTX 2/3-1 (NRC) and TTX. Results corresponded to mouse i.p. studies (Sullivan *et al.*, 1988), with NEO-1 exhibiting the highest potency *in vitro*, and STX-1 and TTX giving almost equivalent mid-range activity. The GTXs produced the lowest activity. A similar study (Jellet *et al.*, 1995) using the cell bioassay for toxicological evaluation of PSP toxins STX, neoSTX, GTX II, GTX II/III and dcSTX in pure form supports these results, with neoSTX>STX>GTXII/III>GTX II>dcSTX.

This simplified MTT assay offers a method requiring minimal instrumentation, utilizes readily available agents, uses less sample material and further reduces possible operator variability. A further modification (Manger *et al.*, 1995b) has facilitated detection of PSP by an antagonistic interaction with a Na^+ channel enhancer within 4-6 h, thus making this a very rapid bioassay capable of processing many samples at the one time (compared with slow sampling rates of the mouse bioassay and chemical analytical techniques such as HPLC). The sensitivity of the MTT assay is also at least 20 times greater than the mouse bioassay (Table 2.1). Results of naturally-occurring low value PSP residues in shellfish were compared to both the standard curve derived for STX and the mouse bioassay. The cell bioassay detected STX in extracts that were negative by mouse bioassay.

Two further neuroblastoma cytotoxicity assays based on the MTT methodology of Manger *et al.* (1993, 1995) have been reported (Truman and Lake 1996; Hamasaki *et al.*, 1996). Both assays use N2a cells cultured in 10% serum. Truman and Lake (1996) report slight modifications in the assay procedure and include a set of HCl control wells to check for non-STX-related activity. Their assay compared fresh samples with samples that had been stored for up to two years and

also with the mouse bioassay. All the fresh samples were below the AOAC regulatory limit, hence the inclusion of stored samples.

Truman and Lake observed, as had Jellet *et al.* (1992) previously, depressed toxicity values in a few non-toxic extracts which had been spiked with known amounts of STX. There was no information given as to whether the extracts had been subjected to further filtration or clean-up procedures. A comparison between the mouse bioassay and cell assay for PSP levels from samples that had been previously stored for up to two years, showed that the cell assay gave higher values than the mouse bioassay. Overall, for both types of extracts, a correlation between the assays for STX equivalent levels in shellfish was 0.867, slightly less than the 0.96 reported by Jellet *et al.* (1992).

The detection limit of this cell bioassay was 0.1 ng STX per well, which the authors report as being comparable to results from the other groups (Table 2.1). The low levels of STX detected by the cell bioassay but not by the mouse bioassay (also reported by Manger *et al.*, 1995b) in some un-spiked samples are suggested not to be false-positive results, but simply to be below the detection limit of the mouse bioassay. The large number of samples that were assayed as being zero STX in both assays implies that this assay could be used as a screening tool to eliminate non-toxic samples, and to be used in conjunction with the mouse bioassay for results that are close to the AOAC regulatory limits. Once again the most limiting factor of the assay is the 18-24 h incubation period after the addition of samples.

The cell bioassay of Hamasaki *et al.* (1996) utilizes a water soluble tetrazolium salt (WST-1) which permits direct measurement without the solubilization step using DMSO that is required for MTT before absorbance is measured. The WST-1 is dissolved in a solution of 1-methoxy-5-methylphenazinium-methylsufate (1-methoxy PMS) which mediates and accelerates the reduction of WST-1. Thus an incubation time of only 4 h for this part of the assay is sufficient, with the possibility of shorter incubation times depending on cell number and other assay conditions (not elaborated further). This shorter incubation period in the final assay procedure, similar to that proposed by Manger *et al.* (1995b), is an encouraging step towards increasing the speed at which the number of samples that can be tested, especially if samples have been collected from commercial beds and are therefore of economic importance to the producer.

So far, the WST-1 assay has been evaluated for response to standard TTX, and in addition has been assessed for possible application as a measurement method for TTX produced by bacteria. There is, however, a problem with overestimation, thought to be due to accelerated formazan formation by the bacterial culture, as well as interference by culture matrix constituents on the electron coupling agents of the 1-methoyx PMS. It is suggested that greater dilution of the samples would resolve these problems, but this remains to be tested. The sensitivity of the assay is equal to the other cell bioassays described previously, and it may prove as sensitive for STX and PSP toxins when fully evaluated.

A simple, rapid, inexpensive and fairly sensitive hemolysis assay which combines the principle of the neuroblastoma tissue culture assay and red blood cells from red tilapia (*S. mossambicus*) shows potential as a screening method for crude PSP extracts (Shimojo and Iwaoka 1997). Results using PSP standards show that it has a detection capability of 0.3 μg/ml STX, 3.5 μg/ml neoSTX, 3.0 μg/ml GTX and 5.0 μg/ml. The sensivity appears to be dose-related and dependent on the concentration of red blood cells. Veratridine and ouabain affect the red blood cells in a similar manner as that observed with the neuroblastoma cells, causing hemolysis if the cells are not protected by the action of sodium channel blocking toxins.

2.4.8. Reconstituted sodium channel method

Trainer *et* al. (1995) have reported to have successfully incorporated, or "reconstituted", purified Na^+ channel membrane proteins from rat brain into single-walled phospholipid bilayer vesicles. The channels are orientated with approximately 75% in an outside-out direction. Normal Na^+ channel kinetic functions were demonstrated using [^{3}H] STX. Binding saturation of channels was evident at 20 nM STX. This high-affinity binding was consistent with the conformational integrity of the Na^+ channel being expressed, providing evidence that in highly purified preparations such as this at least a proportion of the Na^+ channel proteins retain a selective-ion channel that effectively mimics binding sites in native forms. This preparation could therefore provide an experimental biochemical method for the detection of specific toxins such as PSP.

Two different STX-binding proteins have similarly been isolated from rat brain membrane preparations and reconstituted into planar lipid bilayers (Corbett and

Krueger 1990). These proteins formed two distinct Na^+ channel subtypes with different single-channel conductances in the presence of VER and batrachotoxin. Ion flux measurements (Tamkun and Catterall 1981) have shown that purified Na^+ channels retained many of their original functional properties when reconstituted in vesicles.

Sodium channels from rat brain, incorporated in planar lipid bilayers, demonstrated voltage-dependent block by STX in work by French *et al.* (1984). This group examined single channel kinetics of the proteins in the presence of batrachotoxin, which like VER inhibits Na^+ channel inactivation (Table 1.4). Batrachotoxin provided an extended time period to study the voltage-dependent block by STX which would be missed under normal voltage-clamp conditions when inactivation of Na^+ channels occurs within milliseconds of opening.

A study by Strichartz *et al.* (1995) examined the biological activity of synthetic analogues of STX, including (+)- and (-)dcSTX, in terms of the kinetics of block of single rat brain Na^+ channels reconstituted in planar lipid bilayer membranes. They demonstrated that only (+)dcSTX exhibited biological activity. The experiments also showed that, as with natural analogues, synthetic compounds of STX have binding affinities for the Na^+ channel that decrease in an orderly manner with structural changes with (+)dc STX binding with the highest affinity of the six analogues tested.

Whilst these detailed studies are obviously not appropriate for mass screening of PSP samples, they do emphasize the stability of reconstituted Na^+ channel proteins into lipid bilayer membranes which could be utilized as a sensitive assay for detection of biological activity in contaminated shellfish, as suggested by Trainer *et al.* (1995).

2.4.9. Sodium channels expressed in other cells

i) *Xenopus* oocytes - A novel alternative method for screening PSP samples without the need to sacrifice as many animals as with the binding techniques is to isolate the DNA and mRNA encoding for rat brain Na^+ channels and inject this into toad oocytes. The mRNA will be expressed and copies of the Na^+ channel protein will be inserted into the oocyte membrane. These fully-functional proteins can then be used as Na^+ channel inhibition indicators when exposed to contaminated shellfish extracts.

Goldin *et al.* (1986) have already shown that it is possible to isolate mRNA specific for the α-subunit of the Na^+ channel by hybrid selection with a single-strand antisense M13 DNA. Two days after injection into the oocytes, voltage clamp experiments confirmed that the α-subunit RNA alone (in the absence of the two associated β-subunits) was sufficient to encode functional Na^+ channels which exhibited the expected time response and voltage sensitive characteristics and were inhibited by TTX.

Another study involving type II Na^+ channels, implanted from rat brain into *Xenopus* oocytes (Neumcke 1990) has suggested that although fully functional Na^+ channels were expressed, as necessary for a screening method, there was a slight modulation of the inactivation component observed within "the foreign environment of the oocyte membrane".

ii) Kidney cells - A variation on the oocyte method is the expression of Na^+ channel α and β1 subunits in human kidney cells (Trainer *et al.*, 1996) which has demonstrated specific binding properties for STX and the Na^+ channel activator, brevetoxin. The cells were transfected using a constant amount of DNA but with a variable molar ratio of channel α and β1 subunit-encoding plasmid DNA. After transfection the cells were prepared for a binding assay. Whilst this assay is still at the developmental stage it yet again shows the potential of using purified Na^+ channels for toxin evaluation purposes, given the high specificity of the Na^+ channel blocking PSP toxins.

These systems also exhibit great potential as screening assays. A mammalian voltage-dependent Na^+ channel protein that retains native the binding sites for Na^+ channel ligands should provide a more reliable HOP indication than the present mouse bioassay. It is relatively simple and requires the use of fewer animals than the standard mouse bioassay, thus both reducing both costs and overcoming ethical objections.

2.5. SUMMARY

There remains an enormous challenge to develop acceptable analyses or assay methods for routine screening of PSP samples. Whilst analysis techniques are important for the separation and quantification of individual toxins within a sample, thus monitoring which toxins are synthesized or accumulated by various marine

organisms and for the detection of "new" compounds, it is still vitally important to develop methods that measure overall toxicity. Shellfish or other fisheries products are normally eaten as whole parts of flesh by the consumer. Therefore, whilst the mouse bioassay is unsatisfactory for many reasons, it is an indicator of the effects of whole shellfish tissue following consumption.

The emergence of several cell cytotoxicity assays which correlate well with the mouse bioassay offers the possibility of testing many samples at one time using an immortal cell line rather than many animals. The main disadvantage is the long time needed to perform the assay, but this is now being reduced. However, reports that some PSP extracts cause interference or false-positive values not detected by mouse bioassay imply that, to date, no method can yet satisfactorily replace the mouse bioassay, which therefore must remain the main detection method for the foreseeable future. This is especially true when STX levels are near the AOAC regulatory limit, and a false result by cell assay could result in the closure of commercial shellfish beds, severely affecting the income of growers and fishermen alike. Both the solid-phase radioreceptor assay and the competitive binding assays incorporating Na^+ channels from rat brain offer great potential as highly specific and robust assays, and are worthy of further investigation.

Table 2.1. Sensitivity of tissue based bioassays compared to the standard AOAC mouse bioassay

Tissue type	Bioassay	Source	STX eq. detection limit ng/ml	STX eq detection limit µg/100g	MU eq.
Whole animal	Mouse	AOAC, 1984	200	40	200
Neuroblastoma cell line	Morphology	Kogure *et al.*, 1988	0.96*	0.192*	0.96*
Neuroblastoma cell line	Neutral red	Gallacher & Birkbeck 1992	3.2*	0.64*	3.2*
Neuroblastoma cell line	Crystal violet	Jellet *et al.*, 1992	10	0.5-2	10
Neuroblastoma cell line	MTT	Manger *et al.*, 1993, 1994, 1995	10	0.5-2	10
Neuroblastoma cell line	MTT	Truman & Lake 1996	10	2	10
Rat brain membrane	CDA	Davio & Fontelo 1984	0.15 0.8*	0.03 0.16*	0.15 0.8*
Rat brain membrane	CDA	Doucette *et al.*, 1995	4	0.8	4
Rat brain membrane	RRA	Vieytes *et al.*, 1993	0.03-0.3	0.006-0.06	0.03-0.3

* TTX used as the standard for these bioassays

0.1 ng STX eq./10 µl = 2 µg /100 g fresh shellfish tissue
10 ng STX eq./ml = 2.0 µg/100 g tissue
1 ng STXeq./ ml = 0.2 µg/100 g tissue
1 MU = 0.2 µg/100 g tissue

Chapter 3

Crustacean Nerve-Muscle and Nerve Preparations as Potential PSP Bioassays

3.1 INTRODUCTION

The comparative simplicity of the crustacean nervous system and the large size of many of its nerve axons and muscle fibres have permitted investigations that are very difficult to carry out in vertebrates. Such studies have increased our knowledge of membrane conductance, of synaptic transmission and pharmacology, and of the mechanisms of muscle excitation (Yamagishi and Grundfest 1971; Freeman 1976; Atwood 1982). The potential also exists for using crustacean nerves and neuromuscular systems as bioassays for PSP.

3.1.1. Innervation of crustacean muscles

Crustacean muscles are innervated by a relatively small number of motor neurones, and one axon may serve several muscles. Each muscle fibre has polyneuronal innervation, and as many as five motor axons have been recorded innervating one muscle fibre (Atwood 1982). Each axon has multiterminal innervation, with nerve endings occurring profusely over the surface of the muscle fibre. Usually one axon is inhibitory, releasing GABA as a transmitter, and the remainder are excitatory, releasing glutamate, which causes an increase in the Ca^{2+} conductance of the postsynaptic membrane.

Excitatory axons are designated fast or slow according to a number of criteria. Fast axons have larger diameters, higher conduction velocities, and tend to produce large single excitatory post synaptic potentials (epsps). Slow axons have smaller diameters, lower conduction velocities, release less transmitter per action potential and evoke observable epsps in the muscle fibre only when facilitated. These synapses are generally non-fatiguing as the neurotransmitter is released progressively (Leake and Walker 1980).

The inhibitor reduces or antagonises the effect of the excitatory axons through a GABA-mediated increase in the conductance of Cl^- ions, and may have either a presynaptic or a postsynaptic action to the excitatory axons (Dudel and Kuffler 1961; Eccles 1961). Presynaptic inhibition reduces the size of the action potential invading the excitatory terminal, and so reduces the amount of transmitter released (Matthews

1986). Postsynaptic inhibition leads to an increased conductance of Cl^- ions across the muscle membrane, which counteracts depolarisation. Since the equilibrium potential of Cl^- (-70 mV) is normally more negative than the resting membrane potential, the inhibitory post synaptic potential (ipsp) is a small hyperpolarisation of up to 5 mV (Boistel and Fatt 1958; Katz 1966).

Crustacean muscle fibres also vary in their properties, and there is a correlation between fibre type and innervation (Atwood 1982). Phasic muscles are adapted for short term powerful activity, such as limb movement or the tail flip. They tend to possess short sarcomeres (<4 μm in length) with distinct, straight Z lines, well-developed sarcoplasmic reticulum and few mitochondria; they produce twitches upon stimulation (Lehouelleur 1978). Tonic fibres are primarily designed for low speed sustained activity and for maintaining posture. These fibres possess longer sarcomeres (approximately 7-9 μm), irregular Z lines and sparse endoplasmic reticulum; they exhibit slow contractions in response to repetitive stimulation. There is a strong correlation between muscle fibre type and the extent of facilitation and input resistance (Sherman and Atwood 1972). Phasic fibres are supplied by terminals that produce large, non-facilitating epsp's, and tend to have high input resistances. In contrast, tonic fibres are preferentially innervated by nerve terminals which produce small, highly facilitating epsp's. These factors are all relevant when considering a neuromuscular system as a potential bioassay.

3.1.2. Crustacean nerve and neuromuscular systems as bioassays for PSP

Blocking of Na^+ channels in the nerve membrane is known to reduce the amplitude of the propagated action potential (Evans 1972). Therefore, if such a smaller action potential propagates to invade the presynaptic endings, it will lead to the release of a smaller number of neurotransmitter quanta, with the consequence that all subsequent postsynaptic processes will be reduced proportionately. A quantitative estimate of the effect of STX can, in principle, be made by measuring the diminution of activity at any of these points along the nerve-muscle pathway: i.e in terms of either the amplitude of the propagated action potentials, the size of the postsynaptic

epsp, or, in the case of neuromuscular systems, the amount of tension subsequently developed by the muscle fibres.

Although neuronal Na^+ channels are thought to be structurally conserved across wide phylogenetic boundaries, adaptive changes have occurred, and certainly variation exists between species in their sensitivity to TTX and STX (Hille 1992; Neumcke 1990). The nerves of some invertebrates, most especially those frequently exposed to either of these two toxins, are relatively insensitive to them, and are affected by micromolar concentrations (Kao *et al.*, 1980; Hille 1992). Others, such as lobster, show a higher degree of sensitivity to STX, and are affected by nanomolar concentrations (Villegas *et al.*, 1988). This feature makes these crustaceans nerves suitable candidates for screening sodium channel blocking toxins such as PSP (Frietas *et al.*, 1992, 1995; Malpezzi *et al.*, 1993).

Experiments using a leg neuromuscular preparation from the lobster, *Panulirus japonicus*, (Daigo *et al.*, 1989), discussed in **section 2.4.3**, have highlighted the particular sensitivity of such systems for the detection of low levels of PSP toxins. The isolated abdominal superficial flexor (SF) nerve-muscle preparation from the Norway lobster *Nephrops norvegicus* (L.), developed in the Neurobiology Laboratory at the University of Glasgow (Neil and Fowler 1990) and modified here, is a simple and robust preparation. The muscle fibres in the SF muscle comprise two subtypes of tonic fibres, which differ in their biochemical and mechanical properties, and also in their innervation (Neil *et al.*, 1993). The medial SF bundle contains slower-contracting S2 fibres, while the lateral bundle contains mainly S1 fibres which have faster kinetics (Galler and Neil, 1994).

Because of its established properties, and since it retains reliable postsynaptic responses for several hours, the lobster SF preparation was chosen as the system in which to explore further the suitability of a crustacean neuromuscular system for the bioassay of PSP, using epsp size as the primary measure of the effect. Preliminary studies on a crab nerve preparation were also performed.

3.2. METHODS

Adult Norway lobsters (*Nephrops norvegicus,* (L.)) and shore crabs *(Carcinus maenas,* (L.)) were obtained from the Universities Marine Biological Station, Millport, Isle of Cumbrae. Each species was maintained in a communal aquarium tank supplied with circulating seawater.

3.2.1. The lobster neuromuscular preparation

3.2.1.i Dissection of the superficial flexor muscle and its nerve supply

The lobster preparation consists of a superficial flexor (SF) muscle and associated motor nerve supply. The SF muscles form thin sheets along the ventral surface of each abdominal segment, flanking the ventral nerve cord (VNC) and overlaid by the deep flexor muscles. (Figure 3.1.) The motor supply to the SF muscle is carried by a superficial branch of the third ganglionic root (R3), which comprises 5 excitatory axons and 1 inhibitory axon (Prosser 1973; Knox and Neil 1991) of varying diameter. Each SF muscle is divided into distinct medial and lateral bundles which originate on the soft inter-segmental membrane. The medial bundle consists of approximately 30 fibres, which lie as a single layer. The lateral bundle partly overlies the medial bundle and contains about 60 fibres, forming a layer 3-4 fibres thick.

Dissection of the SF muscle and R3 entailed removal of the overlying deep flexor muscles, exercising care not to damage the VNC and ganglionic root branches to the muscle. The VNC was cut approximately 5mm to either side of the central ganglion supplying the motor root, retaining intact the nerve supply to the selected muscle and providing enough cord for anchorage points. The selected muscle was excised intact at its point of insertion, together with small pieces of associated cuticle and membrane. In addition a small square of muscle tissue with its corresponding R3 nerve attached was dissected from the SF muscle opposite, for anchorage.

The SF muscle was pinned by the cuticle, ventral side down onto a Sylgard-lined dish, ensuring the muscle fibres were taut but not stretched. The nerve tissue was pinned out with minimal tension applied, in order not to destroy the synaptic

endings. The cuticle underlying the lateral and medial bundles was removed where possible to improve toxin access.

The dish was mounted onto a metal stage cooled by a Peltier heat pump, and the preparation was perfused at 5 ml min^{-1} by a Gilson Miniplus-3 peristaltic pump with the following *Nephrops* saline (in mM): NaCl 478.9, KCl 12.74, $CaCl_2$ 13.69, $MgSO_4$ 20.47, Na_2SO_4 3.9, HEPES 5.0, buffered to pH 7.45.

3.2.1.ii Extracellular recording and stimulation

A thin platinum-wire electrode was positioned against the R3 nerve by inserting it into the Sylgard base, and at this point the nerve and electrode were electrically isolated from the surrounding saline pool by encapsulating both with a layer of Vaseline. A second platinum-wire electrode inserted into the saline pool was used as a reference electrode. This electrode arrangement recorded spontaneous action potentials in R3 if it was still attached to the central ganglion. Each axon produced an action potential of characteristic amplitude, so that even if all 6 axons were active it was possible to distinguish which axon produced a particular spike (Figure 3.2.). This activity was a useful monitor of the viability of the preparation, and also gave a direct indication of the effect of toxin on the transmission of individual axons (**section 3.3.2.i**).

These electrodes were also used to stimulate the motor nerve, by connecting them to a Digitimer Isolated DS2 stimulator (Digitimer Ltd. UK). The nerve was stimulated at 20 Hz for 1 sec, at a voltage sufficient to recruit all 5 excitatory axons to produce a maximal epsp response in the muscle fibre.

3.2.1.iii Intracellular recordings from muscle fibres

Glass microelectrodes were pulled from capillary glass type GC150 (Clark Electromedical Instruments) on a horizontal, single-stage Industrial Sci. Assoc. Inc. MI puller. Tip resistances measured between 20 - 40 MΩ when filled with 3M KCl. Microelectrodes were inserted into muscle fibres of the medial and lateral bundles of the SF muscle using a micromanipulator (Narashige M4), and recorded the trains of

summating and facilitating epsp's evoked in response to electrical stimulation of the motor nerve, R3.

The stimulating voltage was determined by observing the successive recruitment of individual axons induced by electrical stimulation, using a series of pulse trains (20Hz, 1s) incrementing in steps of 0.5 V per stimulation, from 0 V until no further increase in the evoked signal was observed. This was accepted as being a maximal response. The voltage required to elicit the maximal response was used throughout the experiment. Intracellular responses were recorded on a FM tape (Racal [Store 4]), or plotted on-line using a digital storage oscilloscope (Gould 1640), or digitised using a Cambridge Electronic Design 1401 A/D converter.

3.2.1.iv Experimental series

STX or TTX solutions were superfused over the experimental preparation at concentrations ranging from 10^{-12} M to 10^{-6} M. In addition, a series of experiments using salines with low calcium or variable sodium concentrations was performed.

Each application of toxin or other test solution was followed by a washout period with normal saline (Daigo *et al.*, 1989) of approximately 30 - 40 min. This time did not always lead to a full recovery, which can require a period of 2-3 h (confirmed by Prof. Kawai, *per.s comm.*). In order to overcome this effect, toxin solutions were always applied in a series of increasing concentrations.

3.2.2. The crab nerve preparation

A walking leg was isolated from an adult shore crab, *Carcinus maenas*, by induced autotomy. The proximal leg segments were detached at the meropodite-carpopodite joint by cutting the extensor and flexor muscle tendons, and by breaking its lateral articulations. Using gentle traction, the main leg nerve emerging from the distal leg segments was then exposed (Malpezzi *et al.*, 1993). The preparation was mounted in a rectangular chamber, with the propodite segment fixed in wax. The nerve was drawn across three or more narrow chambers through grooves in the dividing walls, which were then sealed with Vaseline. The outer chambers were filled

with crab physiological saline (in mM: NaCl 470.4, KCL 8.0, $CaCl.2H_2O$ 18.0, $MgCl_2.6H_2O$ 31.5, NaHCO3 6.0, glucose 5.6), and the centre chamber was filled with the various toxin solutions.

Two forms of mechanical stimulation were applied. In one experimental series, controlled drops of saline were released from a height of 10 cm above the fixed dactyl, to produce a standard stimulation of cuticular mechanoreceptors. In a second experimental series, the dactyl was attached to the arm of a galvanometric motor (Laser Lines Inc.) which was driven by a signal generator (Strathkelvin Instruments) to produce sinusoidal or trapezoidal movements about the P-D joint with an angular range of up to 60°, thus stimulating the joint proprioceptors (PD chordotonal organ).

In each case the resulting discharges in the sensory nerves were recorded extracellularly either by a suction electrode attached to the proximal end of the nerve in the final chamber, or by bipolar silver wire electrodes placed in adjacent chambers proximal to the toxin chamber (and in some cases also in two chambers distal to the toxin chamber). The signals were passed to a differential preamplifier (Isleworth 101A) and were displayed on an digital oscilloscope (Gould 400) and in some cases also passed through an A/D converter (CED 1401, Cambridge Electronic Design) for processing by custom software (CED SPIKE2 or Dempster WCP).

3.3. RESULTS

3.3.1. The *Nephrops* neuromuscular preparation

When the motor nerve (R3) remained attached to a small section of the ventral nerve cord containing ganglia, spontaneous activity, involving a variable number of the 6 motor axons, was frequently recorded prior to stimulation (Figure 3.2.a). In conjunction with these extracellular recordings of nerve activity, the presence of intracellularly-recorded epsps in muscle fibres confirmed that the synaptic connections between the motor axons of R3 and the SF muscle were intact (Figure 3.2.b). The medial and lateral muscle bundles produced their own distinctive patterns of epsp facilitation.

3.3.1.i Effects of STX and TTX on muscle epsps

The standard measure of the effect of toxin was the final level of depolarisation of the facilitated muscle fibre epsps in response to a train of stimuli applied to the motor nerve. A degree of variation was observed in this measure, even for a given fibre type under standard conditions (range = 5.8 - 44.1 mV; mean = 14.75 mV ± 8.96 s.e.m., n=22). For this reason, the effects of toxin on epsp size are expressed as the % reduction from the control value. Figures 3.3. and 3.4. show representative results for a series of TTX and STX applications respectively.

In an initial series of experiments with a limited nanomolar range of STX concentrations (15-90 x 10^{-12} M), at least 78% of the responses showed a reduction in epsp amplitude, although little or no proportionality existed between the toxin concentration and epsp size (Figure 3.5.a). Only above 60 nM did the response show a reduction that was persistently below the control value, and indeed in some cases an increase in epsp amplitude was actually recorded following application of STX. These initial results nevertheless indicated that the lobster nerve-muscle preparation can be a very sensitive monitor of Na^+ channel blocking activity.

In another experimental series over a wider range of concentrations (10^{-12} to 10^{-6}M) of both STX and TTX, inhibitory effects were observed as shown in Figure 3.5.b and c. Representative data from several individual TTX and STX experiments are shown in Figures 3.6, 3.7 and 3.8 in the form of both percentage reductions from the original control level (left column) and reductions in each toxin solution from the immediately previous control level (right column). Either no or only a slight reduction in epsp size occurred between 10^{-12}M and 10^{-8}M, but a consistent finding was that at a concentration between 10^{-7}M and 10^{-6}M there was a total loss of response, which most often occurred in a catastrophic manner (Figures 3.6 - 3.8).

Where both medial and lateral muscle fibre bundles were recorded simultaneously in the same preparation (Figure 3.7) a very similar response to the toxin was observed in the two fibre types, and in each a complete inhibition of epsps occurred at the same concentration. These results are not incompatible with the

suppressive effect being presynaptic, as the two bundles receive many common inputs from the pool of motor neurones.

Recovery times of the epsps after exposure to either TTX or STX concentrations between 10^{-12} and 10^{-7} M, were variable. At the lower concentrations of STX (< 10^{-10} M), complete recovery or even a slight increase in epsp size above pre-exposure values was seen within 15 min. However, recovery from STX concentrations of 10^{-8} M or greater was never complete. From a total of 5 preparations, 4 made no recovery following the complete abolition of epsps, while one attained full recovery after a wash period of 45 min.

Following exposure to TTX a greater variation of results was obtained. In some preparations TTX did take longer to wash off. At concentrations between 10^{-12} and 10^{-10} M, results from 9 preparations showed that 4 had recovered by 30 min, whilst 5 had regained only around 75% of normal activity by 30 min. This was double the time period required with STX. At the higher concentrations (10^{-9} to 10^{-7} M), 6 out of 8 preparations showed around a 50% recovery on epsp size by 20-30 min, and the other two preparations failed to recover.

Another effect which was observed in 5 of the experiments was a hyperpolarisation of the membrane potential, recorded as a negative shift in the baseline, following complete block of the epsps by STX (not shown).

3.3.1.ii Effect of reduced sodium and zero calcium on muscle epsps.

The following results were obtained from a set of experiments using lowered concentrations of Na^+ in the perfusate, to reduce the Na^+ conductance (and thus simulate STX or TTX block of neuronal Na^+ channels), and from another set of experiments using Ca^{2+}-free saline, to reduce the Ca^+ conductance across the sarcolemma of a muscle fibre.

Reduced concentrations of Na^+ resulted in a reduction in the post-synaptic epsp (Figure 3.9.2.a, left column). However, these effects were not proportionate, since even with only 10% of the control level of Na^+ in the perfusate, stimulation of the R3 nerve still elicited an epsp of almost 70 % of the control size (Figure 3.9.a,

right column). Curiously, even when Na^+ ions were completely removed (i.e., 0%) a post-synaptic response was still elicited after stimulation. The basis for this persistent activity was not determined.

The experiments in which Ca^{2+} was substituted by HEPES resulted in a progressive, then total inhibition of the evoked epsps within 20 min (Figure 3.9.b). This is consistent with the muscle depolarisation being Ca^{2+}-dependent (Aidley 1989), although this was not tested directly here. The inhibitory effect of the Ca^{2+}-free solution was readily reversed, with an almost complete recovery of the muscle epsps within 30 min after perfusion with normal saline (Figure 3.9.b). A feature of interest under Ca^{2+}-free saline was the occurrence of a hyperpolarisation (Figure 3.9.b), similar to that observed during complete STX blockage.

3.3.2. Nerve preparations

3.3.2.i Lobster motor nerve spontaneous activity

During the course of some of the above experiments, opportunity was taken to monitor the changes in firing frequency and action potential amplitude in the R3 motor nerve produced by STX application. In 8 experiments, exposure to STX for 20 min resulted in a reduction in the average rate of spontaneous activity, ranging from approximately 25% at low toxin (TTX or STX) concentrations (< 40 nM), to almost 90% at toxin concentrations between 10^{-8} M and 10^{-6} M. There were also two other effects: a progressive disappearance of action potentials from the recording, which initially involved the largest axons and later included the smaller axons, and also a suggestion in some cases that there was a reduction in the recorded spike amplitude before this disappearance. Figure 3.10 shows all these effects in a preparation exposed to TTX at 10^{-7} M for 20 min.

The interpretation of such results is not straightforward, since the nerve activity was spontaneously generated from the neuronal network in the attached ganglion. The observed effects could therefore have been due to either a loss of synaptic drive, a loss of the ability of axons to sustain action potentials, or to a combination of the two. To distinguish between these possibilities it is necessary to

use a simplified preparation in which only nerve conduction processes are involved, but not synaptic transmission. Such a system is represented by the crab leg nerve preparation, which can be exposed to toxin while sensory nerve activity is induced by natural stimuli. A preliminary study was therefore made of the potential of this system for monitoring toxin effects.

3.3.2.ii Crab nerve mechanosensory responses

Extracellular recordings were made from the exposed nerve of a crab leg while standardised mechanosensory stimuli (water drops) were applied to the dactyl. The recordings comprised the simultaneous discharge of many primary sensory fibres from various cuticular mechanoreceptors (control in Figure 3.11.a). The extracellular potentials varied considerably in amplitude and in timing, reflecting a mixed population of sensory axons with different diameters and hence conduction velocities. Superfusion of STX at various concentrations (10^{-8} M and above) over the exposed portion of the nerve resulted in a dose-dependent loss of the sensory response, which manifested itself in the progressive disappearance of the larger axons from the burst (Figure 3.11.a). Estimated as a reduction in the size of integrated burst, the STX inhibition after 2 min was 15-20% under 100nM, and 85% under 300nM.

The potential of such a system for detecting PSP was further demonstrated by applying a number of the MAFF shellfish extracts to the exposed nerve. One of these, S58, had a STX eq /100g wet tissue of 104 μg (*ca.* 500 ng/ml, which is close to the AOAC cut-off level) but a bath toxicity of 26 ng/ml. It produced a complete block of the sensory response after 3 min exposure (Figure 3.11.b). The most potent sample, S70, which had a STX eq /100g wet tissue of 565 μg, but a bath toxicity of 143 ng/ml produced an extremely rapid block of the sensory discharge, which was nevertheless reversible (Figure 3.11.c).

3.3.2.iii Crab nerve proprioceptive responses

In order to study the effect of toxins on identifiable axons, advantage was taken of the fact that crab joint proprioceptors are innervated by relatively few axons,

and that a given joint movement recruits only a small proportion of these (Bush and Laverack, 1982). Therefore using the same preparation as above, controlled flexion and extension movements were imposed at the P-D joint using a motorised arm. These stimuli elicited characteristic multi-unit patterns of sensory discharge in which individual axons could be recognised (Figure 3.12.a).

In some cases bipolar recordings were made in normal saline both peripheral and central to the test chamber, providing a further control for any blocking effects (Figure 3.12.b). Using this arrangement, it was found that the application of STX (in this case at 10^{-6} M and above) produced blocking that involved first the largest action potentials, and then progressively the smaller ones (Figure 3.12.b). A very similar effect was also found under reduced Na^{+} saline (not illustrated). A significant finding in all these cases was that the different units disappeared abruptly from the recordings, without any prior reduction in their amplitude, and that during the wash cycle they reappeared again abruptly at full height.

3.4. DISCUSSION

3.4.1. Sensitivity of the lobster SF neuromuscular preparation

The results obtained from the *Nephrops* SF neuromuscular preparation are consistent with previous studies on leg nerve-muscle preparations in lobsters and crayfish (Ogura and Mori 1963; Kawai and Niwa 1980; Daigo *et al.*,1989), and with studies performed using crab nerve-muscles or ganglia (Frietas *et al.*, 1992; Malpezzi *et al.*, 1993) in demonstrating the high sensitivity of crustacean neuromuscular systems to the guanidinium toxins, TTX and STX, and their ability to withstand several wash/toxin/wash cycles.

However, the *Nephrops* SF neuromuscular preparation does not appear to be quite as sensitive as the walking leg preparation as described by Daigo *et al.* (1989). When tested with prepared PSP samples, designated STX-, GTX-, or PX-rich, blocking in the leg preparation occurred from 10^{-13} M to about 10^{-9} M (less than 1 MU) depending on the type of sample. Studies on crustacean axons by Ozeki *et al.* (1966) and Ogura and Mori (1963) found that toxin concentrations required to block

action potentials ranged between 3 x 10^{-12} M and 3 x 10^{-8} M. Ougura and Mori (1963) found that crystalline TTX at 3 x 10^{-8} M caused between 90-100% block of any contractile response of the opener muscle of the isolated crayfish claw, with 3 x 10^{-10} M producing 10-20% inihibition. In this study using the *Nephrops* SF neuromuscular system both STX and TTX produced complete block of neuronal sodium channels at the higher concentration of 10^{-7} M (although the initial inhibitory responses to STX of around 20% occurred at concentrations at 1.5 to 3x10^{-9} M). Nevertheless, the nerve-evoked response of the *Nephrops* SF system is at least an order of magnitude more sensitive than that of the standard mouse bioassay, the detection limit of which is 200 ng/ml (40 μg/100 g tissue).

The results obtained for the recovery of the *Nephrops* nerve-evoked muscle epsps from toxin application agree with previous studies on lobster nerves (Kao 1966; Narahashi *et al.*, 1967; Narahashi 1989) in showing that the the half-time for recovery from STX is about half that for TTX, and that reversibity after exposure to high concentrations of both toxins is incomplete (Evans 1972). These reversibilty results fit with with Na^+ channel, receptor binding times at 20°C of 37s for STX, and 70s for TTX (Hille 1992). In contrast, Dettbarn *et al* (1965) found STX to be rather irreversible in its actions on eel electroplaques.

3.4.2. Hyperpolarisation effects

Hyperpolarisations of the muscle membrane were observed both on STX application, and under zero Ca^{2+}. The first effect was similar to transient hyperpolarisations described by Evans (1972) in both frog and crab nerve preparations exposed to both STX and TTX before blocking conduction. Hyperpolarisation of membrane potential has also been described in a variety of nerve and muscle preparations (Dettbarn *et al.*, 1965; Keynes *et al.*, 1971). It has been suggested that hyperpolarisations between 2 and 5 mV in lobster and squid axons (Freeman 1971; Narahashi 1974) result from a reduction in inward leakage of Na ions, due to TTX and STX inhibiting part of the leakage current (Evans 1972). The transient increase in epsp size observed here on return to normal saline may have a similar explanation in

terms of an increase in sodium conductance, since Hille (1968) reports that g_{Na} may be 10-15% higher on recovery from exposure to low concentrations of STX.

Under zero Ca^{2+} no effect would be expected on the firing of the motor axons, either excitatory or inhibitory. However a possible explanation for the hyperpolarisation observed under these conditions is that the effect of ipsps, being Cl^- -dependent rather than Ca^{2+}-dependent, is unmasked in the absence of epsps. It is unlikely that this effect contributed to the hyperpolarisation seen under STX blockage since the inhibitory nerve in the SF neuromuscular system, f5, is one of the largest axons, and according to the findings of the nerve blockage experiments, would be expected to be one of the first to be blocked by toxin application.

3.4.3. The effects of reduced Na^+

The fact that evoked epsps were observed in the Na^+ -free solution might be attributed to insufficient equilibration time, or to the existence of a Na^+ store close to the axon membrane. A similar persistence of epsp's has been reported in crayfish abdominal muscle fibres after total substitution of Na^+ with sucrose (Takeda 1967) and in a lobster walking leg motor axon after substitution of Ca^{2+} with TRIS (Kawai and Niwa 1980). In these cases it has been proposed that the action potentials have a Ca^+ component as well as a Na^+ current. This possibility was not investigated here, but such components may represent TTX- or STX-resistant elements. Some neuroblastoma cells that depend on Ca^{2+} rather than Na^+ for the generation of action potentials are more resistant to the actions of TTX or STX, and these properties are described in **section7.1.1.**

3.4.4 Evaluation of the crustacean preparations as STX bioassays

The dose-response relationship between STX concentration and epsp size in the SF muscle is shallow below 10^{-7} M, but very steep above this concentration (Figure 3.5). Considered alone, these results do not allow us to identify the underlying responses of the motor nerves to STX, but there are the two main possibilities: 1) the the action potentials in the activated axons become reduced in amplitude, or 2) there

is a progressive transmission failure in the individual axons of the motor neurone pool. These two possibilities are not mutually exclusive, but the extent to which the first may lead to the second, and the possibility that this underlies the transition in the dose-response curve from a shallow to a steep relationship are not known.

Of relevance to such considerations is the degree to which a 'safety factor' exists in the overshoot of the action potential, keeping it above the threshold for regenerative Na^+ influx. Intracellular recordings of action potentials in both squid and crayfish axons under conditions of reduced Na^+ conductance, produced by either a low external Na^+ (Hodgkin and Katz, 1949) or TTX application (Sasner *et al.*, 1984), have shown that action potentials fail when the overshoot reduces to a value close to the reversal potential (0 mV).

Similar factors are relevant to the interpretation of the results obtained in the two crab nerve experiments (Figures 3.11 and 3.12), in which cases the action potentials were recorded beyond the toxin chamber in a normal saline solution. These action potentials might be expected to have regained their full size, since at this point the membrane sodium channels are not blocked by toxin, and the axons are bathed in normal saline. The results obtained confirm this expectation (Figure 3.12): spikes recorded beyond the toxin chamber were either present at their full size, or were absent (but were never of a reduced size). Moreover, this was true both for their disappearance during the toxin application and for their re-appearance during the subsequent wash.

Summary

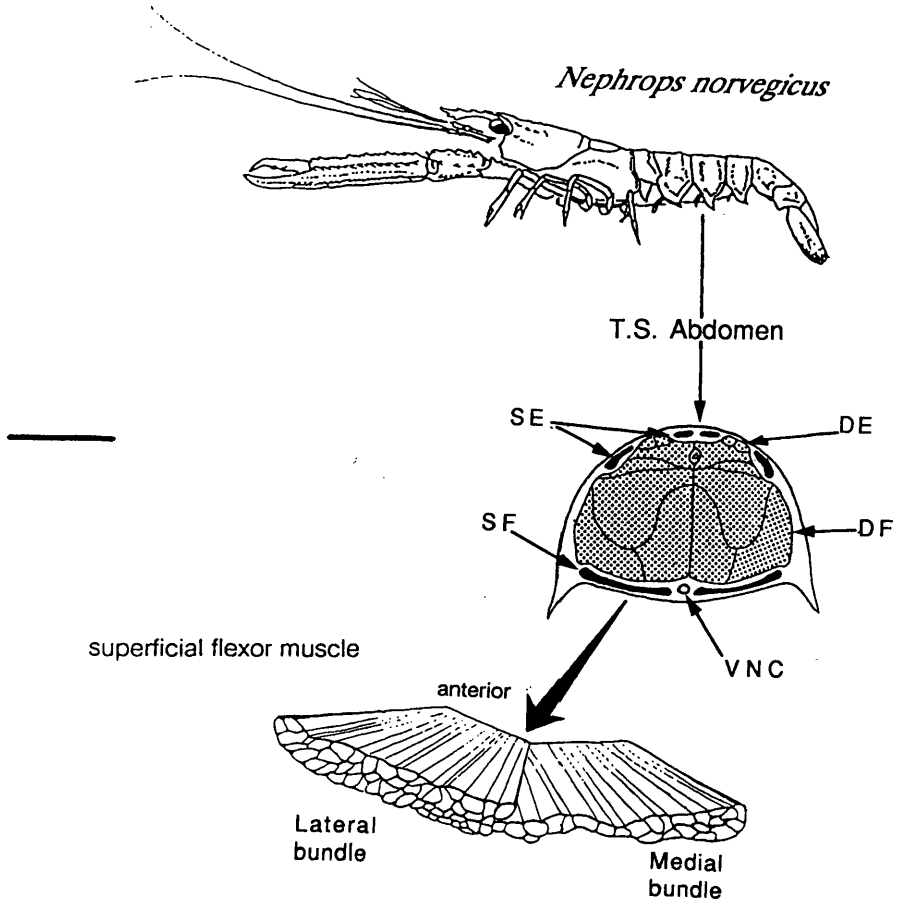
The main conclusion drawn from the experiments performed here is that the *Nephrops* neuromuscular system is convenient to use and relatively high sensitivity, but that the results obtained are difficult to interpret due to the involvement of neuronal, synaptic and muscle membrane properties. This system thus provides no significant advantage over using a nerve preparation, in which the action potentials can be recorded directly. On the other hand, the crab nerve preparations as used in **section 3.3.2.iii** provide a clear indication of the transitions in individual axons from

propagating to non-propagating action potentials under STX, and the size-dependecy of this effect. This could potentially be used as a basis for a bioassay, particularly if electrical stimulation rather than natural stimulation is used in order to activate all the axons in a nerve in a synchronous manner, and a nerve is chosen which contains a large number of axons, so that the progressive drop-out of their action potentials contributes to the changing amplitude of the externally recorded compound action potential. This is the approach which is described in Chapter 5.

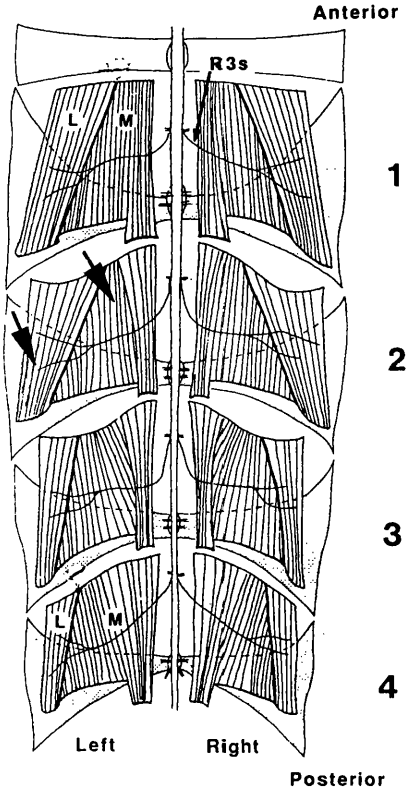
Figure 3.1.

A: Morphology of the abdominal section of the lobster (*Nephrops norvegicus*) showing the ventral arrangement of the superficial flexor (SF) muscles used in the assay preparation. The fibre arrangement of the lateral and medial bundles is shown at the bottom. (SE, superficial extensor muscles; DE, deep extensor muscles; SE, superficial extensor muscles; DF, deep flexor muscles; VNC, ventral nerve cord). Scale bar: top 30 mm; middle, 10 mm; bottom 1.3 mm.

B: Dorsal view of the dissected abdomen showing the arrangement of the SF muscles in segments 1-4. (L, lateral bundle; M, medial bundle; R3s, third superficial root. Arrowheads indicate the positioning of the intracellular recording electrodes). A single muscle with its associated R3 and small section of VNC either side is completely excised for the assay. Scale bar 6.2 mm.


Nephrops norvegicus
T.S. Abdomen
SE
DE
SF
DF
VNC
superficial flexor muscle
anterior
Lateral bundle
Medial bundle

A


Anterior
R3s
L
M
1
2
3
L
M
4
Left
Right
Posterior

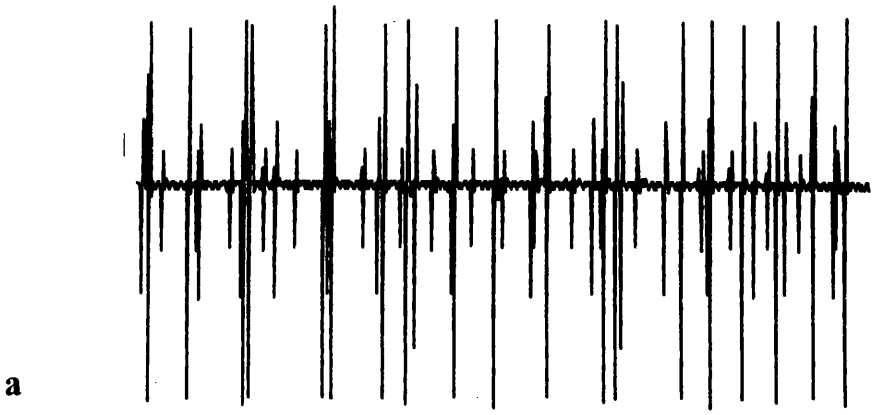
B

Figure 3.2.

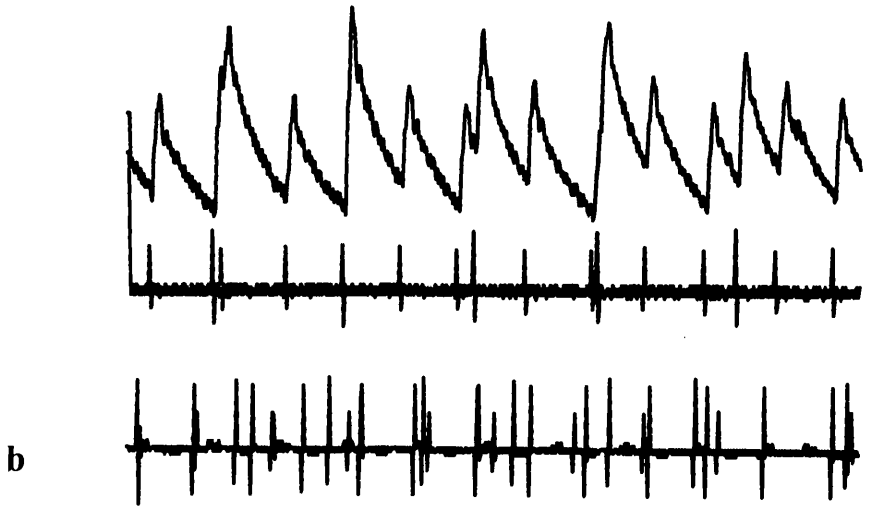
a: Extracellular spontaneous action potentials recorded from R3. In this figure all five excitatory (f-1,2,3,4,6) and the inhibitory axon (f5, the second largest spike) can be identified. F1 is the smallest spike and appears extremely close to f2, with f6 being the largest recorded spike.

b: The top trace is a recording of intracellular muscle potentials (epsp's) elicited by the firing of the two excitatory axons in the middle trace that are active at their own particular frequency here. Where two axons fire very close together, a small summation of muscle epsp's can be seen in the top trace as larger epsp's. Near the middle of the top trace are two epsp's that have not quite summated probably because the axons (middle trace) have not fired close enough. The bottom trace is a recording from an adjoining R3 innervated from the same ganglia, showing a larger number of spontaneously active axons to its partner.

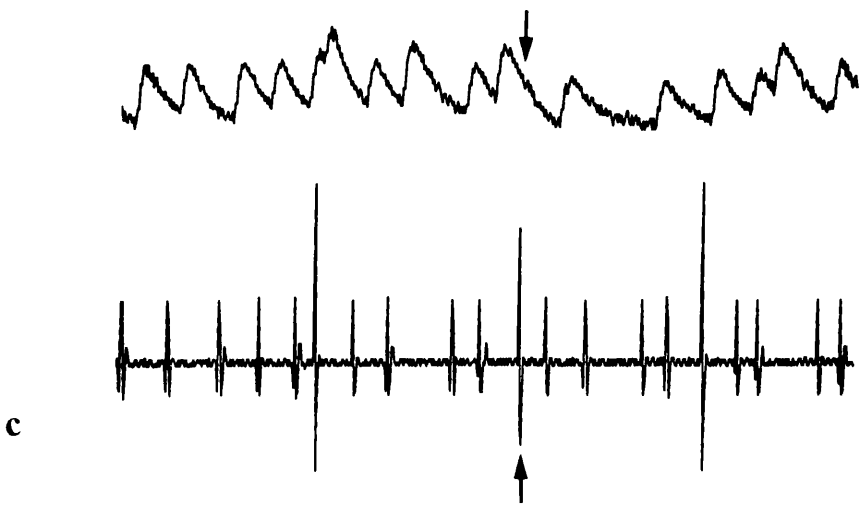
c: In these two traces the inhibitory action of f5 is shown, identified with an arrow (lower trace), and a consequential drop in the epsp baseline, shown by an arrow in the upper trace.



a



b



c

Figure 3.3.

Representative set of results for a series of TTX applications ranging from 10^{-10} M to 10^{-6}M. The traces showing epsp reduction (right column) were recorded at 15 min into TTX perfusion. Washout traces were recorded at 30 min. In this experiment recordings were obtained from both lateral (L) and medial (M) fibres in the same muscle bundle. At 10^{-9}M the response in the lateral fibre was completely inhibited and did not recover during washout. At 10^{-6}M the response in the medial fibre was also completely blocked with no recovery occurring in either fibre. The lateral fibre epsp remained completely inhibited and is therefore not shown

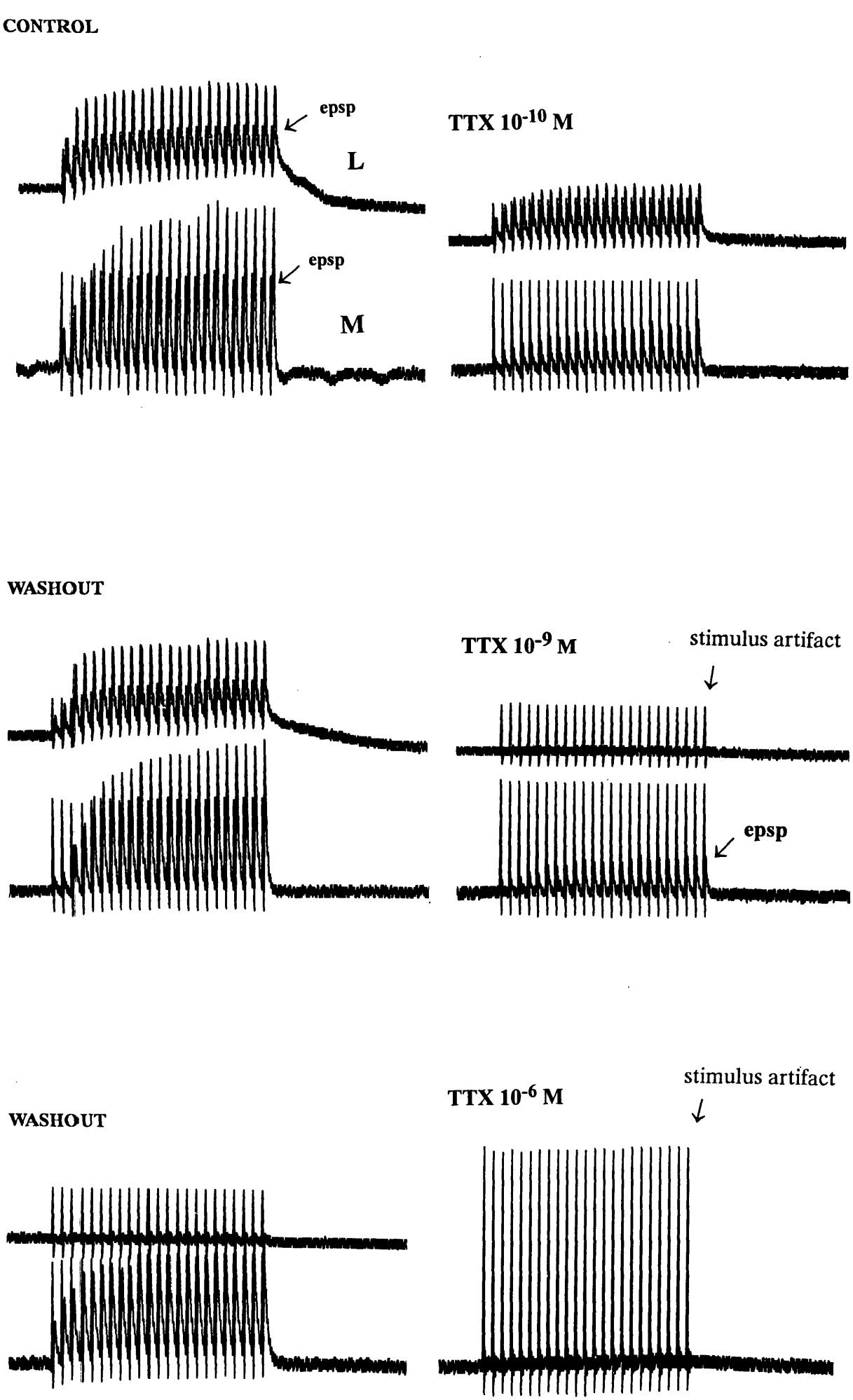

CONTROL
epsp
L
epsp
M
TTX 10-10 M
WASHOUT
TTX 10-9 M
stimulus artifact
epsp
WASHOUT
TTX 10-6 M
stimulus artifact

Figure 3.4.

Representative set of results recorded from a lateral muscle fibre for a series STX applications (30 nM to 80 nM). Traces were recorded at 10 min post STX application and after a 25 min washout period.

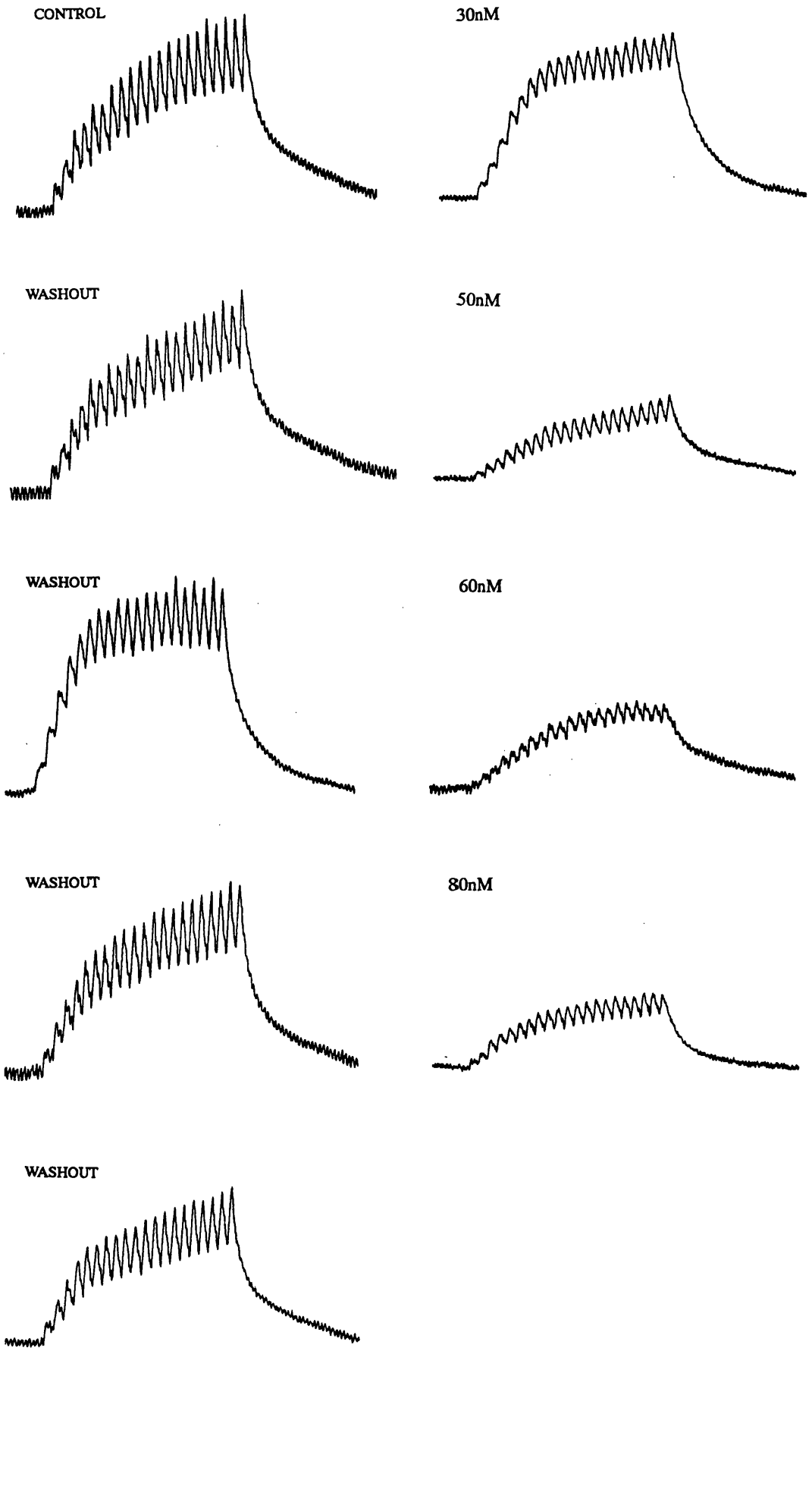
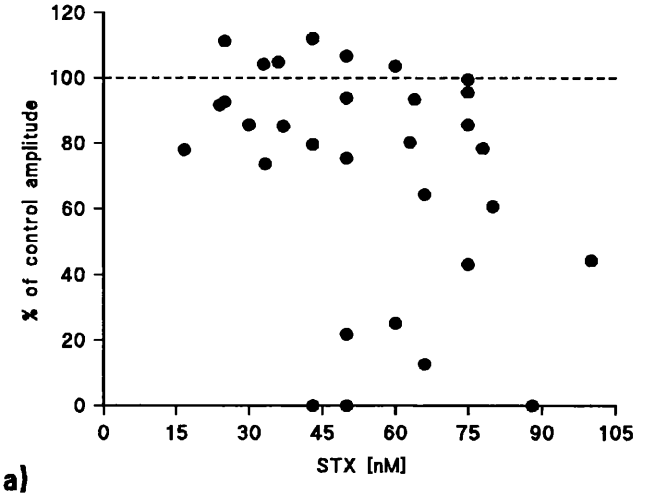

CONTROL
30nM
WASHOUT
50nM
WASHOUT
60nM
WASHOUT
80nM
WASHOUT

Figure 3.5.

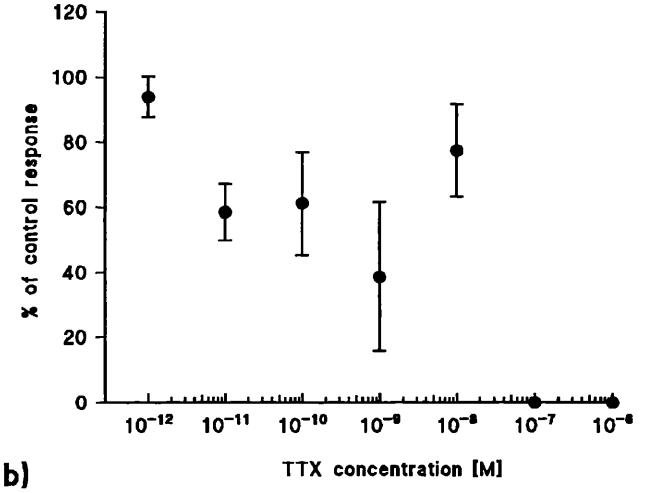
a) Change in epsp amplitude after exposure to STX for 20 min. The series of experiments were performed within one order of magnitude in the nanomolar range (*n* = 8 experiments).

b) Change in epsp amplitude as a percentage of the initial control level after exposure to several series of TTX concentrations at a 15 min time period. Each data point represents the mean ± s.e.m. of 3-6 experiments.

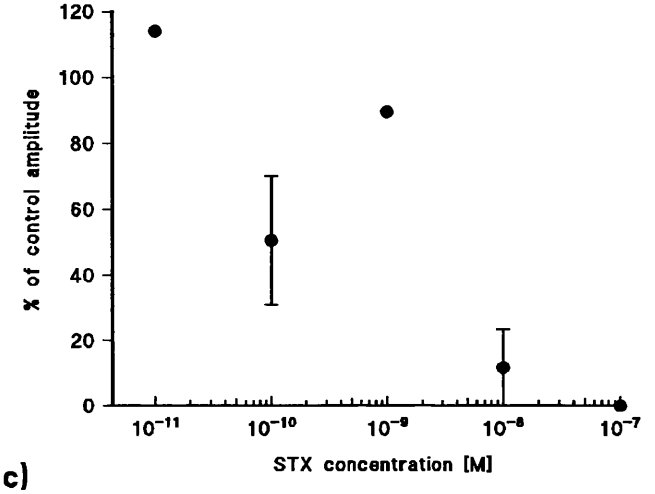
c) The percentage change in epsp amplitude from initial control value following application of STX (10^{-11}M to 10^{-7}M) after 10 min. Each data point represents the mean ± s.e.m. of 2-5 experiments.


120
100
80
60
40
20
0
% of control amplitude
0
15
30
45
60
75
90
105
STX [nM]

a)


120
100
80
60
40
20
0
% of control response
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
TTX concentration [M]

b)


120
100
80
60
40
20
0
% of control amplitude
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
STX concentration [M]

c)

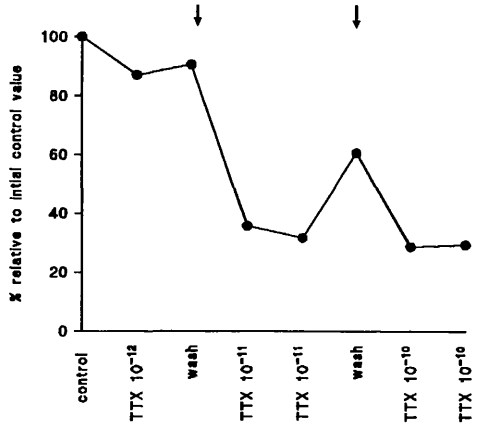
Figure 3.6.

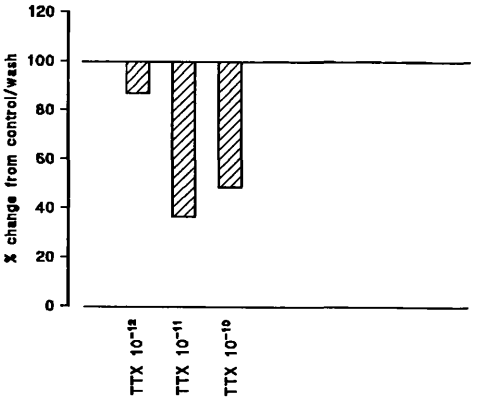
Representative individual experiments showing the percentage change in epsp amplitude from the control value after exposure to a range of TTX concentrations.

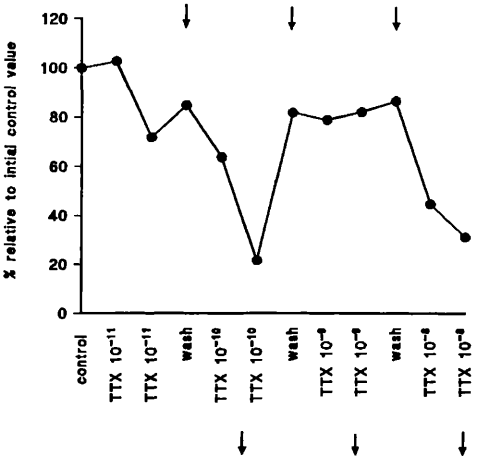
Data from four individual experiments highlight the variable response between preparations to the same TTX concentrations. Complete inhibition was always observed at 10^{-7}M or above. A large increase in epsp amplitude following application of TTX is shown in the third graph (left column), smaller increases are shown in the bottom graph.

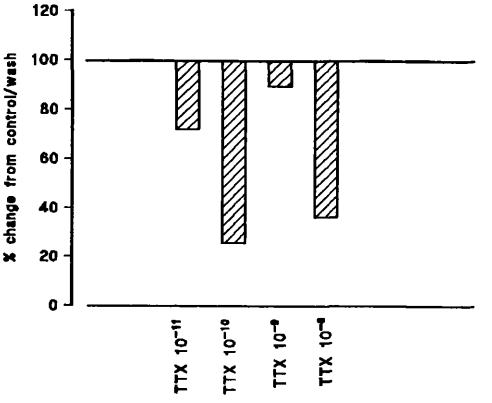
The graphs in left column show the epsp amplitude in relation to the previous amplitude value, either after every 10 min following exposure to TTX, or after a wash period of 30 min.

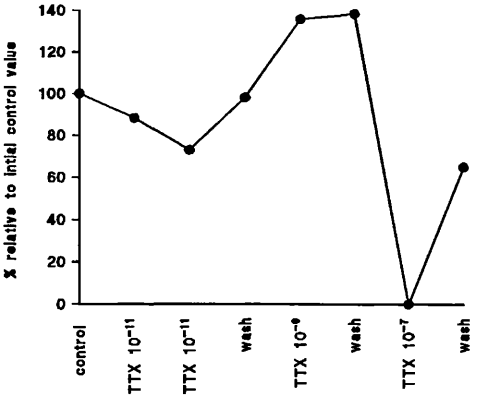
The graphs in the right column show the ultimate percentage of epsp inhibition for each TTX concentration from the control value.

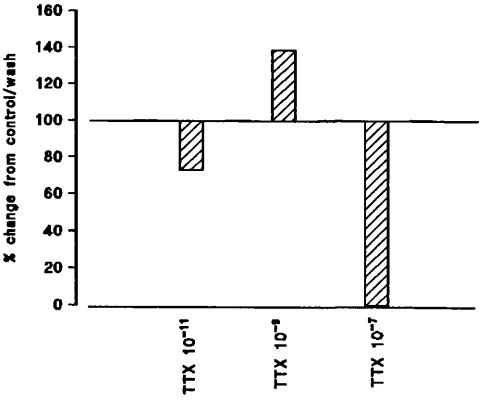

% relative to intial control value
control
TTX 10⁻¹²
wash
TTX 10⁻¹¹
TTX 10⁻¹¹
wash
TTX 10⁻¹⁰
TTX 10⁻¹⁰

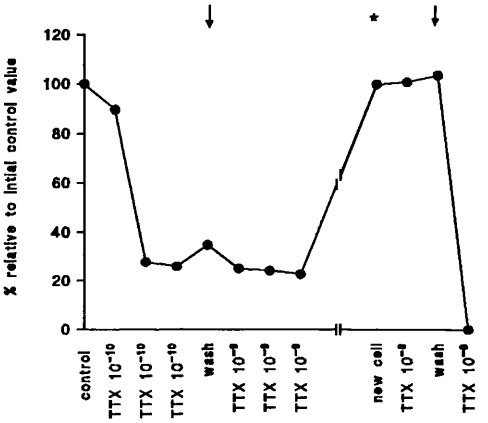

% change from control/wash
TTX 10⁻¹²
TTX 10⁻¹¹
TTX 10⁻¹⁰


% relative to intial control value
control
TTX 10⁻¹¹
TTX 10⁻¹¹
wash
TTX 10⁻¹⁰
TTX 10⁻¹⁰
wash
TTX 10⁻⁹
TTX 10⁻⁹
wash
TTX 10⁻⁸
TTX 10⁻⁸


% change from control/wash
TTX 10⁻¹¹
TTX 10⁻¹⁰
TTX 10⁻⁹
TTX 10⁻⁸


% relative to intial control value
control
TTX 10⁻¹¹
TTX 10⁻¹¹
wash
TTX 10⁻⁹
wash
TTX 10⁻⁷
wash


% change from control/wash
TTX 10⁻¹¹
TTX 10⁻⁹
TTX 10⁻⁷


% relative to intial control value
control
TTX 10⁻¹⁰
TTX 10⁻¹⁰
TTX 10⁻¹⁰
wash
TTX 10⁻⁹
TTX 10⁻⁹
TTX 10⁻⁹
new cell
TTX 10⁻⁸
wash
TTX 10⁻⁸

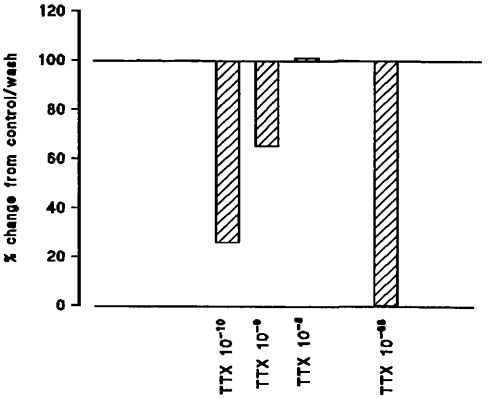
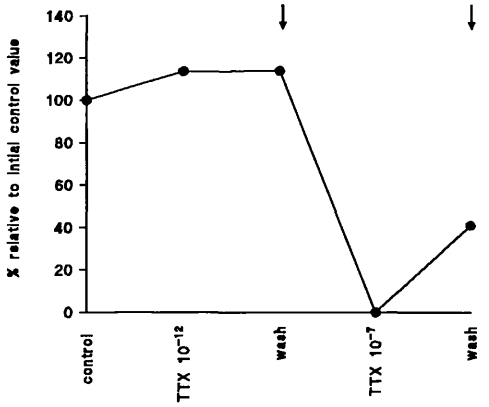

% change from control/wash
TTX 10⁻¹⁰
TTX 10⁻⁹
TTX 10⁻⁸
TTX 10⁻⁴⁸

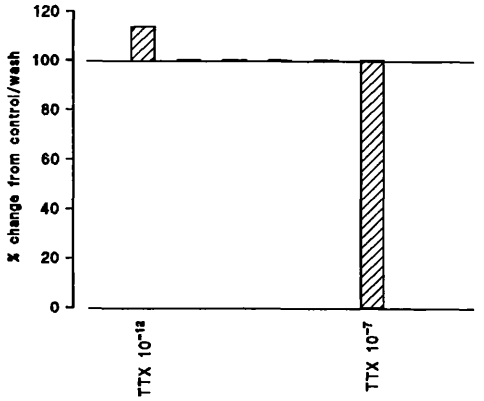
Figure 3.7.

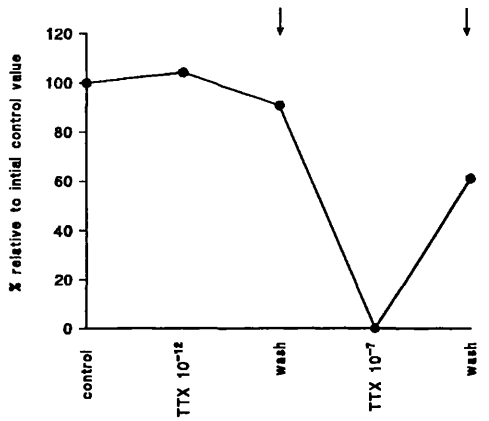
Comparison of the effect of TTX concentrations between medial and lateral fibres in the same preparation show a similar trend. The top two graphs are of the lateral (top) and medial (second) responses in one lobster. The lower two graphs are again lateral and medial epsp amplitudes from another preparation. Complete inhibition is evident at TTX concentrations above 10^{-7}M. Small increases in epsp amplitude are shown in the top two graphs (TTX 10^{-12} M).

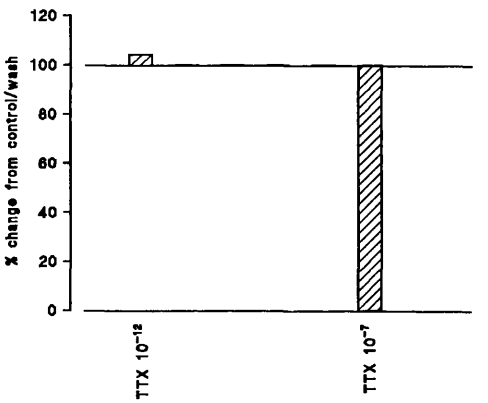
The graphs in left column show the epsp amplitude in relation to the previous amplitude value, either after every 10 min following exposure to TTX, or after a wash period of 30 min.

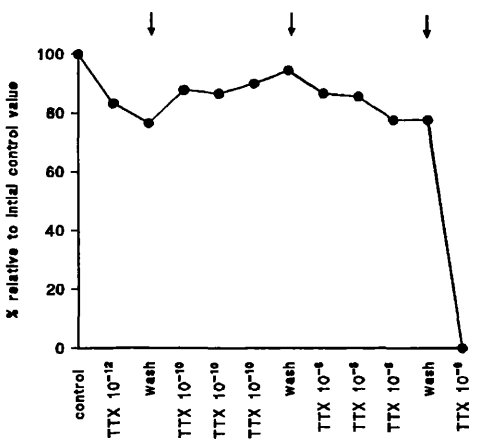
The graphs in the right column show the ultimate percentage of epsp inhibition for each TTX concentration from the control value.

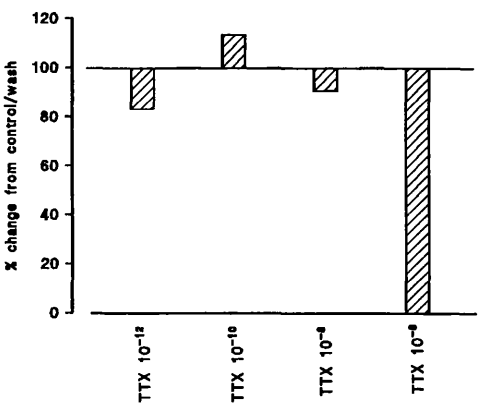

% relative to intial control value
control
TTX 10⁻¹²
wash
TTX 10⁻⁷
wash

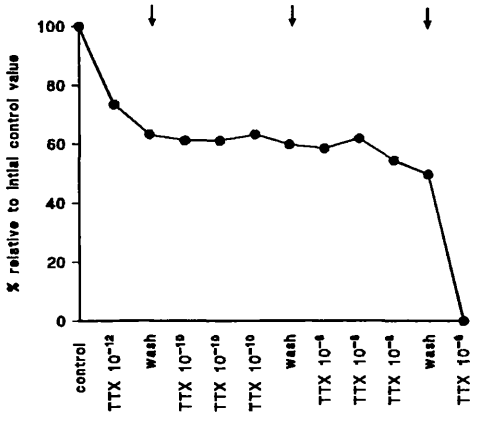

% change from control/wash
TTX 10⁻¹²
TTX 10⁻⁷


% relative to intial control value
control
TTX 10⁻¹²
wash
TTX 10⁻⁷
wash


% change from control/wash
TTX 10⁻¹²
TTX 10⁻⁷


% relative to intial control value
control
TTX 10⁻¹²
wash
TTX 10⁻¹⁰
TTX 10⁻¹⁰
TTX 10⁻¹⁰
wash
TTX 10⁻⁸
TTX 10⁻⁸
TTX 10⁻⁸
wash
TTX 10⁻⁸


% change from control/wash
TTX 10⁻¹²
TTX 10⁻¹⁰
TTX 10⁻⁸
TTX 10⁻⁸


% relative to intial control value
control
TTX 10⁻¹²
wash
TTX 10⁻¹⁰
TTX 10⁻¹⁰
TTX 10⁻¹⁰
wash
TTX 10⁻⁸
TTX 10⁻⁸
TTX 10⁻⁸
wash
TTX 10⁻⁸

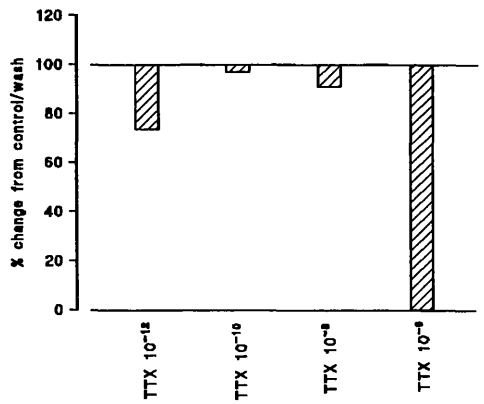
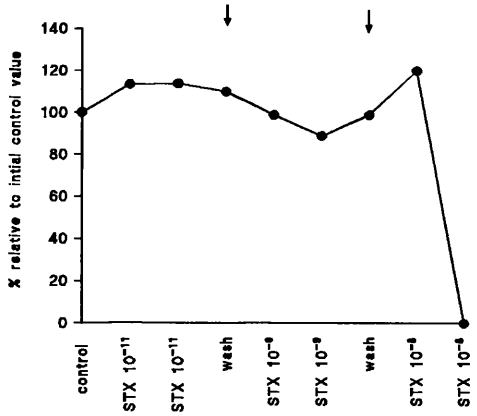

% change from control/wash
TTX 10⁻¹²
TTX 10⁻¹⁰
TTX 10⁻⁸
TTX 10⁻⁸

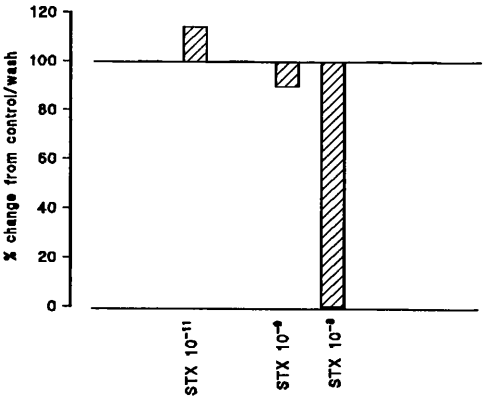
Figure 3.8.

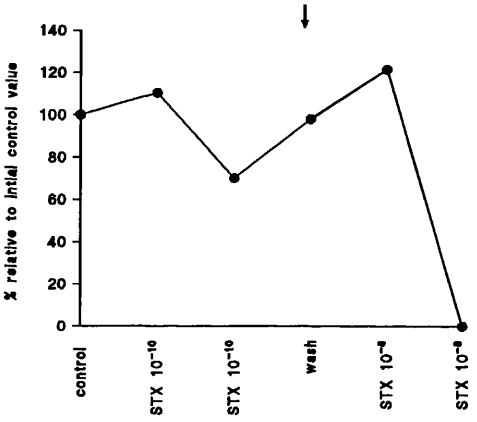
Individual experimental data from some of the STX series show that STX appears to have an consistent inhibitory effect at 10^{-8}M. A variable response similar to TTX is observed at concentrations lower than 10^{-8}M.

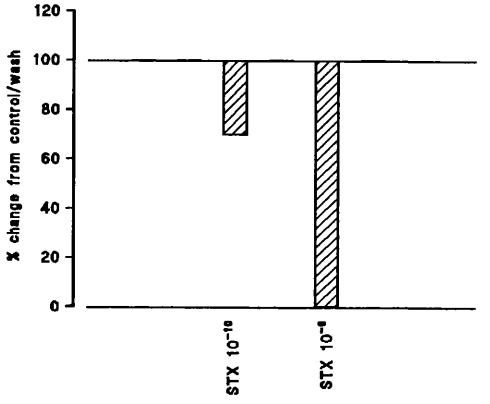
The graphs in left column show the epsp amplitude in relation to the previous amplitude value, either after every 10 min following exposure to STX, or after a wash period of 30 min.

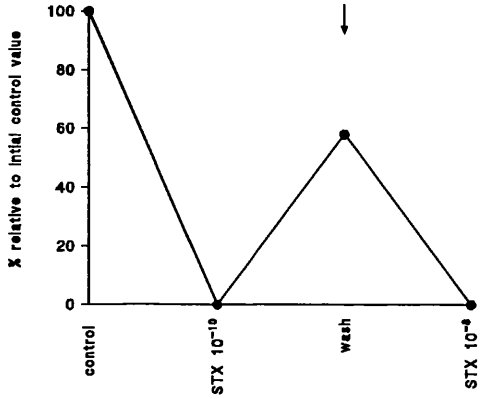
The graphs in the right column show the ultimate percentage of epsp inhibition for each STX concentration from the control value.

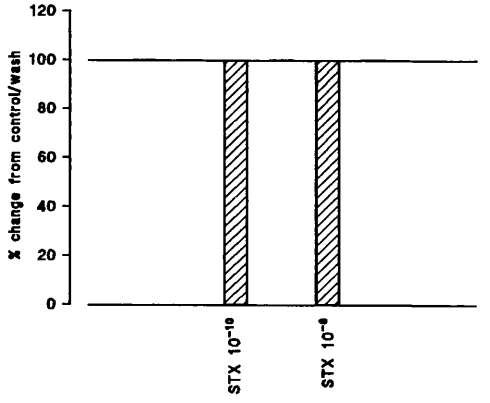

% relative to intial control value
140
120
100
80
60
40
20
0
control
STX 10⁻¹¹
STX 10⁻¹¹
wash
STX 10⁻⁹
STX 10⁻⁹
wash
STX 10⁻⁸
STX 10⁻⁸

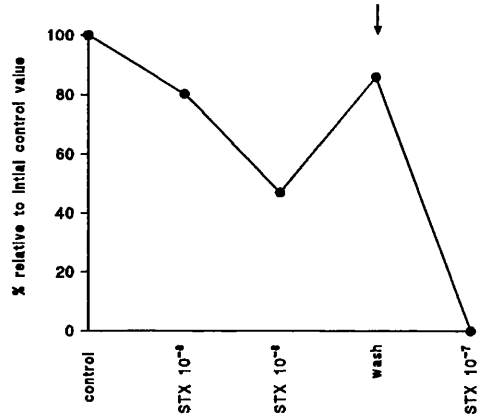

% change from control/wash
120
100
80
60
40
20
0
STX 10⁻¹¹
STX 10⁻⁹
STX 10⁻⁸


% relative to intial control value
140
120
100
80
60
40
20
0
control
STX 10⁻¹⁰
STX 10⁻¹⁰
wash
STX 10⁻⁸
STX 10⁻⁸


% change from control/wash
120
100
80
60
40
20
0
STX 10⁻¹⁰
STX 10⁻⁸


% relative to intial control value
100
80
60
40
20
0
control
STX 10⁻¹⁰
wash
STX 10⁻⁸


% change from control/wash
120
100
80
60
40
20
0
STX 10⁻¹⁰
STX 10⁻⁸


% relative to intial control value
100
80
60
40
20
0
control
STX 10⁻⁹
STX 10⁻⁹
wash
STX 10⁻⁷

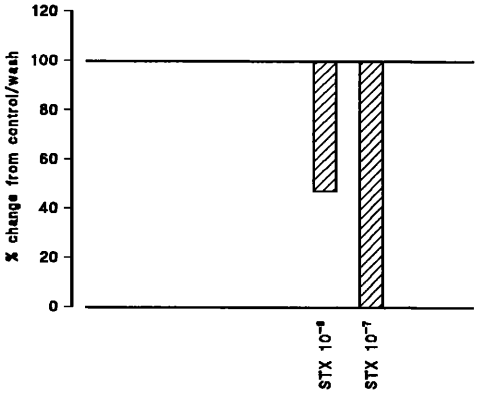
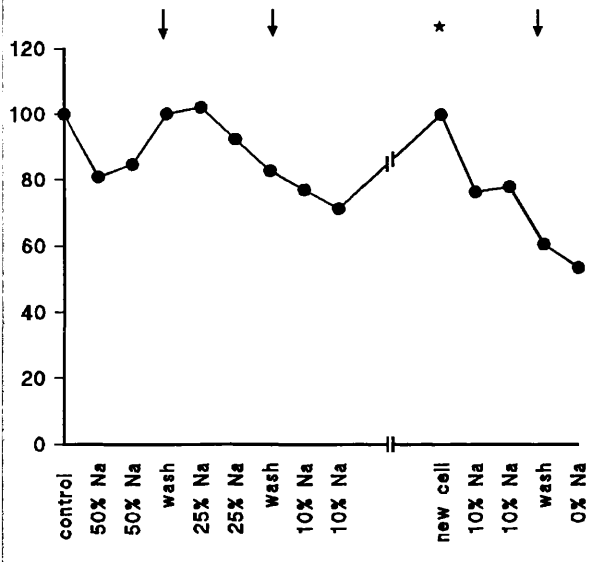
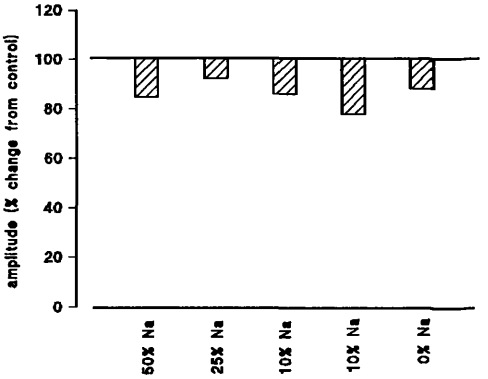

% change from control/wash
120
100
80
60
40
20
0
STX 10⁻⁹
STX 10⁻⁷

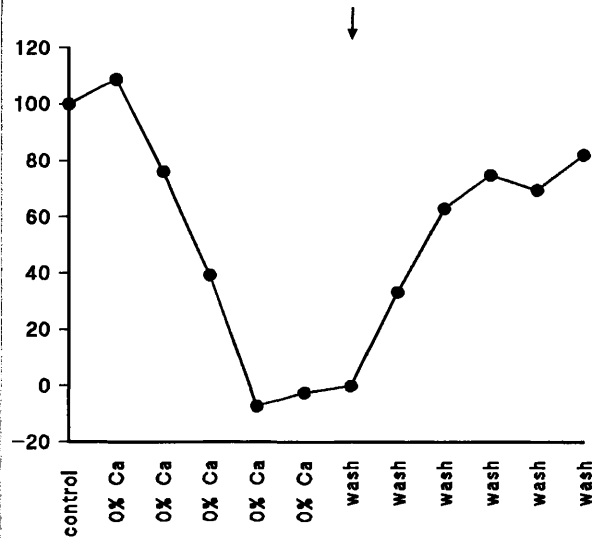
Figure 3.9. Reduced sodium and zero calcium solutions on epsp amplitude.

a) Percentage reduction in epsp amplitude from the original control level after a exposure to a series of reduced Na^+ concentrations in the external perfusate, each followed by a wash period (left column). Each date point for Na^+ represents a 5 min time period. The data points for the washes represent a 10 min time span. Reduction from the immediately previous control level, i.e., following a wash period, is shown in the right column.

b) Percentage reduction and recovery in post synaptic epsp amplitude from an initial control level after exposure to a perfusate with zero Ca^{2+} (left column). Each data point represents a 5 min time period. Percentage change after 10 and 20 min from the initial control value is shown in the right column.


120
100
80
60
40
20
0
control
50% Na
50% Na
wash
25% Na
25% Na
wash
10% Na
10% Na
new cell
10% Na
10% Na
wash
0% Na


amplitude (% change from control)
120
100
80
60
40
20
0
50% Na
25% Na
10% Na
10% Na
0% Na


120
100
80
60
40
20
0
-20
control
0% Ca
0% Ca
0% Ca
0% Ca
0% Ca
wash
wash
wash
wash
wash
wash

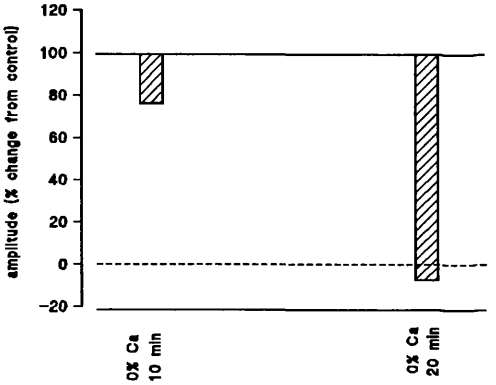

amplitude (% change from control)
120
100
80
60
40
20
0
-20
0% Ca
10 min
0% Ca
20 min

Figure 3.10. Effect of TTX 10^{-7} M on spontaneous action potentials

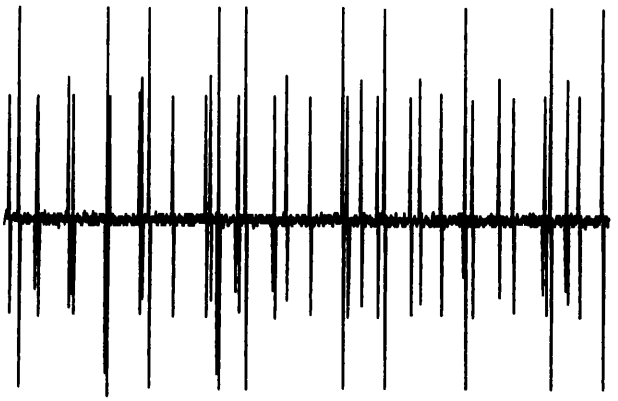
a) Spontaneous action potentials recorded extracellularly from R3 prior to toxin application. At least four axons are firing.

b) Activity after 10 min under 10^{-7} M TTX.

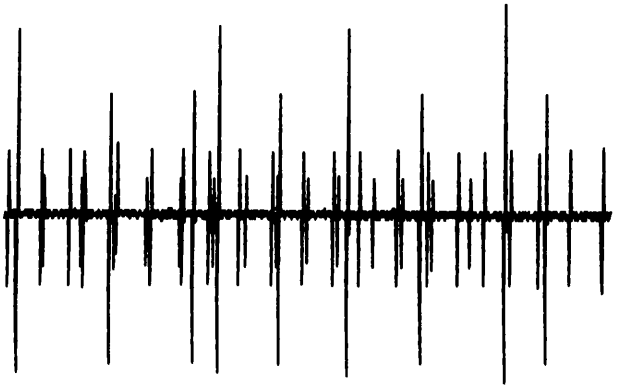
c) Activity after 15 min.

d) Activity after 20 min.

Note the progressive drop-out of the larger axons, and the reduced amplitude of the middle-sized spikes.



a



b

c

d

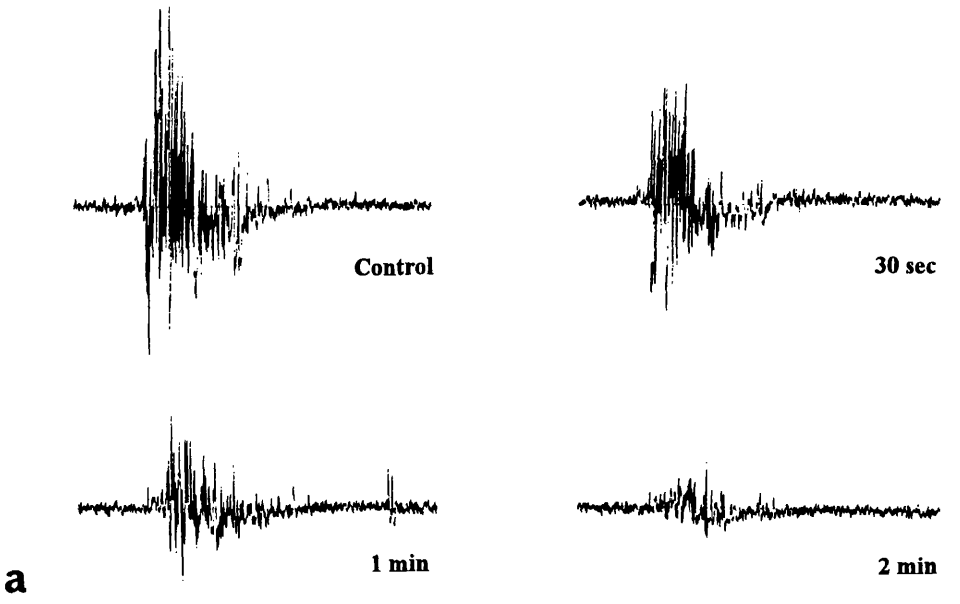
Figure 3.11. Crab nerve mechanoreceptor responses.

a) Extracellularly recorded action potentials of crab sensory axons exposed to 300 nM STX.

b) Extracellularly recorded action potentials of crab sensory axons exposed to PSP sample S58, containing 104 μg STX eq.

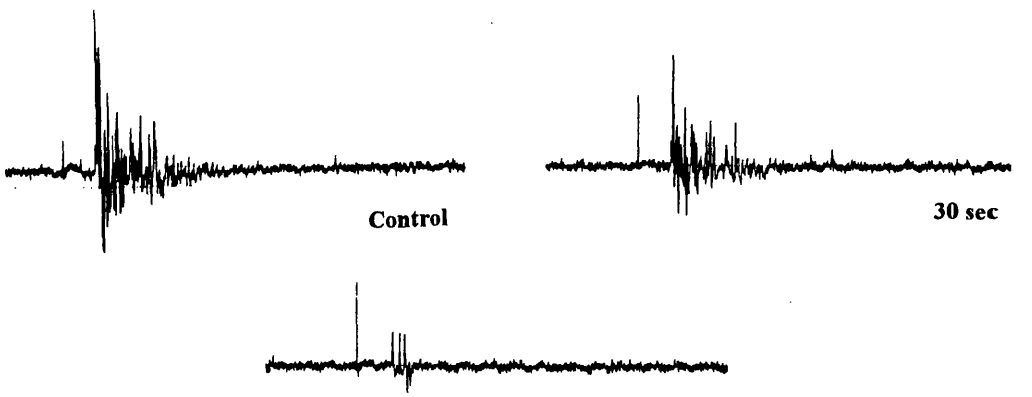
c) Extracellularly recorded action potentials of crab sensory axons exposed to PSP sample S70, containing 565 μg STX eq.

Time scale bars are shown for each set of results.



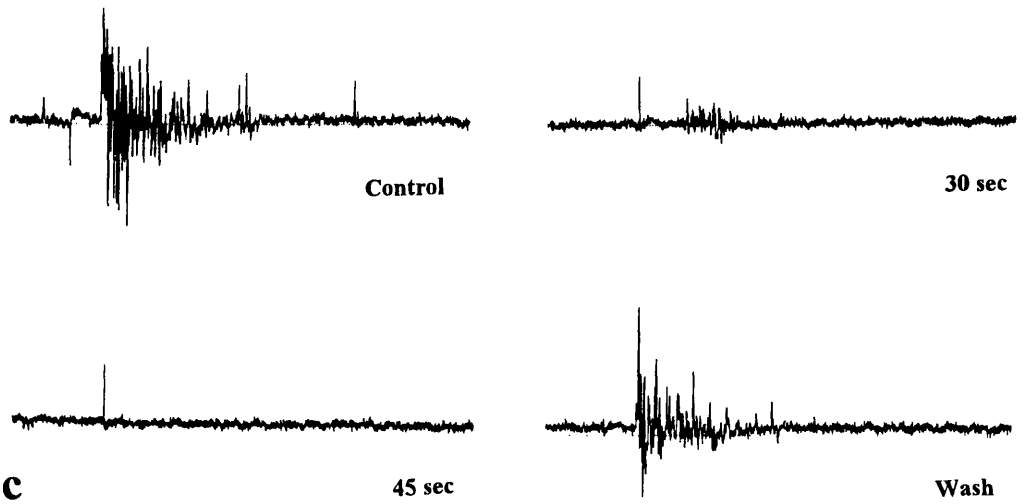
a

51 ms



b

34 ms



c

34 ms

Figure 3.12. Crab nerve proprioreceptor responses.

Multi-unit extracellular potentials recorded from a crab leg nerve using bipolar electrodes, in response to extension (upward on stimulus trace) and flexion (downward on stimulus trace) of the P-D joint through an angular range of 60^{o}. Time scale: 4 s.

a) Control in normal saline, showing distinct extension- and flexion-sensitive units.

b) Simultaneous recordings taken from a peripheral section of nerve (upper traces) and a central section of nerve (lower traces) relative to the test chamber. Left panel: normal saline in the test chamber; right panel: 10^{-6} M STX in the test chamber. The lower traces indicate the effect of the solution in the test chamber.


a)
b)

Chapter 4

Avian Nerve-Muscle Preparation as a Screening Assay for PSP Samples

4.1. INTRODUCTION

PSP intoxication is characterised by clinical symptoms of neuromuscular depression. Death through paralysis of the respiratory muscles is the final clinical action of severe shellfish poisoning. Many early workers mistakenly thought that the paralytic effects of TTX and STX at neuromuscular junctions and synapses were due to a curare-like action, i.e. by binding to the postsynaptic ACh receptors and preventing their activation by acetylcholine (Kandel *et al.*, 1991). However, improvements in electrophysiological techniques made it possible to show that the neuromuscular junction was unaffected by doses of TTX that abolished muscle action potentials (Furukawa *et al.*, 1959; Cheymol *et al.*, 1968, and Katz and Miledi 1967).

High concentrations of TTX or STX have little or no effect on the sensitivity of the end-plate to ACh, and when ACh is released from motor terminals by stimulation, excitatory end plate potentials (epp's) and miniature excitatory end plate potentials (mepp's) can still be recorded (Katz and Miledi 1967, Nishiyama 1967, Narahashi 1974). However, the release of ACh is reduced by these toxins, as their blocking action on voltage-gated Na^+ channels reduces the amplitude of the action potential invading the terminals (Evans 1972). Therefore the paralytic effects of TTX and STX can be ascribed to their action on nerve, and possibly also some muscle membrane Na^+ channels, rather than to effects on the cholinergic receptors of the motor-end plate, or receptor channels at this site.

It has been observed, however, that there is a difference in the behaviour of epp's after exposure to these two toxins. TTX causes an abrupt disappearance of the epp's, whereas STX brings about a graded fall of epp amplitude (Kao and Nishima 1965). The difference may be related to the fact that TTX readily causes axonal block of motor nerves, by binding to Na^+ channels at the nodes of Ranvier; STX may act predominantly at the terminals, reducing impulse amplitude more progressively (Evans 1972, Bowman 1990).

4.1.1. Nerve-muscle systems - phasic and tonic innervation

In multiply-innervated muscle fibres such as the frog rectus abdominis muscle, or the biventor cervicis muscle in domestic fowl, ACh-sensitive regions are dispersed over the whole membrane. This fibre type receives dense innervation from many nerve endings, and the local depolarisations produced by the release of ACh are sufficient to activate the whole fibre, without the need for a propagating action potential. These fibre types do not possess voltage-gated Na^+ channels and their contraction response is not inhibited by STX. These fibre types are termed tonic, and a contraction produced in the manner described is often called a contracture (Ginsborg and Warriner 1960; Bowman 1990).

Many vertebrate skeletal muscle fibres are, however innervated at a focal point on their membrane, usually about midway between the origin and insertion points of the muscle fibre. These focally-innervated fibres receive receive fast conducting Aα type axons, are termed phasic and have a rapid contraction known as a twitch .

The use of both tonic and phasic nerve-muscle preparations to evaluate the effects of numerous venoms and toxins, whose main targets are excitable cells has been extensive: on the cat tibialis anterior muscle (Kao and Nishima 1965); the isolated phrenic nerve-hemidiaphragm of the rat (El-Asmar *et al.*, 1977); the triangularis sterni muscle preparation of the mouse (Brigant and Mallart, 1982; Prior *et al.*, 1993); the intercostal muscle and attached nerve from mouse (Konishi 1985); the chick biventor cervicis nerve-muscle preparation (Ginsborg and Warriner 1960; Watt and Simard 1984; Harvey *et al.*, 1993).

4.1.2. Chick biventor nerve-muscle system

The chick biventor cervicis (CBC) preparation described in detail by Ginsborg and Warriner (1960) comprises the chick biventor cervicis muscle and its associated nerve supply, encapsulated by an attached tendon. The muscle contains both fast twitch, focally innervated fibres and slow tonic, mutiply innervated fibres, making it a useful system to examine both a twitch response (elicited by nerve stimulation) and a

tonic contracture (elicited by the application of compounds such as acetylcholine, carbachol or KCl). As at other vertebrate neuromuscular junctions, neither TTX nor STX prevents acetylcholine or its analogue carbamycholine from evoking a depolarising response (Dettbarn *et al.*, 1965). Thus both pre- and post-synaptic effects of drugs or toxins can be examined in the same preparation. This provides a potentially convenient assay system, since the chicks are inexpensive to buy and maintain, and the preparation is simple to set up. In addition this preparation has already been shown to be TTX sensitive (Marshall *et al.*, 1979)

4.1.3. Compounds employed for the chick biventor preparation

ACh is the natural agonist at nicotinic synapses. It is a hydrolyzable ester of acetic acid with choline. Synthesis of ACh is enhanced by nerve stimulation. Depolarisation of the nerve terminals evokes ACh release if there is sufficient Ca in the external medium. ACh binds to the nicotinic receptors, which lie clustered on the muscle surface membrane in the endplate region, immediately opposite active zones of the pre-synaptic terminal, producing depolarisation of the postjunctional membrane. The exogenous application of ACh will act in a corresponding manner to nerve-evoked release by causing a slow contracture (not twitch) but without nerve stimulation.

Carbachol (carbamycholine) is a synthetic agonist which is not hydrolysed by acetylcholinesterase. This agonist is still able to bind to the nicotinic receptors even if ACh is inhibited by presynaptic blockage of the action potential by STX or TTX. As with ACh, exogenously applied carbachol will cause a contracture in the absence of nerve stimulation due to the widespread chemosensitivity of the muscle fibre membranes to such depolarising substances.

The post-synaptic action of KCl is not a receptor-agonist response as with ACh and carbachol. The application of KCl to the bathing medium causes an increase in $[K^+]_o$ creating a partial depolarisation of the endplate, which leads to the opening of voltage-gated Na^+ channels in the surrounding area. Sodium ions enter the

membrane down their concentration gradient generating a spike potential which initiates the processes of excitation-contraction coupling and results in the muscle fibres contracting

4.2. MATERIALS AND METHODS

4.2.1. Preparation of the biventor muscles

The paired biventor cervicis muscles and their respective nerve supplies were removed from chicks of between 6 and 9 days old, killed by exposure to CO_2, following the methods of Ginsborg and Warriner (1960) and those described in *Pharmacological Experiments on Isolated Preparations* (University of Edinburgh, 1968). The preparations were mounted, in pairs, in 10 ml organ baths (Figure 4.1) attached to force displacement transducers (Grass FT03), under a resting tension of approximately 10 mN. The preparations were maintained at 34°C in a modified Krebs Henseleit solution (in mM): NaCl 118.4, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgSO_4$ 1.4, KH_2PO_4 1.2, glucose 11.1, bubbled with 5% CO_2 and 95% O_2. Under such conditions the preparations remained viable for several hours.

4.2.2. Indirect muscle stimulation and recording

Twitch responses were evoked by stimulating the motor nerve via a ring electrode (pulses of 0.2 ms duration, 0.1 Hz frequency, and at a voltage sufficient to produce a maximal response, using a Grass S88 B stimulator). Both twitches and contractures recorded using the force transducers were plotted on a Washington, Grass model 7 or 79 polygraph.

In the absence of nerve stimulation, contractures in response to sub-maximal concentrations of exogenously applied acetylcholine (1 mM), carbachol (20-40 μM), and KCl (40 mM) were obtained prior to the addition of toxins and at the end of the experiment. Acetylcholine and KCl were allowed to remain in contact with the nerve for 30 sec and carbachol for 60 sec. After each drug application, the preparations were washed with fresh Krebs solution for 15 sec and left until the effect of the drug had

disappeared (see Figure 4.2, top trace). The preparation was then left to stabilise for approximately 20 min before exposure to a PSP sample, at either 1:1000 or 1:200 parts dilution, toxin to Ringer. These dilution factors were chosen in order to determine the workable range of sensitivity of the preparation. Both dilutions could be applied to the same preparation. If, after the second application of agonists at the end of that experimental run, and following a washout, neither had the agonist response changed, nor had twitch amplitude decreased, then the more concentrated sample was added.

4.2.3. Toxicity calculation of PSP samples

The calculation of STX equivalent toxicity in a shellfish sample is determined by converting the estimated mouse units (MU) to μg/ml by multiplying by a conversion factor (CF) as described in the official AOAC (1984) guidelines. Toxicity is based on 1 MU being the dilution in 1 ml that will kill a 19-21 g mouse in 15 min (see **section 2.4.1**). Thus the equation to convert MU to μg STX eq./100g shellfish tissue is:

μg Poison / 100 g meat = (μg/ml) x diln. factor x 200

where: 200 = the 200 ml final volume of solution containing 100 g of shellfish tissue.

This means the STX eq. content in 1 ml of each sample supplied by CSL, Torry, for these experiments will be 0.5% (1/200th) of the 200 ml solution.

e.g., S22 = 40 μg STX eq./ 100 g tissue ÷ 200 = 0.2 μg STX in 1 ml (≡ 200 ng)

S69 = 80 μg STX eq./ 100 g tissue ÷ 200 = 0.4 μg STX in 1 ml (≡ 400 ng)

S32 = 651.6 μg STX eq./100 g tissue ÷ 200 = 3.26 μg STX in 1 ml

These values are further decreased by the dilution factor (e.g., 1:100, 1:200, 1:1000, etc.) used in each particular assay. Thus 0.4μg STX/ml becomes 4×10^{-3} μg/ml (or 4 ng/ml), 2×10^{-3} μg/ml (or2 ng/ml) and 4×10^{-4} μg/ml (or 400 pg/ml) respectively. However, for clarity, the values expressed throughout the complete study refer to the original sample values based on the calculations made by CSL, Torry for 100 g of shellfish tissue. This allows for clear cross-referencing of samples between the

various assay systems. The implications of the absolute sensitivities of each test are discussed in Chapter 8.

Throughout this study a "negative" sample (or, 0 μg STX eq) refers to a sample where no detectable levels of STX were found, either by HPLC or the mouse bioassay. Samples calculated as containing 80 μg STX eq (by mouse bioassay) are referred to as the AOAC safety level value (section **2.4.1**). Above 200 μg STX eq, a sample is referred to as being of a high value. At this toxicity level, not only bivalve shellfish but also species such as crabs, lobsters and whelks, which are higher in the food chain, are tested for contamination at CSL, Torry.

4.2.2. Statistical analysis of data

Values for twitch amplitude are expressed as a percent of the mean ± SEM. Differences between means were tested at n=4, unless otherwise stated. In a Mann-Whitney U test, a value of $P < 0.05$ (two tailed) was taken as indicating statistical significance.

4.3. RESULTS

The PSP samples used in assays with the chick-biventor cervicis (CBC) preparation are listed in order of increasing toxicity in Table 4.1. Representative traces of a negative sample, an AOAC safety level sample (80 μg STX eq.) and a high STX eq content PSP sample are shown in Figures 4.2 - 4.4 respectively. All STX eq values had been previously confirmed by mouse bioassay and HPLC at CSL, Torry. Each figure shows typical twitch heights and contracture recordings prior to and following exposure to a PSP shellfish sample and the postsynaptic indicators ACh, carbachol and KCl.

4.3.1. Effects of PSP samples on twitch height

The results obtained using the PSP samples are plotted on separate graphs in Figures 4.5 - 4.7 and show the blocking action of each sample at the two chosen

dilutions. At the 1:1000 parts dilution there is little difference in blocking action between those at ≈ 80 μg and those at ≈ 600 μg. Only sample S32, the most toxic, showed a clear, but not quite statistically significant ($p = 0.056$) difference at this level of dilution with an IT_{50} of 25 min with a mean block of 60% at 40 min. With the other two most toxic samples S49 caused a 25% block at 40 min but S70 had almost no effect at this dilution

However, at the 1:200 dilution factor, differences between inhibitory times were distinguishable and are described below.

Samples below the level of PSP detectable by other methods (termed "negative samples") had little or no effect on twitch height over at least a 40 minute period (Figure 4.5). These results resemble very closely those reported for time-matched control experiments performed in normal Krebs, where less than 10 % change in amplitude was recorded over a 3-4 h time period (Faheti 1995).

One of the negative samples tested, S101, had previously provided a positive result by cell culture assay (CSL, Ring Trial Report FD94/161). In two cumulative dose experiments, the final concentration of this sample, 1:67 parts, produced less than an 8% change in twitch height over an 80 minute period (not shown), which was consistent with values obtained from time-matched control experiments where no toxins had been applied to the preparation. Similarly sample S97 had also produced a positive value in the cell bioassay, but had no effect on the CBC preparation. These results from the CBC assay agree with the HPLC and mouse bioassays performed by CSL, Torry.

Two samples (S164 and S138, see Table 4.1) with values of 45.8 μg and 74.5 μg STX eq respectively, were applied to CBC preparations. Neither sample produced a significantly detectable inhibitory activity in relation to their respective control twitch amplitudes. Sample 164, obtained from King scallops, produced an IT_{50} of approximately 30 min. ($p = 0.057$) whilst sample S138, (Razor clam),with an STX eq. content just below the AOAC safety level, showed no greater than a 40% block after 60 min (Figure 4.6).

Samples S69 (80 μg) and S176 (81.6 μg) showed significant twitch blocking action ($p = 0.050$ and 0.028 respectively) but there was a considerable difference between the IT_{50} values for the samples (Table 4.1 and Figure 4.6). Sample S176 from Queen scallops, had a blocking rate approximately twice that of sample S69 from King scallops. However S176, had only a 50% inhibitory effect on one preparation compared to a complete block shown in the other preparations, and was therefore excluded from the results. A third sample (S58, mussel) with a higher value of 104 μg STX, produced only a 30% blocking action (Figure 4.6), which was less than S164 (45.8 μg) which caused about a 60% block.

The three highest value STX eq. samples obtained from CSL, Torry, and tested, were S70, S49 and S32 (Table 4.1). All three produced very rapid and significant blocking action ($p = 0.029$, 0.028, 0.029 respectively). Sample S70 had an IT_{50} of approximately 13 min, whilst both S49 and S32, had IT_{50}s of < 7 min, with complete block of twitch response at approximately 15 min (Figure 4.7). When the mean IT_{50} results for all the samples are plotted against their respective STX eq value (Figure 4.8) a relatively large scatter is seen for those with a lower STX eq content, but those samples with a high STX eq value showed closely similar values of about 6 min between the IT_{50} times. A linear regression fitted to the data yielded $r^2 = 0.638$.

4.3.2. PSP sample responses to ACh, carbachol and KCl

Application of ACh, carbachol and KCl to preparations exposed to PSP samples at the dilution factor of 1:1000, resulted in a general trend for contractures to show a small decrease in amplitude for all three compounds (Figures 4.9 - 4.11). The most obvious exceptions to this overall trend were seen with two negative samples, S97 and S101 where some increases in contracture sizes were recorded (Figure 4.9). Carbachol produced an increase in amplitude with both these negative samples, but there was a low *n* number (n=2) for S101. The other notable change was recorded with S70. Both ACh and carbachol caused a rise in contracture amplitude (Figure 4.11) compared to control values.

The application of ACh, carbachol and KCl to preparations exposed to PSP samples at the dilution factor of 1:200 also resulted in a general trend for contractures to be slightly reduced in amplitude compared to control values. Overall, the size of the decreases for all three compounds were slightly greater than those observed when the samples were diluted at 1:1000 parts. However, samples at 1:1000 parts dilution showed a higher proportion of decreases (10 out of 11 samples tested) than those at 1:200 parts dilution (6 out of 11 samples); the other 5 showing almost no change from control values. Increases in contracture amplitude appeared to be less marked than those observed at the 1:1000 parts dilution. ACh had a minimal effect on contracture size with the three most toxic samples (S70, S49 and S 32, Figure 4.11). Carbachol only appeared to increase/influence contracture size with sample S49. The most variable reactions were measured following KCl application.

4.4. DISCUSSION

The primary objective in these experiments was to record changes in twitch height (a nerve-induced muscle contraction) as an indicator of sensitivity and reliability, not only as a novel and alternative assay method, but also as a preliminary examination of the effects of a range of crude PSP extractions on a nerve-muscle system. To a great extent the system has successfully achieved this objective. The IT_{50} scores in Table 4.1 for the 1:200 dilution series showed that the CBC preparation was sufficiently sensitive to differentiate between toxic and non-toxic samples.

4.4.1. Muscle twitch responses

Results from the twitch experiments for the negative samples showed good agreement with the mouse and HPLC assays. None of the three negative samples (S1, S97 and S101) tested showed a sufficient decrease in amplitude to indicate the presence of STX compounds, in contrast to the results of a cell culture assay (CSL, Ring Trial 1994, Report FD94/161), which indicated that two out of these three samples contained Na^+ channel blocking activity (see **section 2.4.7**).

For those samples which produced an inhibitory response, the reduction in twitch amplitudes is indicative that STX-like compounds are present in these samples. The twitch is a nerve-evoked event and thus dependent on the invasion of an action potential of sufficient amplitude into the nerve terminals to trigger ACh release. A reduction in twitch height reflects the pre-synaptic blocking of Na^+ channels and a corresponding decrease in quanta of ACh released.

The concentrations of STX used in these experiments were very low. Each sample arrived in a volume of approximately 1 ml, which represents about 1/200 of the original sample volume (**section 4.2.3**) and therefore was only 5% of the original STX eq value. Subsequently there was a further dilution of either 1:200 or 1:1000 parts sample to dilutent. The experimental results indicate that the 1:1000 dilution factor was bordering on the lower detection limit of sensitivity of the CBC preparation. For samples with an original STX value of 80 μg, this dilution now represents a value of 400 pg/ml. Thus the results show that the nerve-muscle system was able to detect STX at several orders of magnitude more sensitive than the mouse bioassay, yet was robust enough to measure the most toxic samples, with original STX values of *ca.* 651 μg.

4.4.2. Muscle contracture responses

The results of the contracture responses (Figures 4.9 - 4.11) indicate that there may be changes in post synaptic sensitivity caused by some samples, the most likely reason being the presence of unidentified compounds co-extracted with the STX compounds (Sato *et al.*, 1988; Jellet *et al.*, 1992). It is also possible that the small changes reflect a run-down of the preparation over the time period in which the assay is run. A reduction in both twitch height and contracture amplitude of up to 10 % has been reported for this particular nerve-muscle preparation with a continuous stimulation regime of 0.1 Hz (0.2 ms duration) (Faheti, 1995), although this occurred over a 3-4 h period. Slight changes in tension of the preparation may also be a possible explanation.

In addition to this it must also be noted that there was a short 15 sec wash period with fresh Krebs solution after each agonist application. Whilst there would be no dilution of a PSP sample concentration for the ACh response, a small dilution of the toxin would have occurred prior to application of carbachol, and more so before that of KCl application. As KCl was probably the most pertinent agonist being tested in the context of these experiments examining blockage of voltage gated Na^+ channel activity, KCl should perhaps have been applied first. For future experiments the application of only one agonist per experiment would be a more rigorous protocol.

The mechanism of action of the compounds in the PSP samples, i.e., whether they act pre- or post- synaptically, or both, thus cannot be completely resolved by the initial experiments performed in this study. STX is reported (Evans 1972) not to affect the sensitivity of the end-plate area to ACh and therefore, also carbachol. Thus if ACh is applied exogenously, as here in the presence of a PSP sample, little or no effect on contractures should be observed. The fact that both increases and decreases in amplituded were recorded in these experiments does not conform to previous findings, and implies there could be compounds other than STX present in some of the samples which are affecting the cholinergic receptors of the motor end-plate or receptor channels at this site or even on the muscle membrane.

Brevetoxin another marine algal toxin is reported to produce concentration dependent depolarisation and contraction in canine trachealis smooth muscle (Richards *et al.*, 1990), whilst okadaic acid a major component of the DSP complex is known to cause a long-lasting contraction of isolated smooth muscle and aorta tissue, and heart muscle (Cohen *et al.*, 1990; Kodama *et al.*, 1986; Hescheler *et al.*, 1988; Takai 1988) by inhibiting the activity of myosin protein phosphatases. This particular toxin is found in shellfish in British waters and the possibility exists that trace amounts of DSP may be present in the PSP samples tested here, as has been previously observed in mussel samples from other coastal waters (Martinez and Miguez 1995).

The effects of KCl on contracture height are also difficult to evaluate. As will be shown later (**section 7.3**) patch clamp recordings of ensemble currents in nerve cells show that the K currents are normally so large and powerful that they mask the Ca^{2+} currents. However, if an unidentified compound is present within a sample that could affect the Ca^{2+} channels on the muscle then this may be reflected as a change in the shape or size of the K^{+} currents and an overall modification of the contracture response or amplitude.

Direct muscle stimulation before and after exposure to toxin would be the most reliable method to assess whether the changes in post synaptic events have any pharmacological significance previously missed by earlier studies. Prior to this it would be necessary to rigorously determine whether the decreases in post synaptic results are a run-down effect of ACh release given the preparation has been repeatedly stimulated for at least 3 hours from the start of the experiment.

In addition to direct muscle stimulation, the use of perineural recording techniques to study ion channels at motor nerve terminals would be valuable in assessing the pharmacological activity of compounds which alter neuromuscular transmission (Brigant and Mallart 1982; Konishi 1985; Harvey *et al.*, 1993). Techniques such as this would enhance the presently available knowledge of mixed compound PSP samples.

The variability in responsiveness of some preparations to the same PSP sample. (e.g. S176) which caused complete inhibition in three preparations, yet only a 50% block in a fourth (**section 4.3.1**) can best be explained by the presence of a safety factor margin for the quantity of ACh transmitter produced. The amount of ACh released by a single isolated nerve impulse is usually considerably in excess (estimated to be about 5 fold) of that required to evoke the critical degree of endplate depolarisation needed to cause a muscle contraction (Bowman 1990). Variation in the margin of this safety factor in some preparations, could result in a corresponding degree of difference in sensitivity to STX.

Summary

Overall, the CBC preparation has been found to be sufficiently sensitive to reliably detect PSP samples that contain the STX-equivalent-toxicity which represents the AOAC safety limit of 80 μg STX/100 g shellfish tissue. In addition to this, the use of agonists has highlighted additional postsynaptic sensitivities that could influence the results of this or indeed other assay systems, and should be considered in the development of assays measuring the toxic effects of PSP extracts. Some of the results obtained have therefore been difficult to interpret due to the involvement of neuronal, synaptic and muscle membrane properties. This part of the system thus provides no significant advantage over using either just the muscle twitch responses or a simple nerve preparation, in which the action potentials can be recorded directly. This is the approach which is described in Chapter 5, where an isolated section of frog sciatic nerve is used.

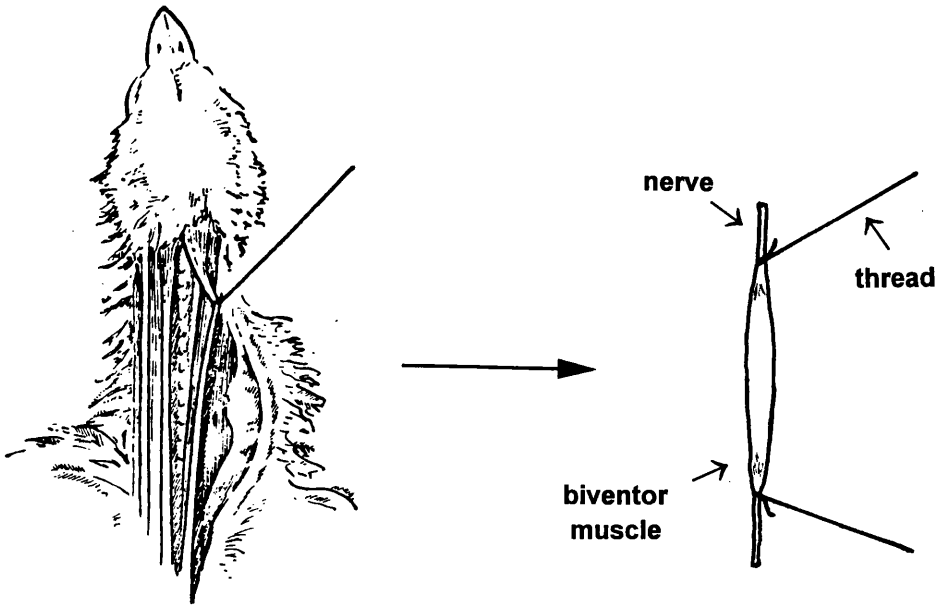
Table 4.1. Summary of the PSP samples used in the chick biventor cervicis (CBC) bioassay, including the STX eq. for each sample as detected by the mouse bioassay (CSL, Torry), the species from which the samples were obtained, and the 50% inhibitory time (IT_{50}) values on muscle twitch height.

PSP sample	µg STX eq.	Species	IT_{50} (min)
1	0	Mussel	-
97	0	Brown crab	-
101	0	Oyster	-
164	45.8	King scallop	30
138	75.4	Razor clam	-
69	80	King scallop (g)	50
176	81.6	Queen scallop	20
58	104	Mussel	60^{+}
70	565	Mussel	13
49	606.8	Mussel	7
32	651.6	Mussel	6

Figure 4.1.

The upper part of the figure shows the dissection of the biventor muscles. The back of the neck is exposed and a thread is tied round each upper end of the biventors muscles where they join the tendon. They are then dissected along their length and a second thread is tied round near the lower part of each muscle. They are then cut free and the preparations transferred to a container with fresh Krebs solution.

The pairs of biventor muscles are then mounted in holders and placed in an organ bath (A) as shown in a typical apparatus set-up in the lower diagram. One end is fastened to a fixed pin (P) with a ring electrode attached to it. If hollow the pin can serve as an aerator. The other end of the preparation is attached to a force-displacement transducer (F). The bath solution can be changed when required from the reservoir (R) containing continuously gassed Krebs solution, which is warmed as it passes along the coils (C) submerged in the main water tank heated by a thermostat (T).


nerve
thread
biventor
muscle

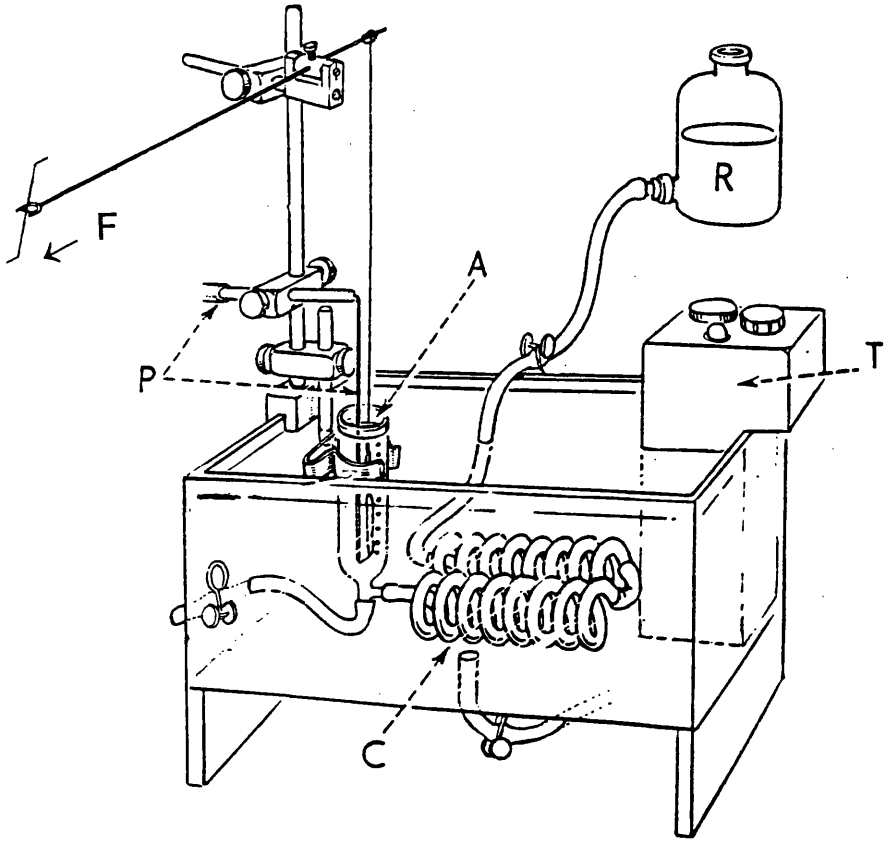
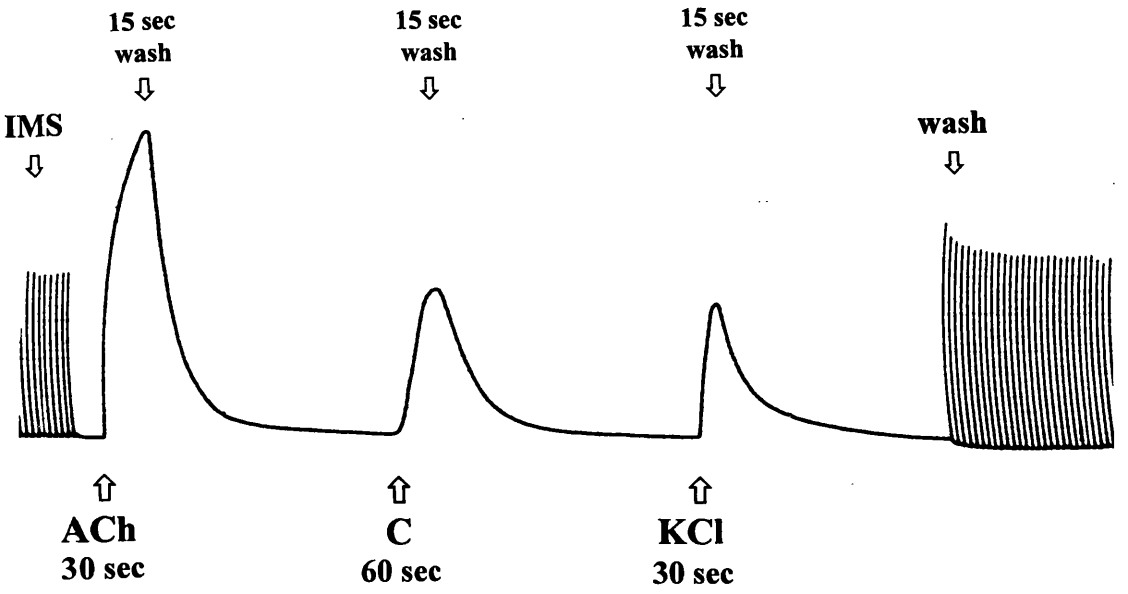
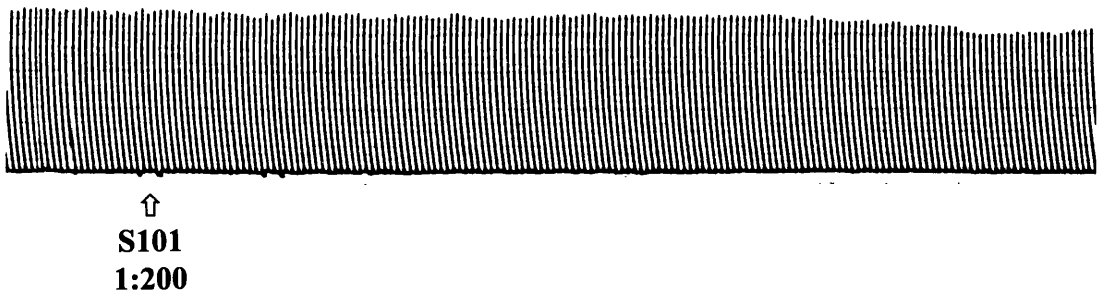

F
A
R
P
T
C

Figure 4.2. A typical recording from a chick biventor cervicis muscle preparation after exposure to a PSP sample (S101) containing no detectable levels of STX by mouse bioassay. The dilution factor was 1:200 parts toxin to Krebs.

Twitch responses are evoked by indirect muscle stimulation (IMS). In the absence of stimulation contractures in response to exogenously applied acetylcholine (ACh), carbachol (C) and potassium chloride (KCl) are obtained. Following application of S101 (1:200 parts dilution) in the middle trace, no significant change in twitch amplitude was observed over a 65 min time period.

- Wash times (15 sec) and agonist application times (e.g.ACh 30 sec) apply to each set of traces in each of the Figures 4.2 - 4.4. (not shown later for figure clarity).


15 sec
wash
15 sec
wash
15 sec
wash
IMS
wash
ACh
30 sec
C
60 sec
KCl
30 sec


S101
1:200

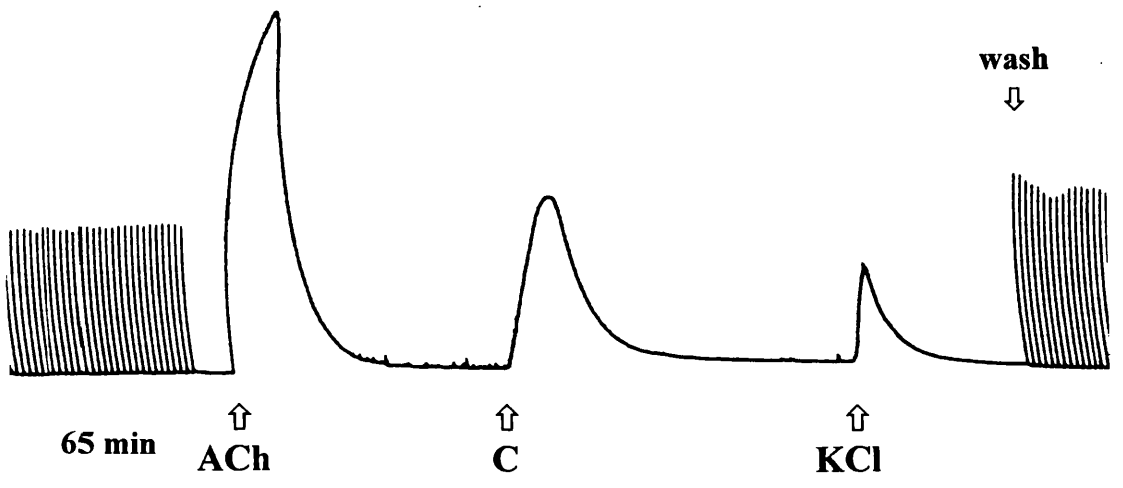

wash
65 min
ACh
C
KCl
5 min

Figure 4.3.

Typical biventor muscle twitch and contracture recording showing a progressive decrease in twitch amplitude over a period of 65 min, following exposure to the PSP sample (S69) calculated by mouse bioassay to contain 80 μg STX eq. A significant block ($p = 0.050$) was seen after approximately 50 min. No significant change was measured in contracture heights following exogenously applied ACh, carbachol (C), or KCl after the muscle was exposed to the PSP sample. The dilution factor for the sample was 1:200 parts toxin to Krebs.

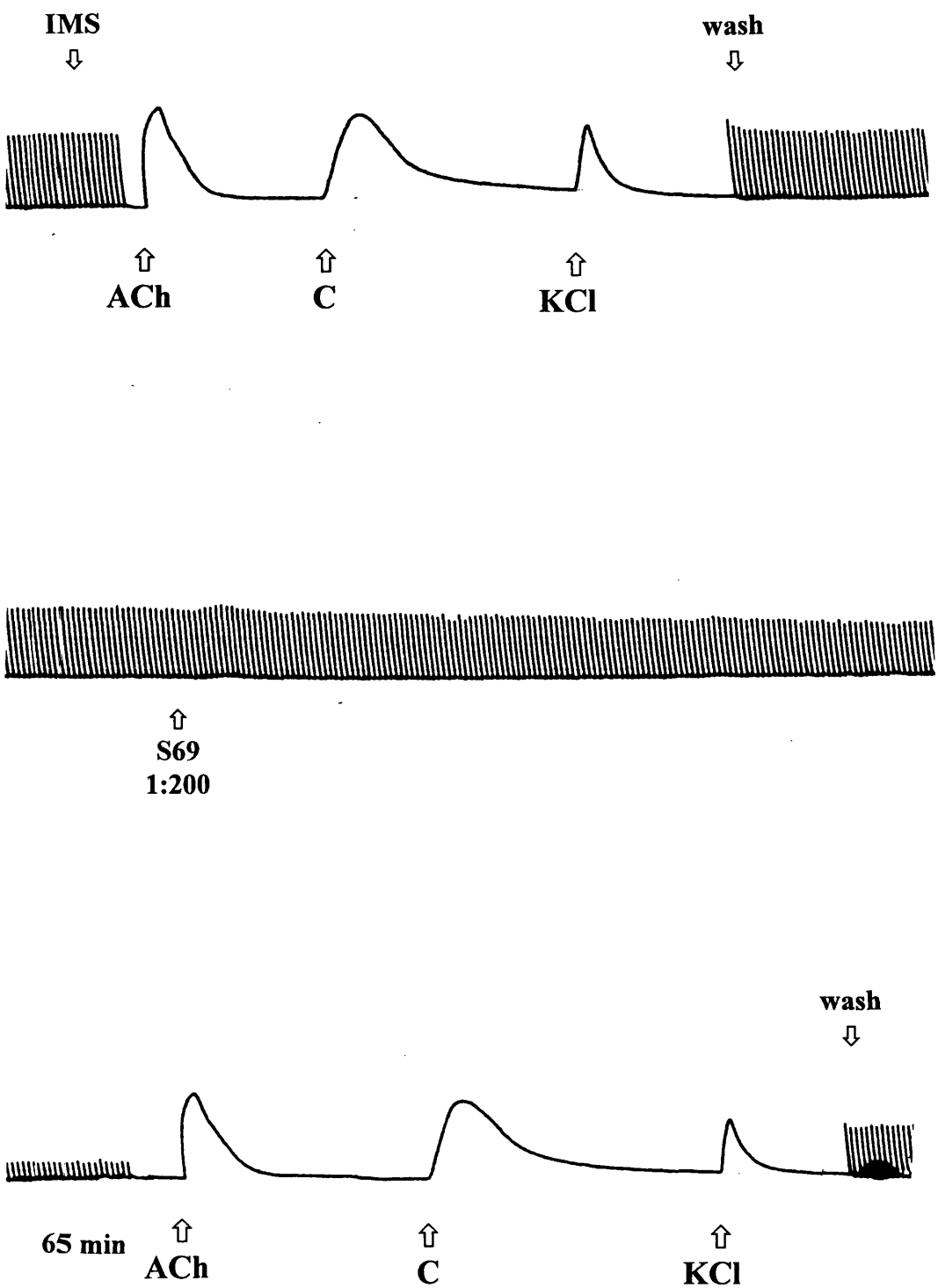
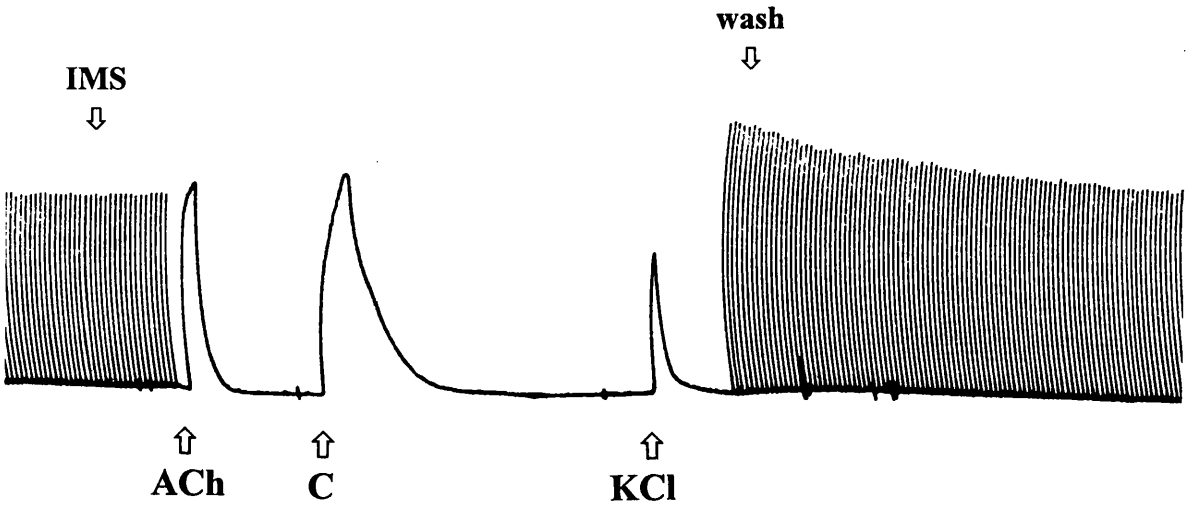
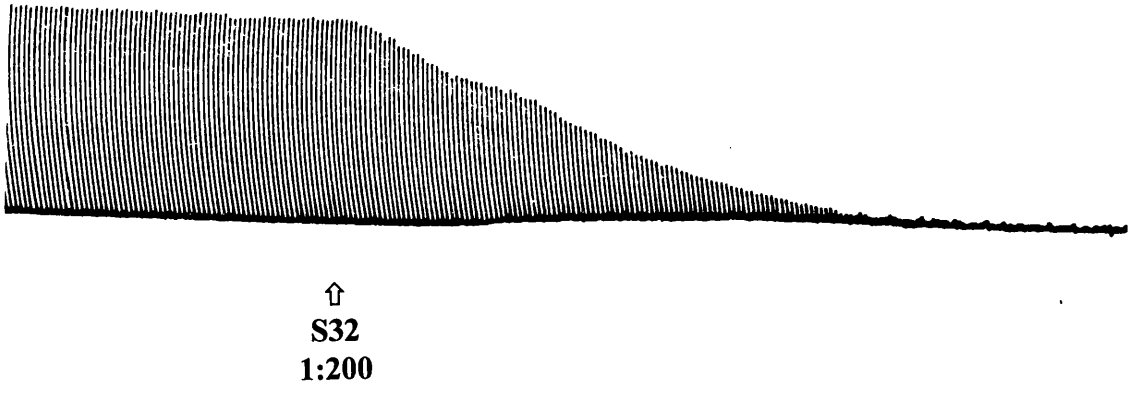

IMS
wash
ACh
C
KCl
S69
1:200
wash
65 min
ACh
C
KCl
5 min

Figure 4.4.

Recording of a biventor muscle preparation after exposure to a PSP sample (S32) containing 651.6 μg STX eq. The sample dilution factor was 1:200 parts toxin to Krebs. A complete block in twitch amplitude was observed within 15 min ($p = 0.029$), with a mean IT_{50} of 6 min. No significant changes in contracture height after exogenous application of ACh, carbachol (C), or KCl were measured for this sample.


wash
IMS
ACh
C
KCl


S32
1:200

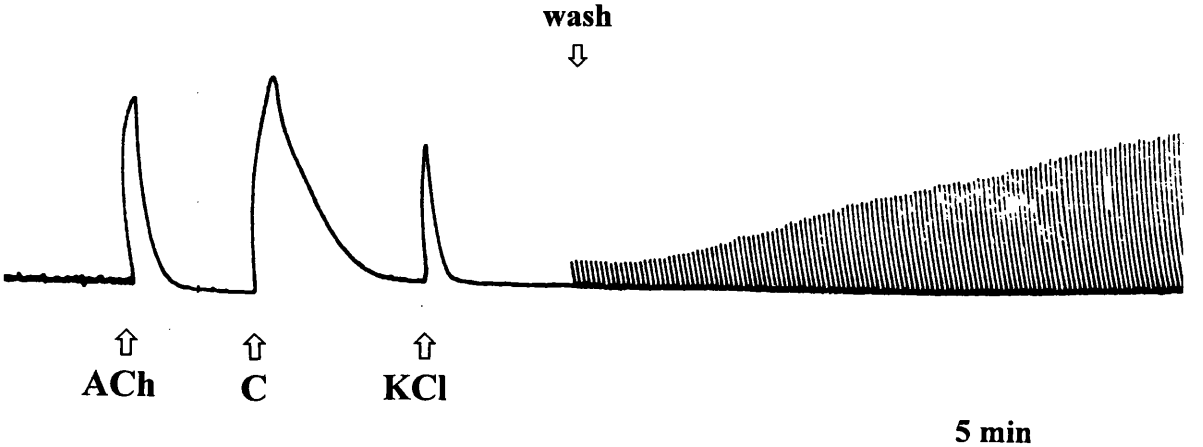
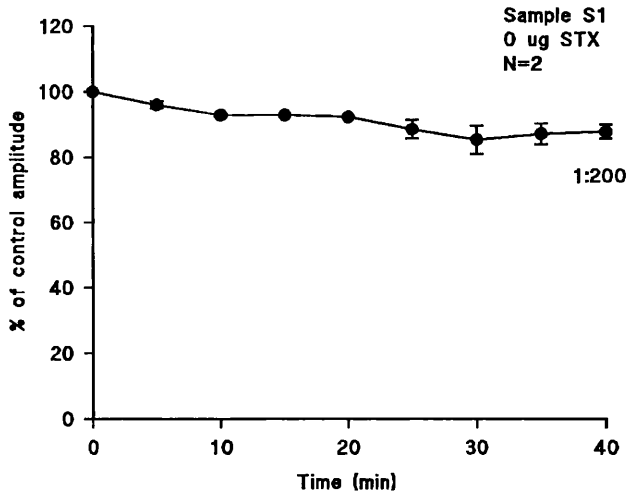
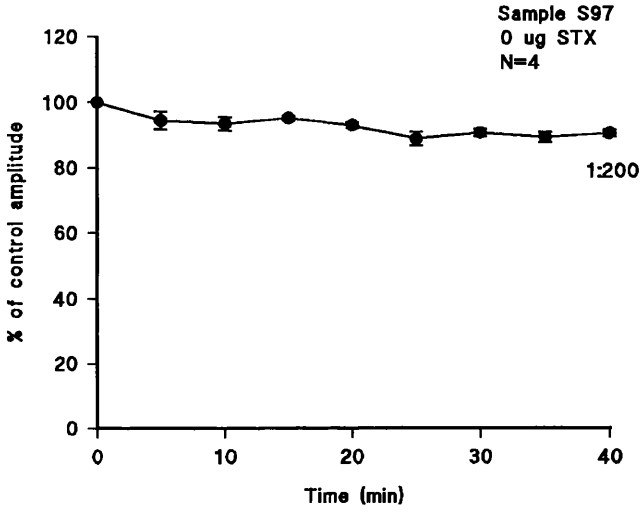

wash
ACh
C
KCl
5 min

Figure 4.5.

The application of three negative PSP samples, S1, S97 and S101, (top, middle and bottom traces respectively) tested as a 1:200 dilution (• – •) had little or no inhibitory action on the amplitude of IMS evoked twitches compared to a mean control value in the chick biventor cervicis preparation over a period of 40 min. Each data point represents the percent of the mean ± s.e.m. of 2 or 4 experiments.


Sample S1
0 ug STX
N=2
1:200
% of control amplitude
Time (min)
0
20
40
60
80
100
120
0
10
20
30
40


Sample S97
0 ug STX
N=4
1:200
% of control amplitude
Time (min)
0
20
40
60
80
100
120
0
10
20
30
40

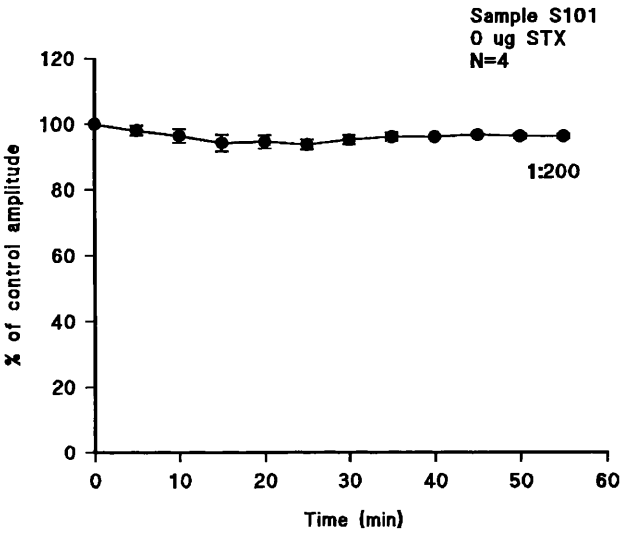
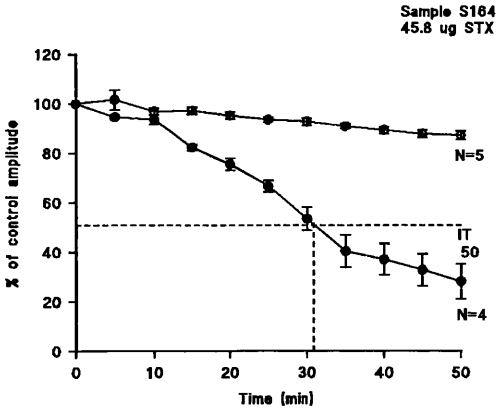
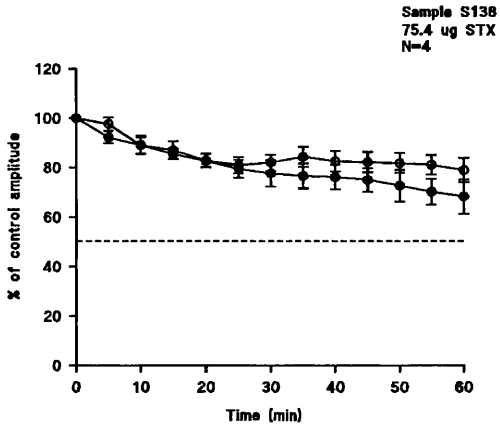
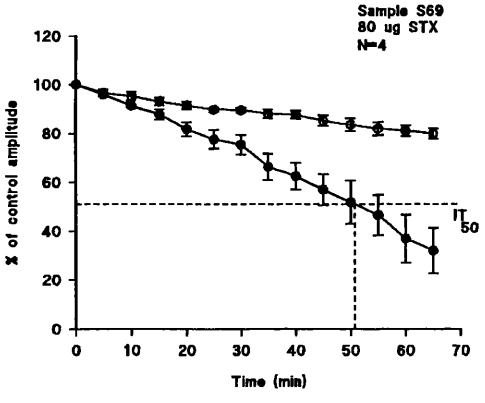

Sample S101
0 ug STX
N=4
1:200
% of control amplitude
Time (min)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

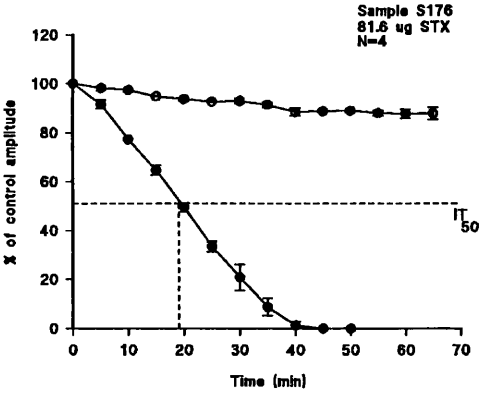
Figure 4.6.

A set of graphs showing the inhibitory action of five PSP samples of increasing toxicity, ranging from 45.8 μg STX eq. (S164, top left hand) to 104 μg STX eq. (bottom graph). The response curves were obtained from exposure to samples at 1:1000 parts (o – o) dilution and 1:200 parts (• – •) dilution of the samples. The top two sets of data were obtained in the presence of samples below the AOAC safety level. Samples S69 and S176 are equivalent to the safety level. Sample (S58) containing 104 μg STX eq.is slightly above the AOAC safety level. Data points are means ± s.e.m.


Sample S164
45.8 ug STX
% of control amplitude
Time (min)
N=5
N=4
IT
50


Sample S138
75.4 ug STX
N=4
% of control amplitude
Time (min)


Sample S69
80 ug STX
N=4
% of control amplitude
Time (min)
IT
50


Sample S176
81.6 ug STX
N=4
% of control amplitude
Time (min)
IT
50

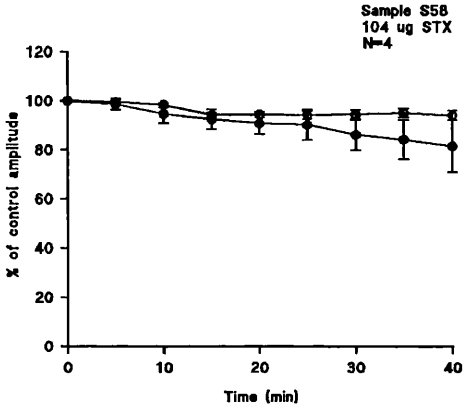
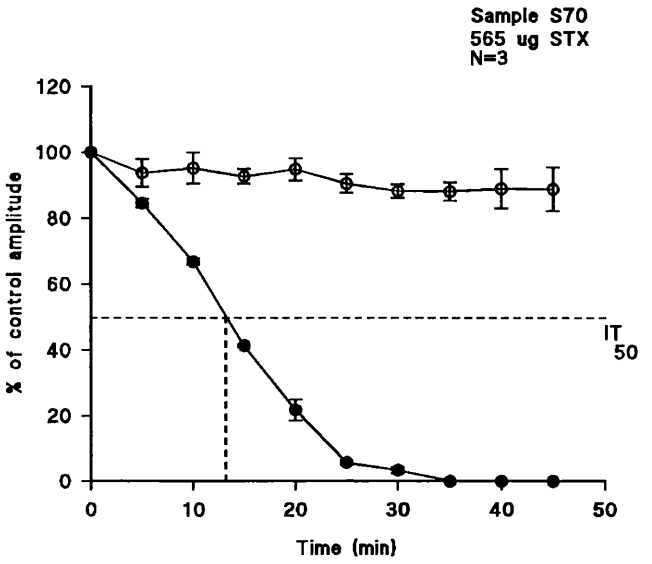
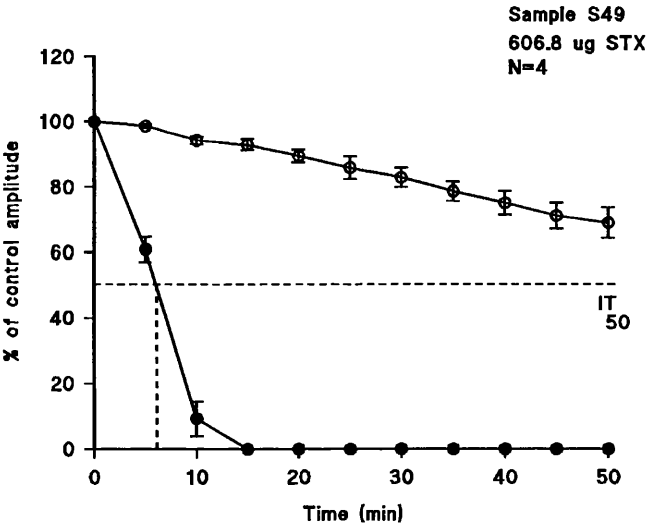

Sample S58
104 ug STX
N=4
% of control amplitude
Time (min)

Figure 4.7.

Twitch inhibition response curves obtained in the presence of the three most potent PSP samples supplied by CSL, Torry. Samples S70, S49 and S32 (top, middle and bottom graphs), were calculated as containing 565 μg, 606.8 μg and 651.6 μg STX eq. respectively. The response curves were obtained from exposure to samples at 1:1000 parts (o – o) dilution and 1:200 parts (• – •) dilution of the samples. Exposure at the 1:200 dilution showed significant blocking action. The two most potent samples, S49 and S32, completely inhibited twitch responses within 15 min ($p = 0.028$ and $p = 0.029$, respectively). Data points are means ± s.e.m.


Sample S70
565 ug STX
N=3
120
100
80
60
40
20
0
% of control amplitude
IT
50
0
10
20
30
40
50
Time (min)


Sample S49
606.8 ug STX
N=4
120
100
80
60
40
20
0
% of control amplitude
IT
50
0
10
20
30
40
50
Time (min)

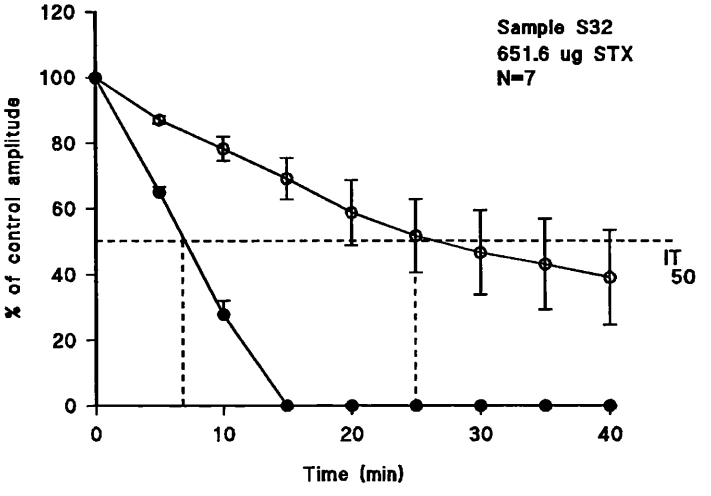

Sample S32
651.6 ug STX
N=7
120
100
80
60
40
20
0
% of control amplitude
IT
50
0
10
20
30
40
Time (min)

Figure 4.8.

The mean IT_{50} values for the PSP samples used in the chick biventor cervicis assay taken from Table 4.1. showing that at a dilution of 1:200 there is a considerable scatter in blocking time rates for samples close to the regulatory AOAC safety level of 80 μg STX eq. A much closer agreement in inhibitory times is seen with the more potent samples. The linear regression is fitted to the mean data ($r^2 = 0.63$).

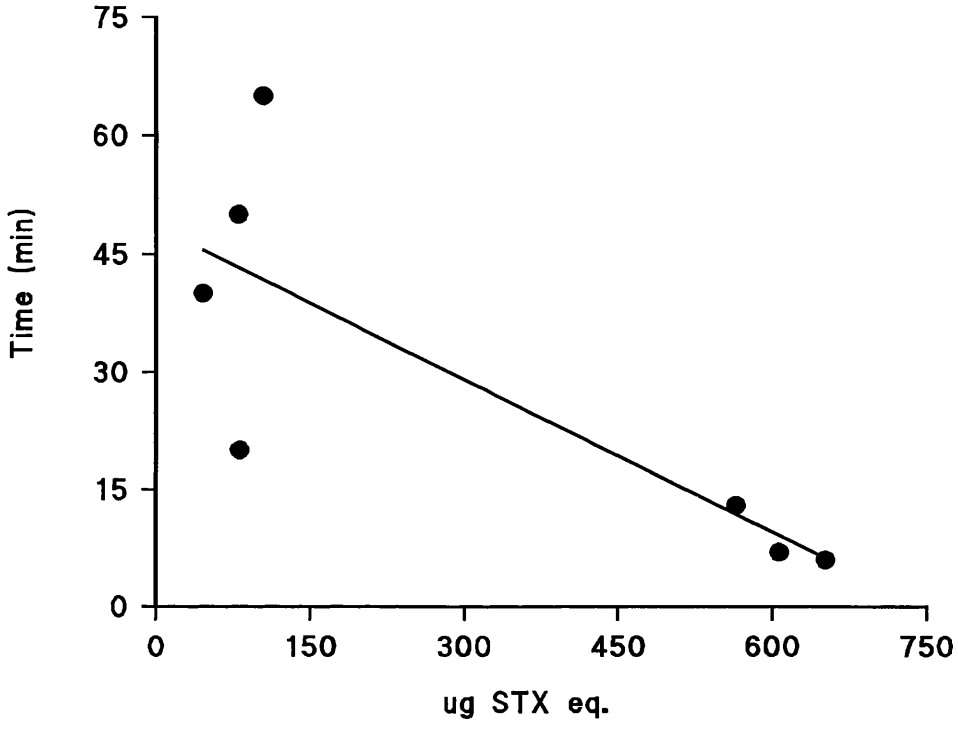
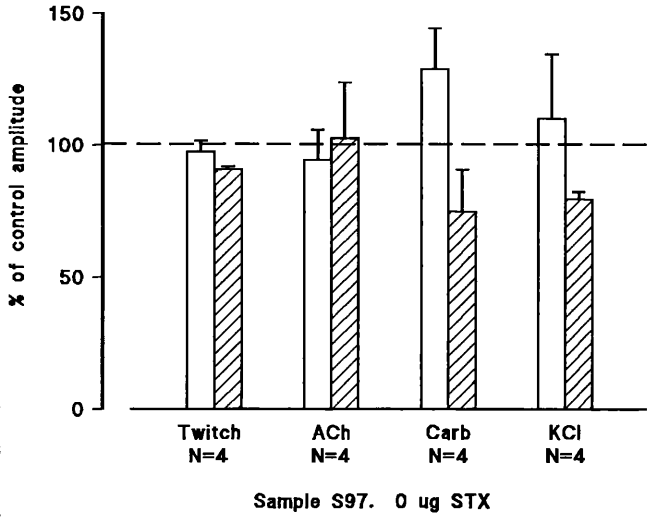
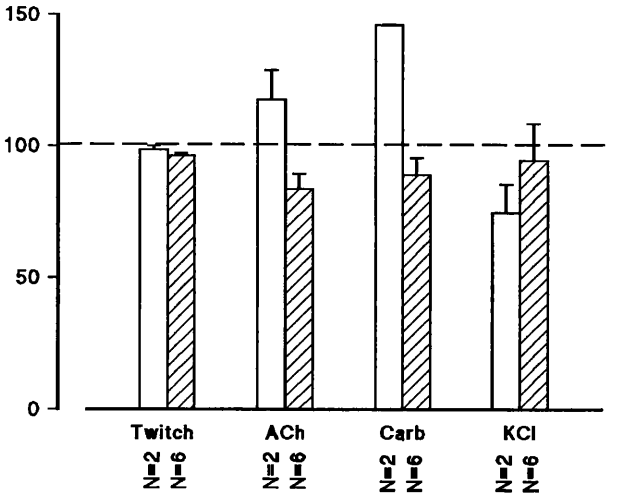

75
60
45
30
15
0
Time (min)
0
150
300
450
600
750
ug STX eq.

Figure 4.9.

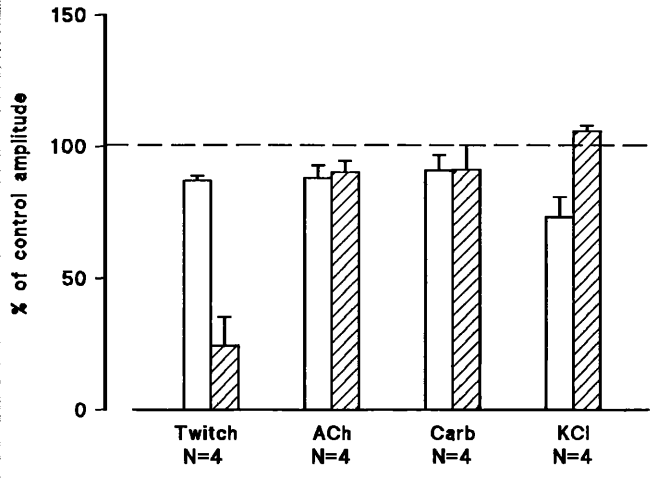
The effects of the agonists acetylcholine (ACh), carbachol (Carb) and KCl on contracture amplitude following exposure to four PSP sample at the dilutions, 1:1000 (plain bars) and 1:200 (striped bars). Identical bars for percentage reduction in twitch height from control amplitude are also shown for each sample. The top two data sets were obtained from two negative PSP samples (S97 and S101). The bottom two sets were obtained from preparations exposed to two samples (S164 and S138) containing a lower STX eq. content than the AOAC safety level. The bars represent mean values ± s.e.m. with *n* numbers per sample indicated under the bars.



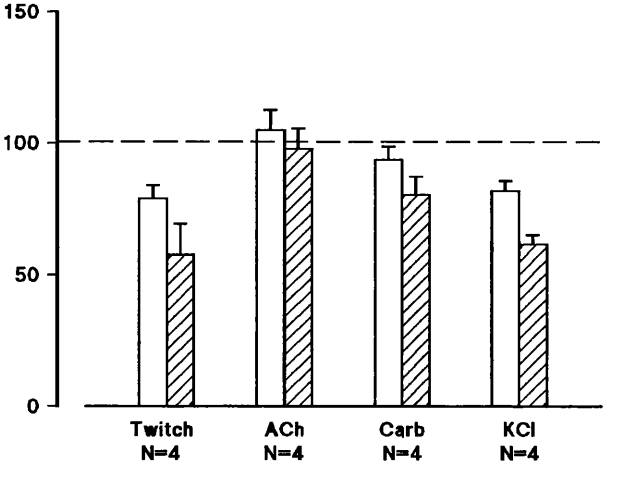
Sample S97. 0 ug STX



Sample S101. 0 ug STX



Sample S164. 45.8 ug STX

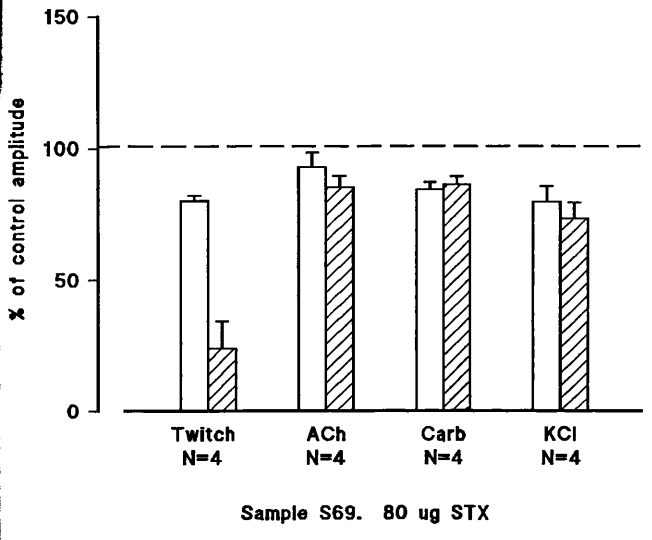


Sample S138. 75.4 ug STX

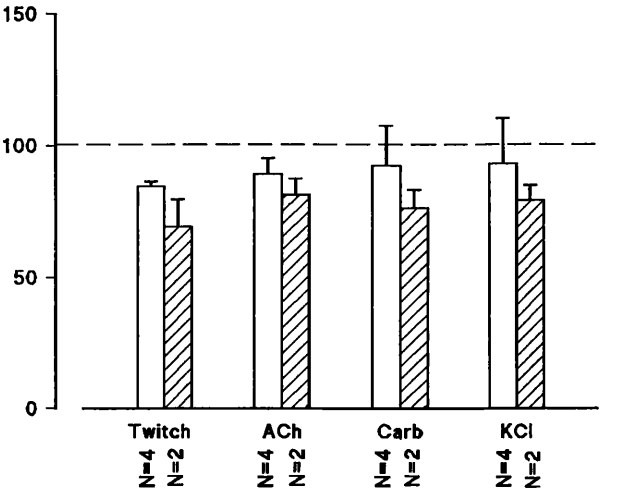
Figure 4.10.

Bargraphs of contracture responses obtained from preparations exposed to two samples, S69 and S82, at the AOAC safety level of 80 μg STX eq. (top graphs), and two samples, S176 and S58 (bottom graphs) containing an STX eq. of 81.8 μg and 104 μg respectively. S58 just exceed the safety level. Each bar represents the percentage mean value ± s.e.m. of experiments per sample with *n* numbers for each sample indicated under the bars.

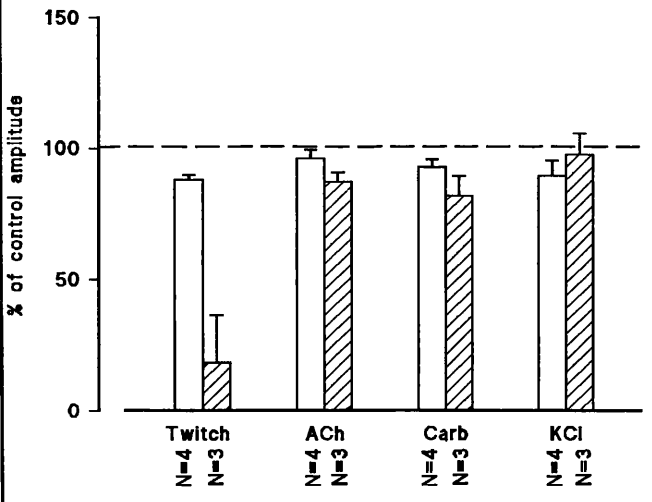
PSP samples were applied in two dilutions, 1:1000 (plain bars) and 1:200 (striped bars). Identical bars for percentage reduction in twitch height from control amplitude are also shown for each sample.



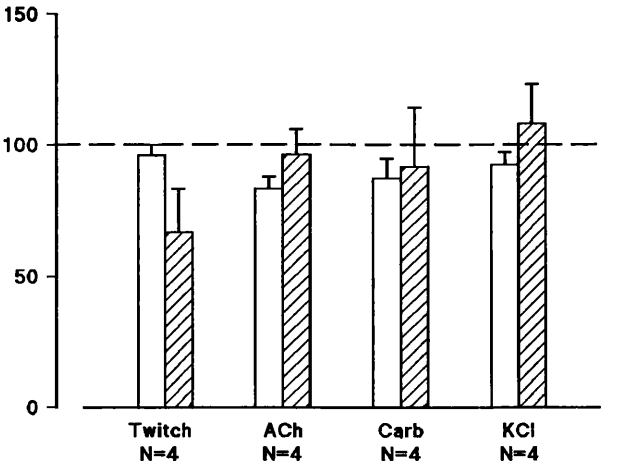
Sample S69. 80 ug STX



Sample S82. 80 ug STX



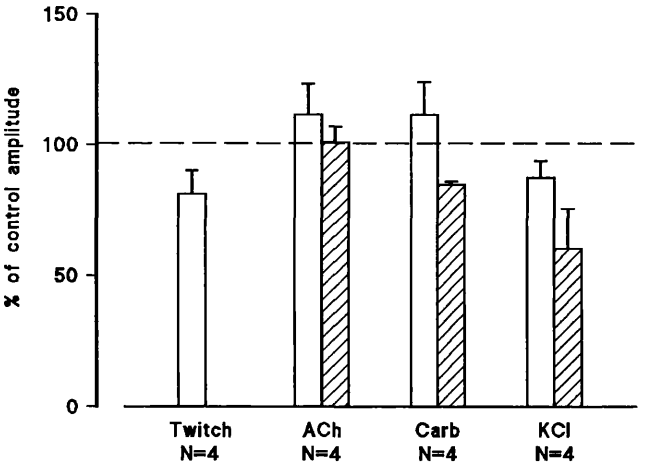
Sample S176. 81.8 ug STX



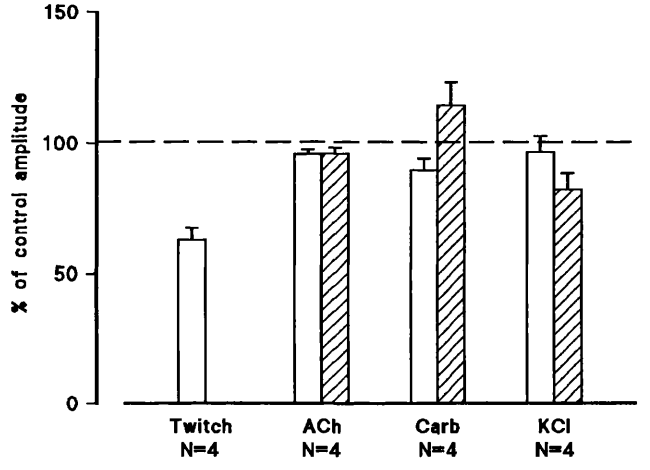
Sample S58. 104 ug STX

Figure 4.11.

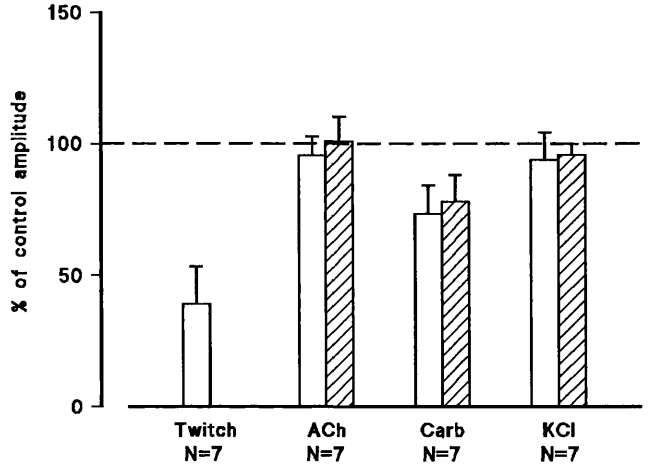
The effects of the agonists acetylcholine (ACh), carbachol (Carb) and KCl on contracture amplitude following exposure to the three most potent PSP samples S70, S49 and S32 at the dilutions, 1:1000 (plain bars) and 1:200 (striped bars). Block in twitch amplitude is shown only for the 1:1000 parts dilution. Complete inhibition of twitches occurred at the 1:200 parts dilution and is therefore not seen on the ordinate axis. The bars represent mean values ± s.e.m. with *n* numbers per sample indicated under the bars.



Sample S70. 565 ug STX



Sample S49. 606.8 ug STX



Sample S32. 651.6 ug STX

Chapter 5

Frog Sciatic Nerve Preparation: A Simple Nerve Assay

5.1. INTRODUCTION

5.1.1. Action of shellfish toxin on frog sciatic nerve

Several early investigations used the sciatic nerve of the frog to study the action of semi-purified extracts from mussels contaminated with PSP toxins on the sodium-dependent processes in nerve membranes. Kellaway (1935) used mussel extracts in powder form to demonstrate that PSP could block the electrical activity in both nerve and muscle. Conduction in the sciatic nerve was abolished in 4½ hours. The poisoning was attributed to a powerful curarizing action on the unmyelinated nerve endings, which were thought to be more susceptible to the toxin than the nerve or muscle. Fingerman *et al.* (1953) arrived at the same conclusion that the toxin from shellfish had a curare-like action, chiefly because poison-treated muscle did not respond to exogenously applied ACh. However, epp's that were large enough to produce a twitch could still be recorded for a period after toxin application.

This assumption was repudiated by Evans (1964). Experiments using a purified extract of toxin from *S. giganteus* on conduction and contraction in frog nerve and muscle revealed no evidence of curarizing action at the neuromuscular junction. Applied to intact frog sciatic nerve, the toxin also required several hours to cause a significant block in nerve conduction. Such resistance of the sciatic nerve to blocking was attributed to the perineural sheath, which was shown to be an effective barrier against externally applied STX or TTX. Reversibility of the toxic effect on the isolated preparations was also impaired by this barrier, and the toxin required more than an hour before significant recovery was observed. However, desheathing the nerve produced a partial block within 4-5 min with very low concentrations of the toxin, accompanied by an increase in the latency of the compound action potential. Block occurred without altering the resting membrane potential, and with 0.01 μg/ml of pure toxin extract it was completely reversible, and could be repeated as many as 8 times without permanent damage, if complete recovery was allowed between applications (Evans 1964; Kao and Nishiyama 1965).

5.1.2. The compound action potential of the frog sciatic nerve

The frog sciatic nerve trunk is composed of around 800 nerve axons of varying diameter, which fall into three categories. The most rapidly-conducting ones (about 30 m/sec in the frog at 20°C) are the large diameter, myelinated, A fibres, which produce the alpha component of the compound action potential (CAP) (see Figure 5.1.b.c). A population of more slowly conducting A fibres of smaller diameter produces the beta component of the CAP. B and C fibres represent populations of smaller, more thinly myelinated axons with slower conduction velocities (Brazier 1977; Aidley 1989). A study of the CAP A and C components in bullfrog sciatic nerve (Kobayashi *et al.,* 1993) found that the A fibres were TTX-sensitive (TTX_S) whereas the C fibres were TTX-resistant (TTX_R), and this was attributed to the existence of slow-conducting TTX_R Na^+ channels in C-type fibres.

In an isolated organ bath, although the fibre responses are initiated together at the point of electrical stimulation, the impulses will reach a distant point in the order of their conduction velocities. Therefore, in this type of mixed nerve, if all the fibres are stimulated, the height and shape of the CAP will be determined by the number of fibres of each type and their conduction velocities. If these nerve axons behave in the same fashion as the crustacean fibres, being recorded full-size up to the point that they disappear, then the population response of this large nerve might be expected to show a relatively smooth (but not necessarily linear) proportionality to toxin concentration, as axons progressively drop out from contributing to the CAP.

The sciatic nerve preparation also offers the advantage of simplicity over the chick biventor and lobster neuromuscular preparations as an assay system, being composed solely of nerve axons, without synapses or postsynaptic cells. The apparent robustness of the nerve to several repetitive applications of toxin, combined with the sensitivity of the action potential to STX, with an EC_{50} of 8.0 nM (Strichartz *et al.*, 1986) and a minimum block of 3 nM (Kao and Fuhrman 1967), contribute to its suitability for testing toxins with Na^+ channel blocking activity. For these reasons the sciatic nerve is a potentially useful assay, and was therefore investigated here. Since

there appears to be no recent data available on the action of crude shellfish extracts on nerve preparations, particular attention was paid to the action of crude PSP extracts from various shellfish species on the CAP.

5.2. MATERIALS AND METHODS

5.2.1. Dissection of the sciatic nerve

The sciatic nerve of the British frog, *Rana temporaria*, was dissected following the methods described in *Pharmacological Experiments on Isolated Preparations* (University of Edinburgh, 1968). The nerve was removed from the top of the spinal cord to the knee joint, producing a length of nerve of approximately 4 cm. The middle section of the nerve was partially desheathed and the underlying fibres were exposed by inserting fine pointed scissors just under the surrounding perineural tissue and cutting along the sheath about 3 cm.

The preparation was placed across a 3 chamber "Vaseline" gap bath, with small grooves lined with silicone grease between each well. The grooves were then plugged with more silicone grease to electrically isolate each chamber. A small square of glass cut from a cover slip, was then pressed gently over the silicone to improve electrical isolation. The preparation was maintained in a frog-Ringer solution composed of (mM): NaCl 115, KCl 2.5, $CaCl_2$ 1.8, HEPES 3.0, and buffered to pH7.0 with 1M HCl (Masukawa and Livengood 1982).

5.2.2. Electrophysiological recordings

A set of 3 Ag/AgCl half cell electrodes consisting of a stimulator, ground and recording electrode were connected to the baths by a magnetic strip. Compound action potentials were evoked by stimulating the nerve at 0.4 Hz for 0.05 ms, at a voltage sufficient to produce a maximal response using a stimulator (Grass S48, Grass Med. Instr., Quincy, MA) via a stimulus isolation unit (Grass S1U5). Signals were recorded using a CED 1902 transducer with a CED 1401 A/D converter and analysed on a PC computer (Vanilla 286-12) using Dempster WCP software. Experiments were

performed at room temperature (22-25°C). The nerve could be stimulated over a period of several hours without decrement of the amplitude of the compound action potential (CAP), and proved to be a very reliable and robust preparation. The toxicity of STX standards and shellfish extracts were estimated from their effects on both the amplitude and the delay to peak of the principal waveform (alpha component) of the CAP.

5.2.3. Experimental protocol

A PSP sample diluted 1:100 parts in saline was applied to the middle chamber for 5 min. The preparation was then washed until the action potential regained its original amplitude, normally after 25 min. After another 15 minutes, as long as the signal retained its original shape and height, further samples were added then washed off as above.

Prior to each set of experiments per preparation, the section of nerve spanning the middle chamber was desheathed to allow easy accessibilty of the toxins. The reliability of the desheathing process was determined by the application of an internal control. A high STX eq. value sample (S70) known to produce 50% inhibition within 60 sec was applied to a desheathed preparation. Any preparations that did not exhibit the appropriate block within this time (± 5 sec) were discarded (n=2).

5.3. RESULTS

A total of 16 PSP samples ranging from 0 µg to 606.8 µg STX eq/100 g tissue were tested at a dilution of 1:100 parts, based on the inhibition response of the CAP to a 200 nM STX standard applied during initial experiments. Table 5.1 lists these samples in descending order of toxicity of µg STX eq/100 g tissue, and their calculated IT_{50} and IT_{90} values obtained from the inhibitory action of the sample on the desheathed nerve. The equivalent IT_{50} values for samples 1, 101, 138, 69, 176, 58, 70, 49, had been previously evaluated on the CBC nerve-muscle preparation, (described in Chapter 4).

5.3.1. Sensitivity and robustness of the frog sciatic nerve preparation

Toxin accessibility to the nerve axons was a prime consideration of this assay. As Figure 5.2.a clearly shows, prior to partial desheathing, a PSP sample of 80 μg STX eq. had almost no inhibitory effect in the given time period. Following the desheathing protocol as described in **section 5.2.1**, the same toxin caused a 50% inhibition of evoked action potentials by 1.5 min. These findings agree with Evans (1964). A negative sample (not shown) produced no change in CAP amplitude. Partial desheathing was therefore considered necessary, in order to increase the sensitivity of the preparation to low value samples.

Robustness together with reliability were also tested. Figure 5.2.b shows an experiment in which S70 was applied as the first toxin. Following this, 5 other samples of varying toxicity were tested on the same preparation, each following a wash-recovery period. At the end of the experiment S70 was applied again. There was less than a 15 sec difference in IT_{50} values (≈20% change) between the two samples applied with a 4 hour interval, and with 5 intervening test samples.

The recovery rates of the CAP for a typical experiment involving several toxin applications is shown in Figure 5.3. Following a wash period of between 25-30 min, the action potential recovered > 95% each time. Rise time and shape of the CAP remained consistent with those from control recordings.

The consistency and reproducibility of the sciatic nerve CAP is illustrated in Figure 5.4 (top trace, left column) as a set of four superimposed records extracted from a typical series of 150 control records that were collected before each experiment. This figure, together with the following 11 sets of traces in Figures 5.4. and 5.5., contain four records captured at four time points (control, 0.25, 0.5, and 1.0 min), the last record in each set representing the time to total block with the most potent sample (S49).

These 12 sets of traces show that as the toxicity of the sample increased there was a corresponding increase in its blocking action on the CAP. No decrease in action potential height was observed (Figure 5.4, middle two traces) when the nerve was

exposed to negative PSP samples. However, when the toxicity level reached 80 μg STX eq. (the AOAC safety level), the CAP was inhibited by *ca.* 50% within 1.5 to 2.0 min (Figure 5.5, top two traces). This decrease is similar to the values obtained for 200 nM STX (Figure 5.4, top trace, right column). Inhibition by S49 (606.8 μg) showed an almost linear decline with time (Figure 5.5, bottom trace, right column).

These traces also show that the desheathed nerve is sensitive enough to detect toxicity levels at least half that of the AOAC safety level as shown in Figure 5.4 (bottom trace, left column) with S22 at 40 μg STX eq.

5.3.2. The effect of PSP samples on CAP rise time

The effect of the PSP samples on the generation of the CAP is also seen in Figures 5.4 and 5.5, in terms of the absolute delay (ms) between the peak amplitudes of the control record and the 1 min time point record. In general, the delay to peak increased with the increasing toxicity of the sample. For example, at 80 μg STX eq., the delay to peak was ≈ 0.18 ms, whereas at 606 μg the delay was twice this value at ≈ 0.36ms. These values were then plotted against the CAP inhibition rates for their matching sample (Figure 5.6) which shows the proportionality between these two measures for different STX eq concentration in the sample.

5.3.3. Blocking effect of PSP samples on CAP amplitude

Mean data (± sem) of the change in CAP amplitude for exposures to each toxin sample are plotted as the % change from the control values obtained immediately prior to each toxin application in Figures 5.7-5.11 (in order of increasing toxicity).

Samples S4, S15 and S101, defined as 'negative' in **section 4.2.1**, produced no inhibitory action on the CAP. On the contrary, a slight, progressive increase in CAP amplitude was recorded following application of S15 and S101. The latter sample, S101 had previously given a positive STX result with a cell culture assay (CSL Ring

Trial 94 Report), but proved negative when tested on the CBC preparation (**section 4.3.1**), HPLC and the mouse bioassay.

Application of S22 (40 μg) caused a 30% reduction in amplitude within the 4.5 min time period, with an IT_{50} of *ca.* 6 min (samples were left to run for 15 min), finally stabilising with a 93% blocking effect at 13 min. Sample 138 produced a similar blocking rate to S22 (Table 5.1, Figure 5.8) despite being calculated at almost 80 μg. STX eq. Neither of these two samples therefore caused a significant difference in the CAP amplitude within the experimental time limit of 4.5 min ($p = 0.312$ and 0.194 respectively).

At 80 μg the IT_{50} rates radically shortened to 1.9 min or less, with IT_{90} rates at 5.0 min or less (Table 5.1). These samples containing 80 μg STX eq or more all caused a significant reduction in the amplitude of the CAP within the 4.5 min experimental time period ($p = <0.05$). The mean IT_{50} value for 80 μg was 1.53 min (s.e.m ±0.35). The inhibition rate continued to shorten as toxicity increased, for example, at *ca.* 300 μg the mean IT_{50} was 1.03 min (s.e.m ±0.12), whilst the IT_{50} of the most toxic sample at 606.8 μg was 0.6 min. This corresponding shortening was reflected in the shape of the inhibition curves (Figures 5.10 and 5.11) which became steeper as toxicity increased. Comparison with the IT_{50} values for samples used in the CBC preparation showed a similar pattern of increasing steepness with greater toxicity.

Inhibition of the CAP, calculated from the data in Figures 5.8-5.10 and Table 5.1, are plotted as the mean IT_{50} and IT_{90} values for each sample and shown in Figure 5.12, firstly as a linear plot (Figure 5.12.a and c) and then as a logarithmic transformation (Figure 5.12.b and d). A regression analysis of the mean data, including the two outliers yielded correlations of $r^2 = 0.452$ for IT_{50} values, and $r^2 = 0.308$ for IT_{90} values. However, a regression analysis of PSP samples > 80 μg STX eq plotted separately (not shown), exhibited a more evident linear inhibition trend with correlations of $r^2 = 0.638$ and 0.613, respectively.

Toxicity in mussels is regarded by the AOAC as the most appropriate monitor of toxicity for all other species. Thus the inhibitory action of mussel extracts was compared to those from the mixed species results. A simple 2x increase in sample concentration using 5 separate samples from mussels showed a progressive inhibitory action on the CAP as potency increased (Figure 5.13.a). A linear regression fitted to the

$IT_{50.}$ values yielded a correlation of $r^2 = 0.989$ (Figure 5.13.b). As with the mixed species samples, the mussel sample with an STX eq value of < 80 μg STX eq, did not exhibit the same graded inhibition proportionality as those samples > 80 μg STX.

5.3.4. Recovery rates of the CAP

Reversibility or dissociation rates are an indication of the potency of a toxin molecule binding to a membrane receptor. TTX has a residency time of 70s, whilst that for STX is 37 s, and subsequently these long residency times place TTX and STX in the "very slow" drug class (Hille 1992).

The recovery rates of the CAP during the washout period appeared to reflect the potency of the samples as illustrated in the reversibility curves in Figures 5.14 and 5.15. Recovery of the CAP with samples that contained around 80 μg STX eq., was initially by a rapid increase to about 50% of the control amplitude within 2.5-3.0 min, followed by a more gradual recovery rate, most obvious after about 80% recovery had occurred. With the more highly potent samples (> 300 μg STX eq) there was a period of no response for about 1 min, followed by an almost linear dissociation rate (Figure 5.15). The RT_{50} values being approximately 8 min.

Preparations exposed to S49, the most toxic sample (606.8 μg STX eq) showed the greatest variation once initial recovery had started (Figure 5.15). All the preparations exposed to this sample remained completely inhibited, showing no signs of recovery for 3-4 min, then as with the other samples, recovery followed a linear pattern. The RT_{50} value was 11 min.

In a manner reflecting the inhibition of the CAP, the recovery of the action potential also exhibited a linear trend, with the most potent samples taking the longest to wash off (Figure 5.16). A regression analysis of the mean RT_{50} recovery values showed a correlation of $r^2 = 0.918$.

5.4. DISCUSSION

The results of the experiments described here show that MAFF shellfish extracts previously determined by mouse bioassay and HPLC as containing varying concentrations of STX eq., had a direct neurotoxic effect on the desheathed sciatic nerve of the frog in the same manner as that described for STX. These findings agree with those for semi-pure extracts (Fingerman *et al.*, 1953; Evans 1964, 1969; Kao and Nishiyama 1965). The closest comparison in terms of the sample source is with Evans (1970). Extracts from mussels (*M. edulis*) taken from a batch caught during the 1968 PSP outbreak off the north east coast of Britain reduced conduction in the frog nerve.

The results also confirm that the myelin sheath surrounding the nerve trunk acts as a diffusion barrier so that far greater concentrations of toxins are needed to block conduction of CAPs, especially in peripheral nerves, unless the nerve is desheathed (Fingerman *et al.*, 1953; Evans 1972). Desheathing was therefore necessary to produce both sufficient sensitivity and rapid monitoring rates to allow several samples to be tested within a few hours.

5.4.1. Inhibitory actions of PSP samples on the CAP

The degree of block (within the assay time of 4-5 min) depended upon the potency of the sample. At levels >80 μg STX this occurred in a concentration-dependent manner. The desheathed nerve was sufficiently sensitive to allow a clear distinction between samples containing no detectable levels of STX, those with STX levels considered to be at the safety cut off limit, and samples with high levels of STX eq., remembering this now represents values of *ca.* 2 ng/ml to 33 ng/ml for samples

with original STX values of 80 μg and 651 μg. This sensitivity was consistent for both the assay containing samples obtained from a variety of species or when increasing levels of mussel extracts only were tested; blocking response time shortened with toxicity. Synthetic STX and six of its analogues were similarly found to inhibit the CAP of the frog sciatic nerve in a concentration-dependent manner (Strichartz *et al.*, 1995). The robustness yet sensitivity of the nerve preparation permitted a measure of absolute potency, expressed as EC_{50} values for CAP inhibition and of relative potencies, determined by matching approximately equipotent concentrations of the toxins.

Nerve preparations from cockroach, squid and crayfish have similarly been employed to study the effects of not only STX (Evans 1974; Henderson *et al.*, 1974; Ritchie and Rogart 1977; Kao and Walker 1982) but also other marine toxins such as palytoxin and brevetoxin (Parmentier *et al.*, 1978; Pichon 1982; Huang *et al.*, 1984). The advantages of using just a section of nerve are that it greatly reduces or eliminates factors such as whole animal metabolism, immune reactions or behavioural responses (Parmentier *et al.*, 1978), as well as pre- or post-synaptic dependencies or influences. The latter points were in evidence with the lobster and the CBC preparations, in which the method of measurement encompassed both pre- and post-synaptic events.

In all but those samples containing <80 μg STX eq, inhibition of the CAP occurred in a graded manner. Such a progressive blocking action response of this large nerve implies that the population of axons contributing to the CAP dropped out in proportion to toxin concentration. The non-linearity with samples <80 μg STX may be connected with the range of axon diameters. Also, in a nerve, depolarisation produced by a normally propagated impulse far exceeds the threshold for all-or none behaviour; this excess is known as the safety factor (see also section **4.4**) and can be as great as 10:1 (Brazier 1977). It seems reasonable to assume that this safety factor level would also influence the degree of block produced by a toxin sample.

The slight increase in CAP observed with S15 and S101 is difficult to interpret and may be a natural increment. However, the "positive" results (i.e., there was a

response similar to that expected if STX were present in the sample) for the same samples that were observed in the cell bioassay in the CSL Ring Trial (1994) may imply that these samples contain a compound(s) that modifies or interacts with the cell membrane or its channel proteins. The AOAC sample extraction procedure is somewhat crude, and observations by Jellet *et al.* (1992) of damage to neuroblastoma cells by some PSP extracts again suggests that an unidentified compound(s) has been co-extracted with the PSP compounds and is acting upon the membrane.

A comparison with the post-synaptic agonists results for S101 from the CBC preparation showed that at a low concentration (1:1000), S101 caused an increase in contracture height following ACh and carbachol application, but a decrease in the contracture effect of KCl, thus supporting the possibility that other compounds may be present in S101.

Another sample that exhibited a different to expected response was S138 (75.4 μg STX eq). The slow inhibition rate of the CAP had also been obtained with the twitch recordings in the CBC assay. In addition in the CBC experiments, the induced muscle contracture responses were reduced by both dilutions of the sample. Unfortunately, as S138 was the only razor clam sample to be tested it is difficult to make assumptions about the modest blocking potency of the sample compared to others with an equal STX eq content.

This diversity in the results could also be linked to the samples having been derived from a variety of species. The sequestration and conversion of dinoflagellate toxins by the shellfish will therefore be species specific (**sections 1.2 and 1.3**). This would result in each sample having different complements of PSP compounds. Depending on the toxin profile, the cryptic toxicity of these compounds may be revealed through the acidic AOAC extraction procedure in 0.1 N HCl.These may then be further modified when diluted in a Krebs solution of pH 7, (remembering the mouse bioassay is performed with solutions of pH 2.5-4.5), although Strichartz *et al.* (1995) also tested their toxins at pH 7.2.

This composition of different STX compounds could also apply to the mussels samples used in the one experiment, but being a single species source, toxin profiles

may be closer thus reacting in a similar way under the same experimental conditions. The stronger correlation between STX eq content of the sample and inhibitory action on the CAP found for the mussel extracts requires further investigation before such a proposal could be confirmed.

Various other factors should also be considered regarding the relationship between sample toxicity and the sciatic nerve response. The large scatter (Figure 5.12) around the samples at 80 μg STX eq or less, combined with the regression analysis, has shown that little correlation exists between the toxicity defined by the mouse bioassay and that in the nerve assay at this level of potency. This may be due to several reasons. The methods of testing the samples, both preparation and procedure (i.p. versus direct application) are very different, and the mouse assay has a long established, predefined, toxicity curve (see **section 2.4.1**) derived from the testing procedure.

Toxin profiles within the samples may affect one bioassay differently to the other (see also sample S138 in the chick biventor assay) leading to dissimilar toxicity evaluation. This may be especially true with the samples below and around the AOAC level. Sample S138 also gave a toxicity value different to the mouse bioassay when evaluated by the cell bioassay (MAFF Report FD94/161). As the toxicity of the sample increases, presumably, the ratio of the two most potent Na^+ channel blockers, STX or neoSTX present will also increase. Inhibition of the sciatic nerve CAP become very consistent with the more toxic samples.

5.4.2. Changes in rise time of the CAP

In several of the traces where an increase in rise time of the CAP from baseline to peak amplitude was observed, the slope of the falling or recovery phase remained unchanged. These results correspond to changes seen in the action potential when a sodium deficiency is introduced to an external test solution (Hodgkin 1964). The rising or depolarising phase of the action potential is associated with a transient increase in Na^+ conductance. The results suggest therefore that the shellfish samples block the Na^+-dependent depolarising phase without affecting the K^+-dependent repolarisation phase. These findings are very similar to those reported for the toxin effect from *Aphanizomenon flos-aquae*, a fresh water Cyanobacterium on crayfish giant axons (Sasner *et al.*, 1984).

Thus, where such a change in threshold has occurred (see Figure 5.5), CAP conduction velocity has been slowed by the blocking of a percentage of the Na^+ channels. As the point of recording is between the ground electrode situated in the bath containing the sample and the recording electrode in the adjacent bath the CAP amplitude has not had chance to recover. Therefore recordings were measured at a time when both amplitude and delay are similarly affected. The consistency of the way the two sets of data correspond implies that the delay could equally well be used as an evaluation criterion in circumstances where it may not be feasible to measure changes in amplitude. However, further experiments are required to explain the observations of the large increase in both inhibition and delay rates up to about 80 μg-100 μg followed by a plateau-like effect between those values and values up to about 500 μg, above which a sharp increase again occurred.

5.4.3. Recovery of the CAP

The ability of the sciatic nerve to withstand several toxin applications, each followed by a full recovery of the CAP agrees with results from other studies using STX extracted from *S giganteus* (Kao and Nishima 1965) or extracts of unknown toxicity from mussels (Evans 1969, 1970; Frietas *et al.,* 1992).

The recovery times of the CAP also showed good correlation with the potency of the sample.The fact that recovery reflected STX eq content so closely ($r^2 = 0.918$) strongly implies that as a measuring tool, recovery from inhibition would be equally as reliable as measuring inhibition of the CAP. In fact the two measures together should give a very reliable assessment measure.

Summary

The isolated frog sciatic nerve has proved to be a simple robust preparation which can be maintained for several hours and is capable of sustaining multiple sample applications without loss of the recovery response of the CAP. The sciatic nerve preparation has the advantage over the chick biventor and lobster neuromuscular preparations of being an assay system composed solely of nerve axons, without synapses or postsynaptic cells. The sensitivity of the CAP to the PSP samples yielded a lower detection range of *ca.* 2 ng/ml, making it more sensitive than the mouse bioassay (200 ng/ml) and equally as sensitive as the tissue culture bioassays (2.5 to 10 ng/ml).

Table 5.1. Summary of the PSP sample results obtained using the frog sciatic nerve compound action potential bioassay. Samples are listed in order of potency (μg STX) by the mouse bioassay, with their corresponding IT_{50} and IT_{90} values obtained from the sciatic nerve preparation.

Sample	μg STX	IT_{50} min	IT_{90} min	Species
49 †	606.8	0.6	1.25	Mussel
70 †	565.0	0.9	1.9	Mussel
180	312.0	1.2	2.85	Queen Scallop
30	310.8	0.9	2.85	Mussel
134	303.2	1.0	2.9	King Scallop
58 †	104.0	1.4	3.3	Mussel
176 †	81.6	1.2	2.5	Queen Scallop
69 †	80.0	1.5	3.5	King Scallop (g).
82	80.0	1.9	5.0	Mussel
138 †	75.4	6.0*	13.0*	Razor clam
22	40.0	6.0*	-	Mussel
1 †	0	-	-	Mussel
4	0	-	-	Mussel
15	0	-	-	King Scallop
101 †	0	-	-	Oyster

* extrapolated from available data

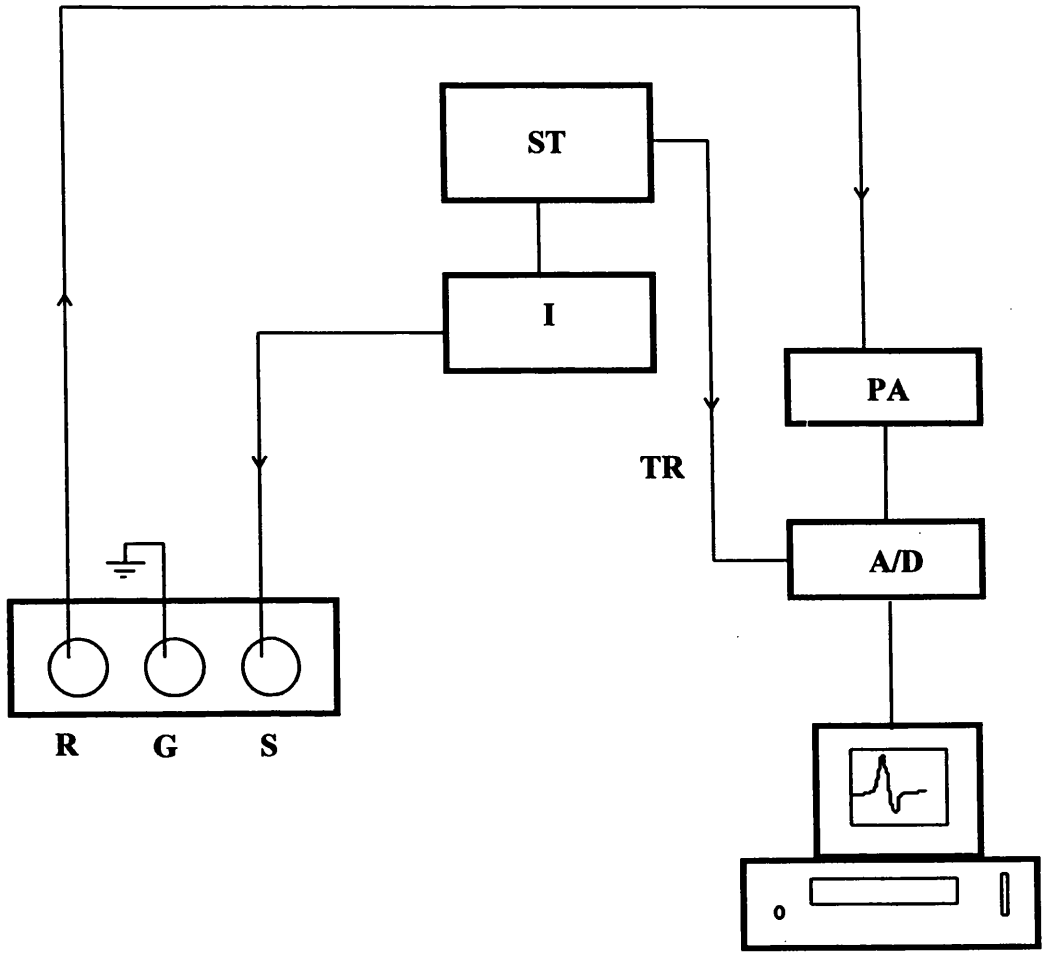
† these samples were also tested in the CBC assay (section 4)

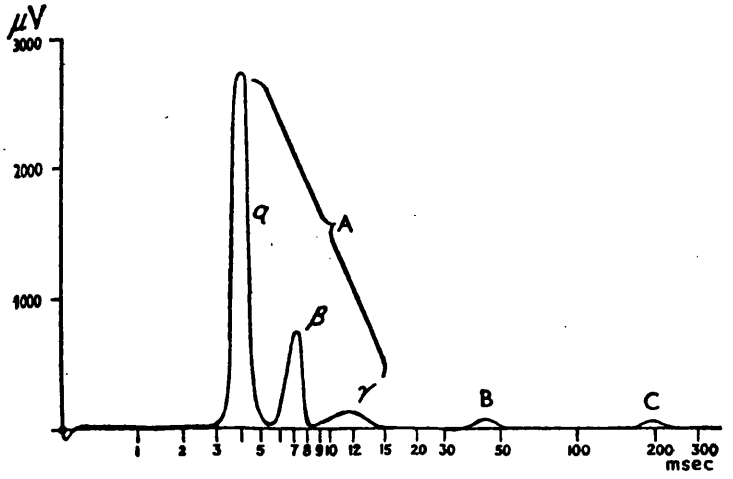
Figure 5.1.

a) Diagrammatic representation of the apparatus and wiring used in recording compound action potentials from the frog sciatic nerve using the Vaseline-gap method.

A/D....analogue/digital interface, **G**....ground electrode, **I**....Isolation unit, **PA**....preamplifier, **R**....recording electrode, **S**.... stimulating electrode, **ST**....stimulator, **T**....trigger.

Inset: (b) the alpha, beta and gamma components of the *A* complex of the sciatic nerve compound action potential. These are followed by the *B* and finally the *C* elevation spikes. **(c)** the action potential wave form generated in this study from recruiting only the *A* complex fibres.


ST
I
PA
TR
A/D
R
G
S
a)


μV
3000
2000
1000
q
A
β
γ
B
C
1
2
3
5
7
8
9
10
12
15
20
30
50
100
200
300
msec
b)

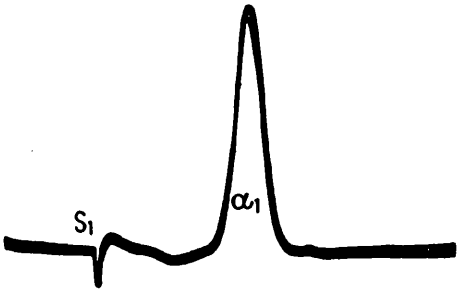
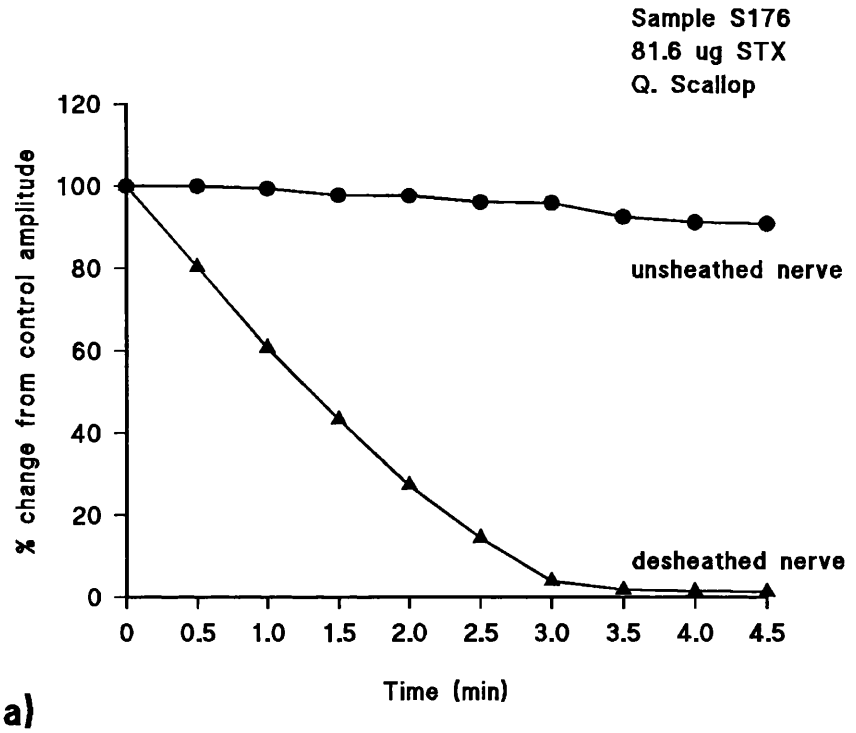

S1
α1
c)

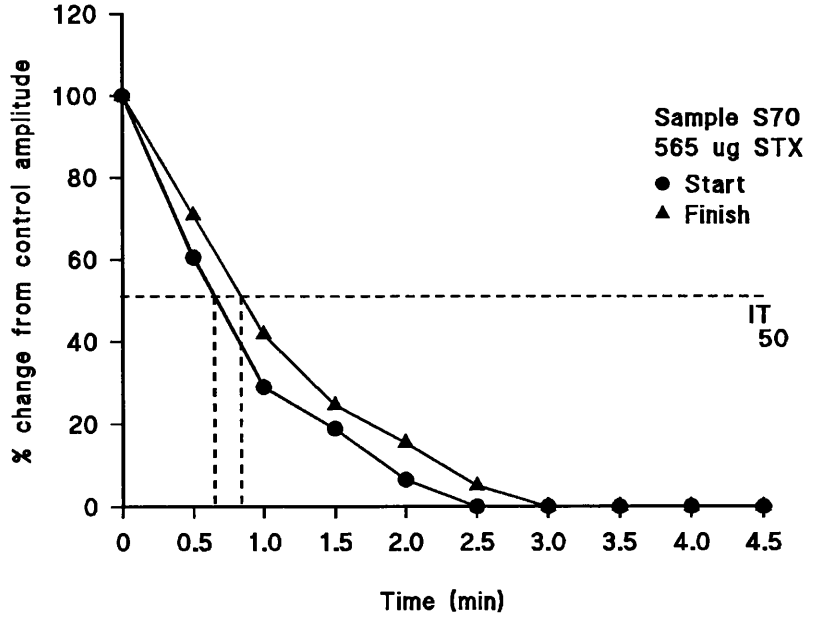
Figure 5.2.

a) The percentage changes from control values over a given time period of the amplitude of evoked action potentials from an unsheathed (•) and partially desheathed (♦) frog sciatic nerve when exposed to a PSP sample (S176) containing 81.6 μg STX eq.

b) The IT_{50} inhibition rates following application sample S70 (565 μg STX eq) as the first toxin applied at the start of the experiment and again at the end of the experiment after a 4 hr interval, during which five other samples were tested.


Sample S176
81.6 ug STX
Q. Scallop
% change from control amplitude
120
100
80
60
40
20
0
unsheathed nerve
desheathed nerve
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

a)


% change from control amplitude
120
100
80
60
40
20
0
Sample S70
565 ug STX
Start
Finish
IT
50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

b)

Figure 5.3.

Results from typical experiment showing the inhibition (plain bars) and recovery values (striped bars) of the compound action potential when a partially desheathed nerve was exposed to both STX and a number of PSP samples of varying toxicities. Recovery values (R) are in excess of 95% each time.

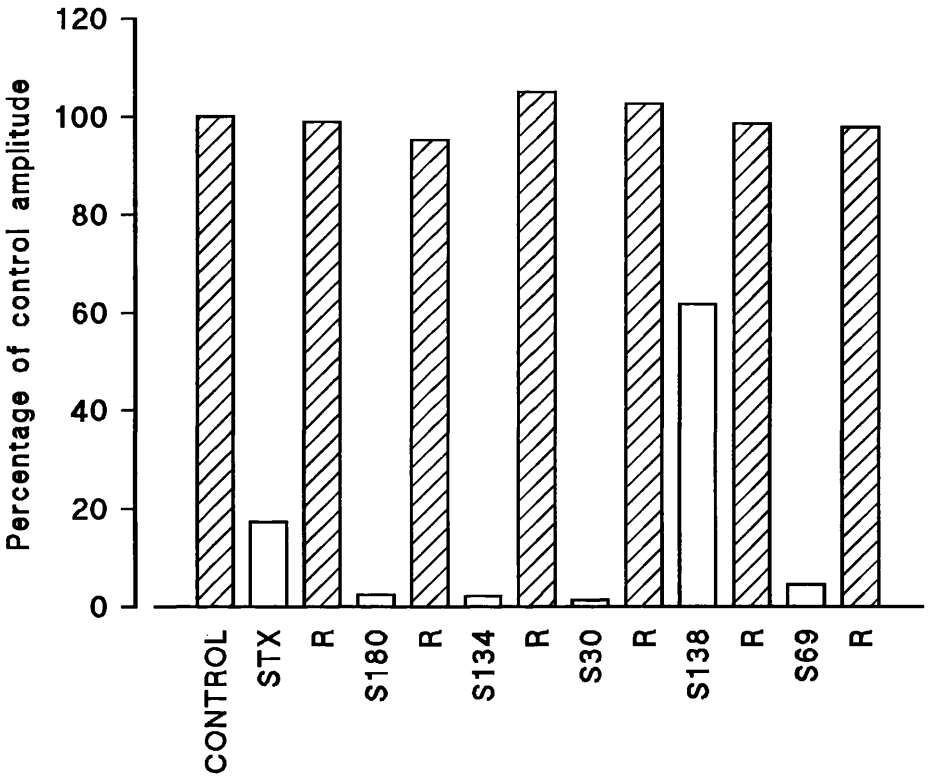

Percentage of control amplitude
120
100
80
60
40
20
0
CONTROL
STX
R
S180
R
S134
R
S30
R
S138
R
S69
R

Figure 5.4.

Representative sets of compound action potentials (CAPs) recorded from the frog sciatic nerve. Each set comprises four traces: a control, 0.25, 0.5 and 1.0 min time points. The last record (1.0 min) corresponds to the time to total block of the CAP by the most potent PSP sample S49.

Top left set: control (crtl). Top right set: 100 nM STX (STX). The remaining four sets are of CAPs recorded in the presence of S4, S101, S22 and S138.

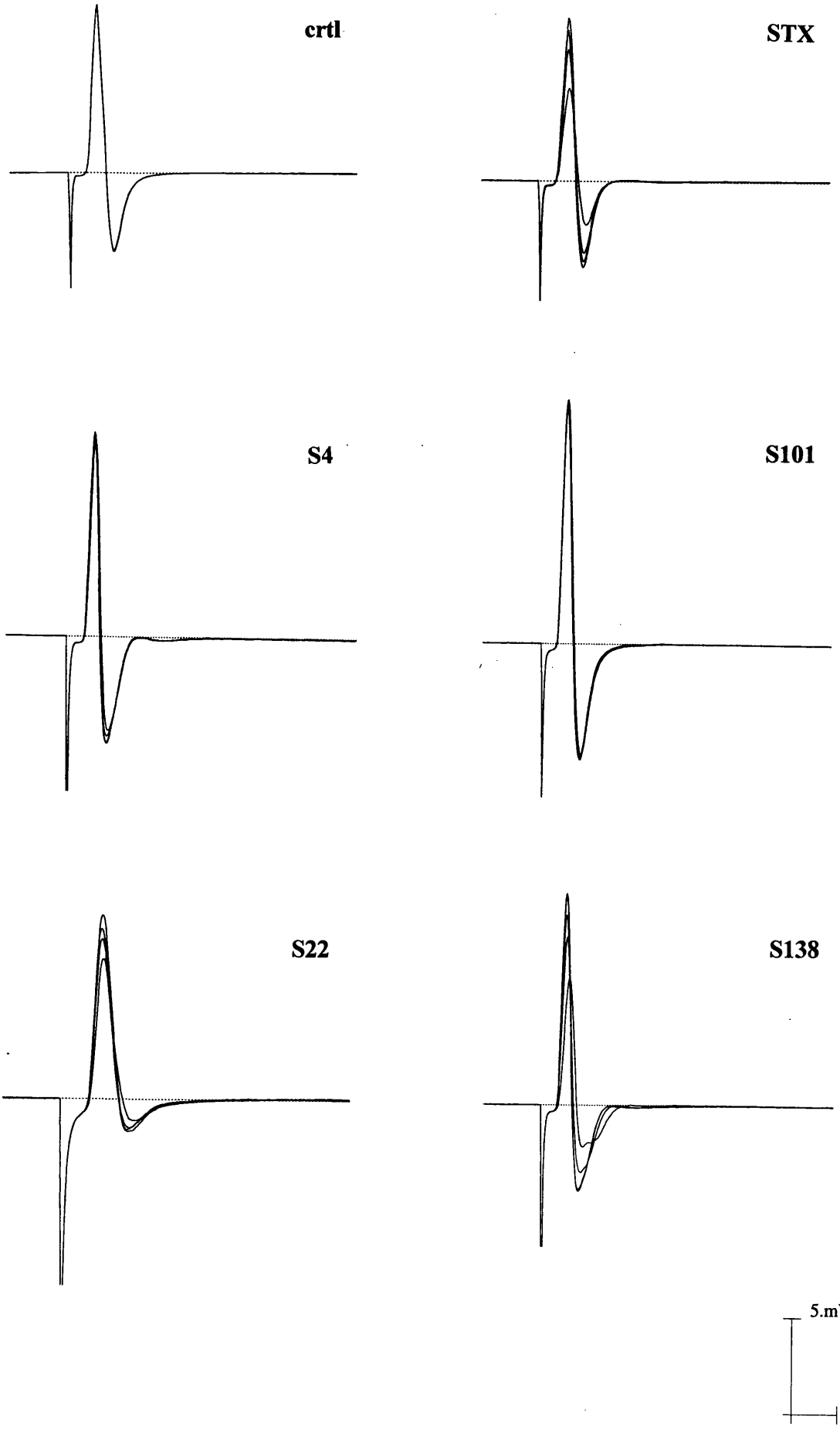

crtl
STX
S4
S101
S22
S138
5.mV
2.05ms

Figure 5.5.

Six representative sets of frog sciatic nerve CAPs each composed of four superimposed records as described in Figure 5.4. The sets are laid out in order of increasing PSP toxicity (left to right and down the page). The final set, S49 (606 8 μg STX eq.), shows a graded decrease in the CAP, which is then abolished by the 1.0 min time.

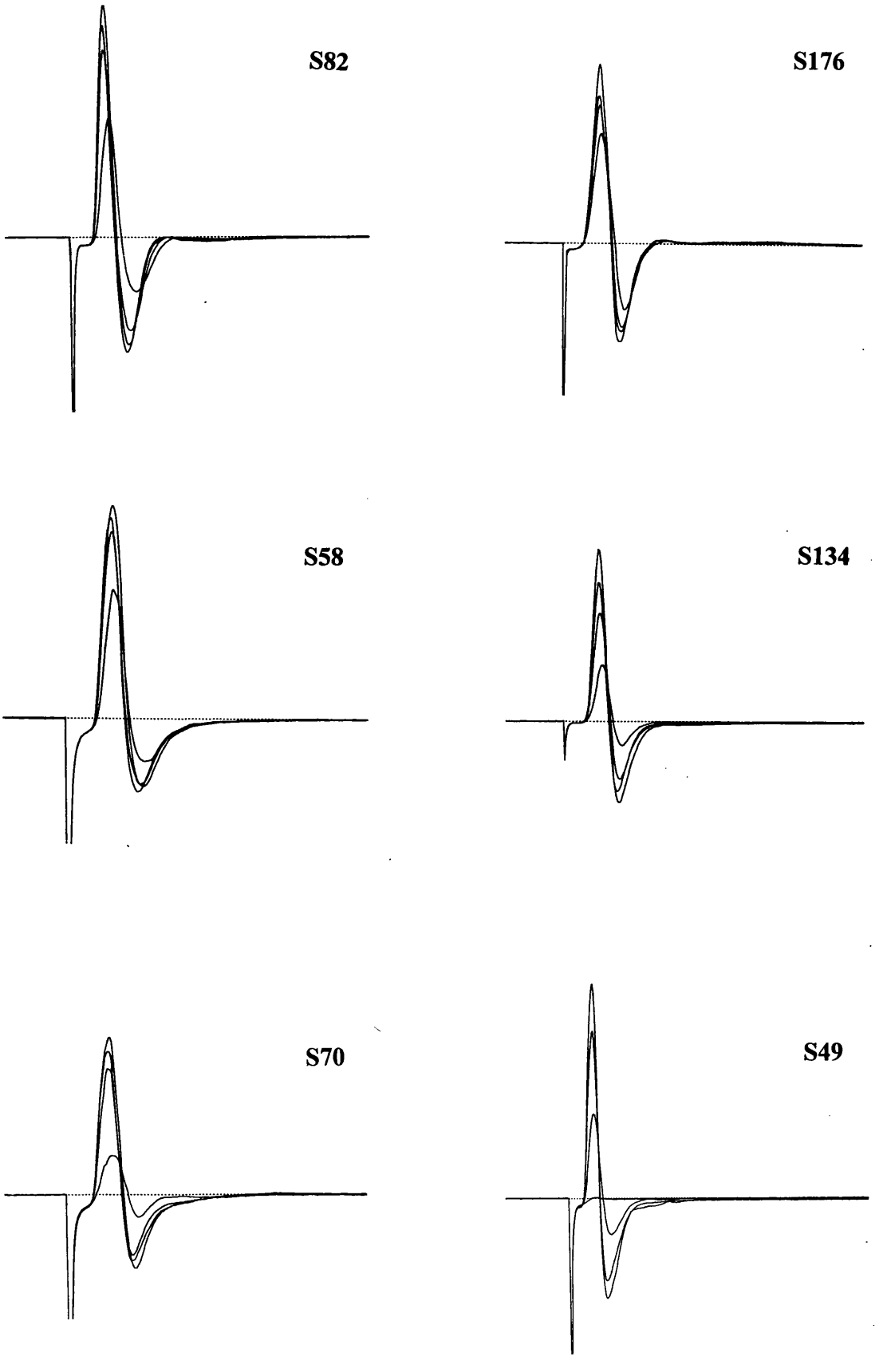

S82
S176
S58
S134
S70
S49
5.mV
2.05ms

Figure 5.6.

The relationship between the CAP inhibition rate (•), the delay in rise time (generation) of the CAP (*), and the STX eq. value in the sample.

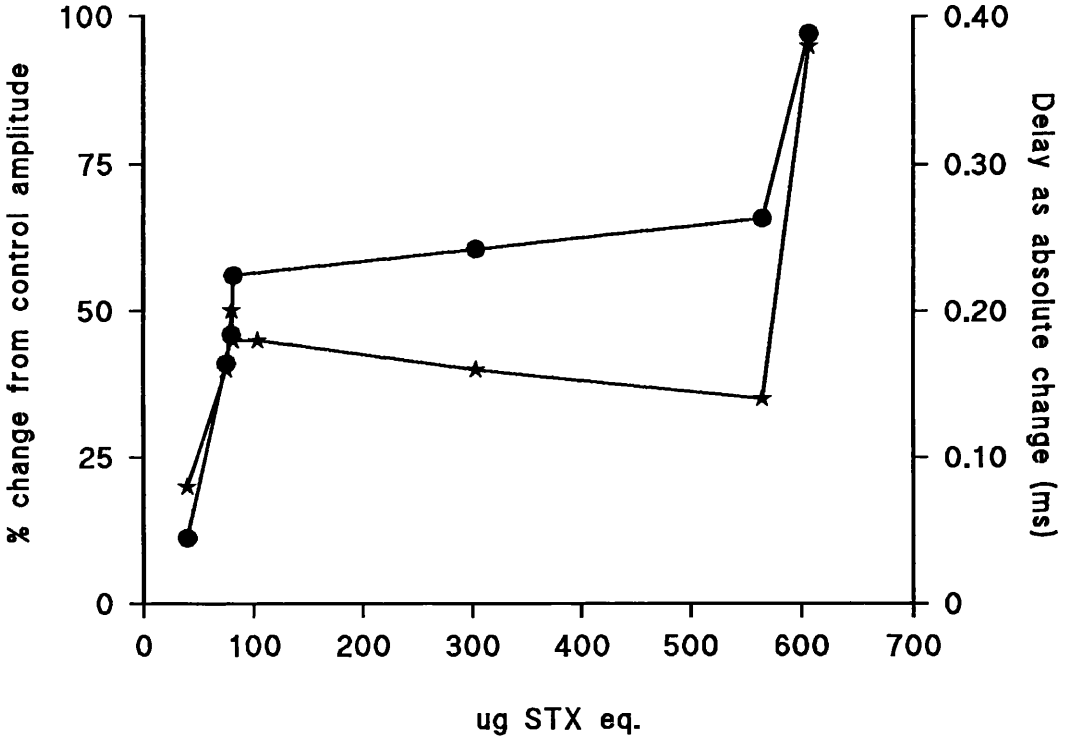
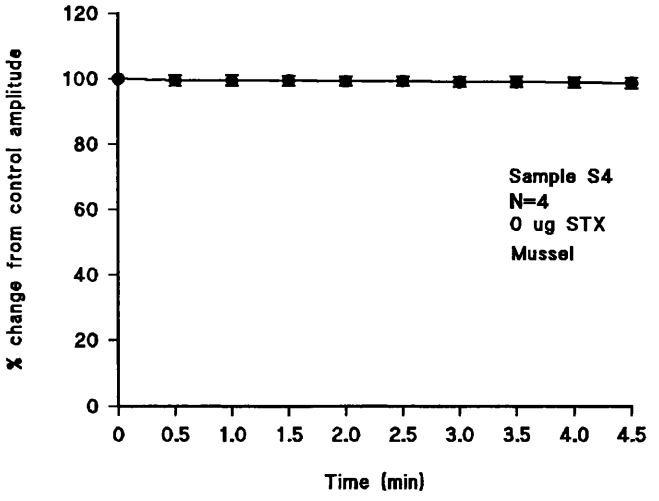
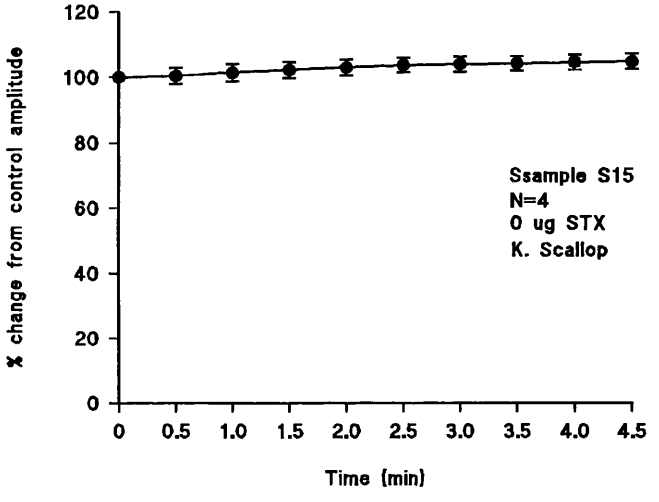

100
75
50
25
0
% change from control amplitude
0.40
0.30
0.20
0.10
0
Delay as absolute change (ms)
0
100
200
300
400
500
600
700
ug STX eq.

Figure 5.7.

The application of three negative PSP samples S4, S15, and S101 (top, middle and bottom traces respectively) had no inhibitory effect on the frog nerve CAP when compared with control values recorded over the same time period. Data points are means ± s.e.m.


% change from control amplitude
120
100
80
60
40
20
0
0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
Time (min)
Sample S4
N=4
0 ug STX
Mussel


% change from control amplitude
120
100
80
60
40
20
0
0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
Time (min)
Ssample S15
N=4
0 ug STX
K. Scallop

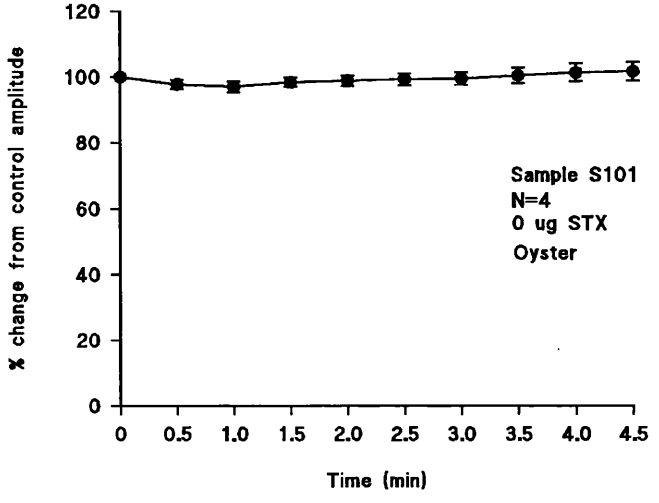
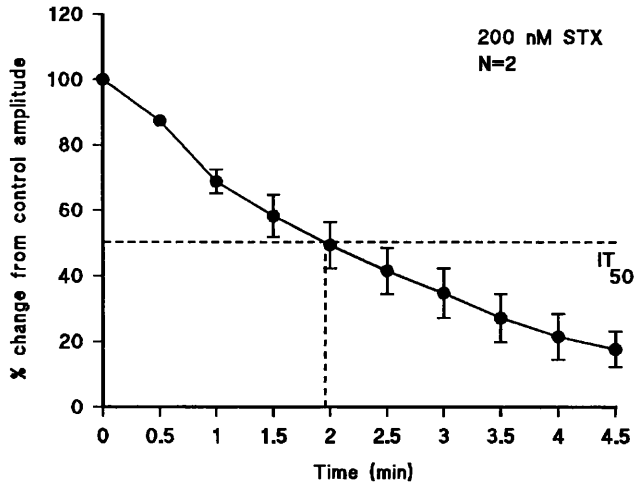
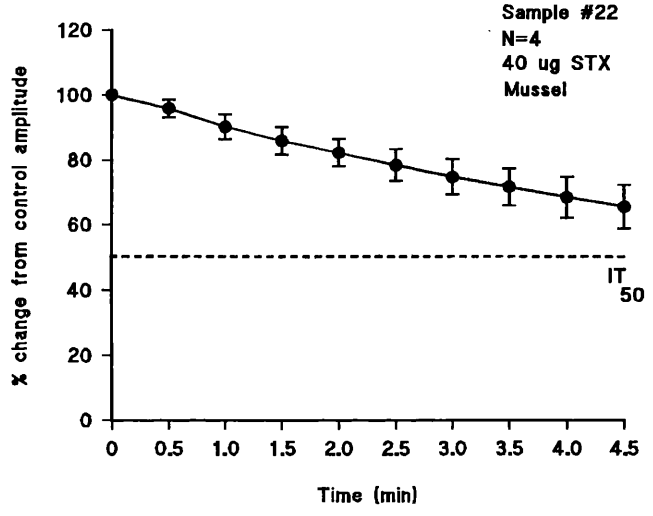

% change from control amplitude
120
100
80
60
40
20
0
0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
Time (min)
Sample S101
N=4
0 ug STX
Oyster

Figure 5.8.

The inhibitory effect on the frog CAP of 200 nM STX (top), sample S22 (40 μg STX eq) (middle) and sample S138 containing (75.4 μg STX eq) (bottom). Data points are means ± s.e.m. Note that the IT_{50} point for 200 nM STX is 2.0 min, but that no IT_{50} was measurable for the two samples.


200 nM STX
N=2
% change from control amplitude
120
100
80
60
40
20
0
IT50
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Time (min)


Sample #22
N=4
40 ug STX
Mussel
% change from control amplitude
120
100
80
60
40
20
0
IT50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

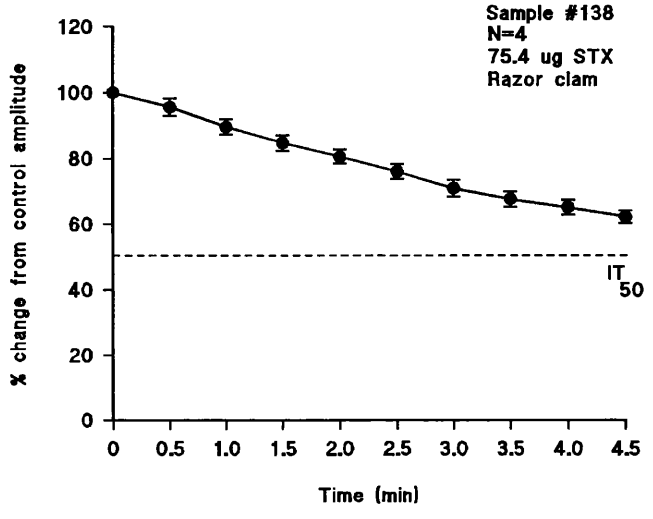
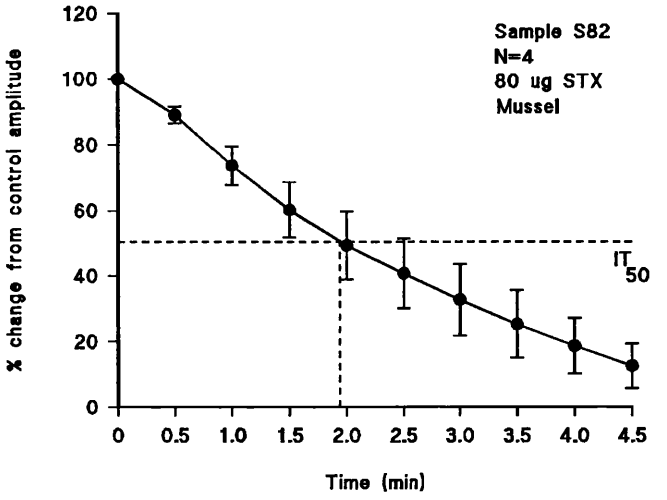
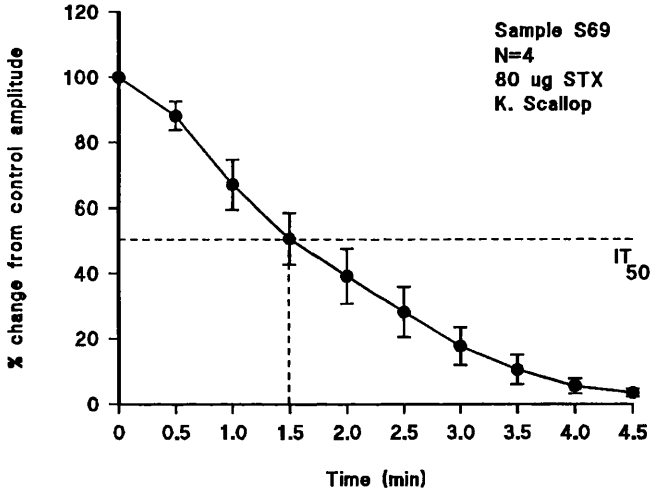

Sample #138
N=4
75.4 ug STX
Razor clam
% change from control amplitude
120
100
80
60
40
20
0
IT50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

Figure 5.9.

The top and middle data sets show the inhibition curves for two samples, S82 and S69, whose toxicity corresponded with that of the official AOAC (1984) safety level for PSP contamination of 80 μg STX. The bottom data set shows the inhibition curve for sample S176 (81.6 μg STX) which just exceeds the AOAC toxicity safety level. Data points are means ± s.e.m.


Sample S82
N=4
80 ug STX
Mussel
% change from control amplitude
120
100
80
60
40
20
0
IT50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)


Sample S69
N=4
80 ug STX
K. Scallop
% change from control amplitude
120
100
80
60
40
20
0
IT50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

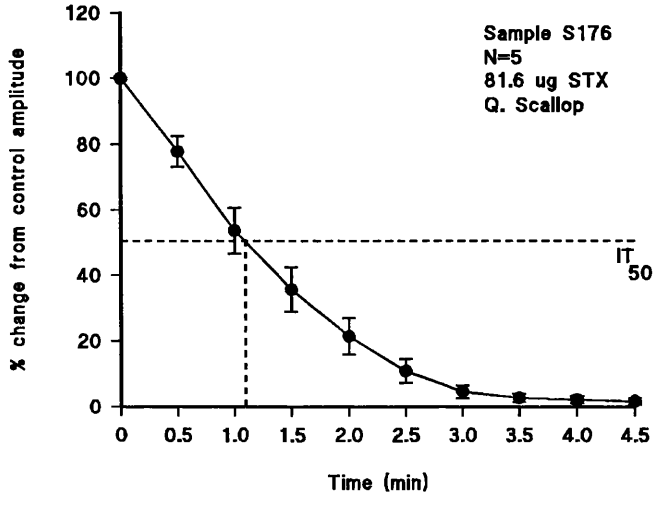
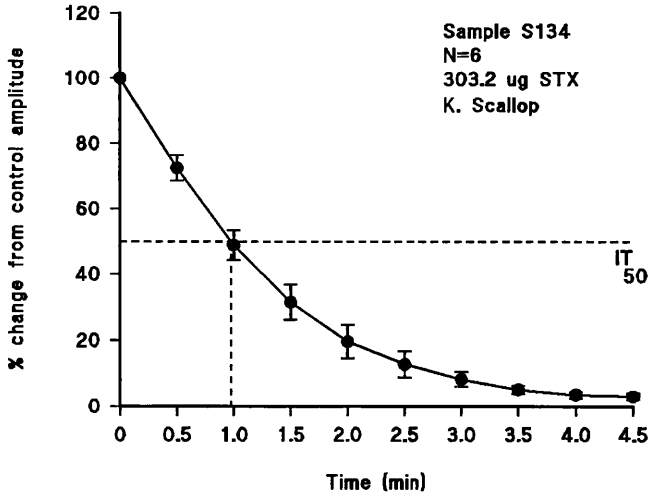
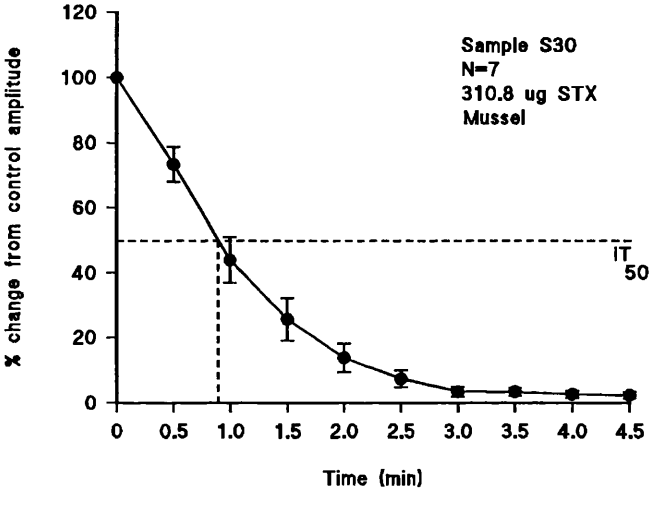

Sample S176
N=5
81.6 ug STX
Q. Scallop
% change from control amplitude
120
100
80
60
40
20
0
IT50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

Figure 5.10.

Inhibition curves for three PSP samples that contain a minimum of 300 μg STX eq., by mouse bioassay. Points are means ± s.e.m. The top and middle data sets for S134 (303.2 μg STX eq.) and S30 (310.8 μg STX eq.) have an IT_{50} value of just less than 1 min. Sample S180 with an STX eq., of 312 μg has an IT_{50} of 1.2 min.


Sample S134
N=6
303.2 ug STX
K. Scallop
% change from control amplitude
120
100
80
60
40
20
0
IT
50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)


Sample S30
N=7
310.8 ug STX
Mussel
% change from control amplitude
120
100
80
60
40
20
0
IT
50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

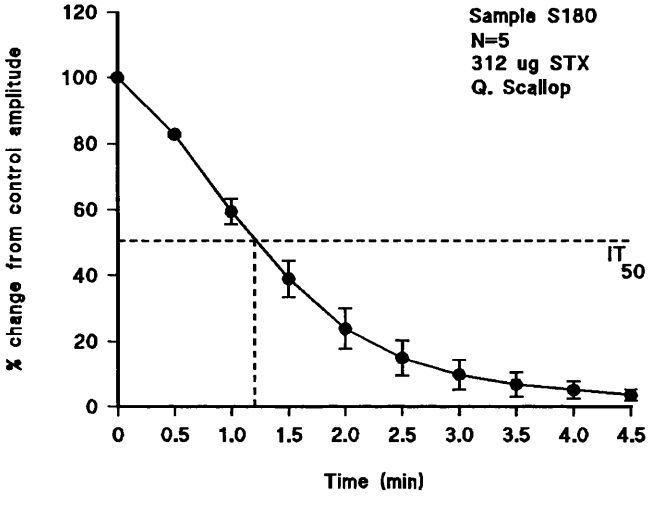
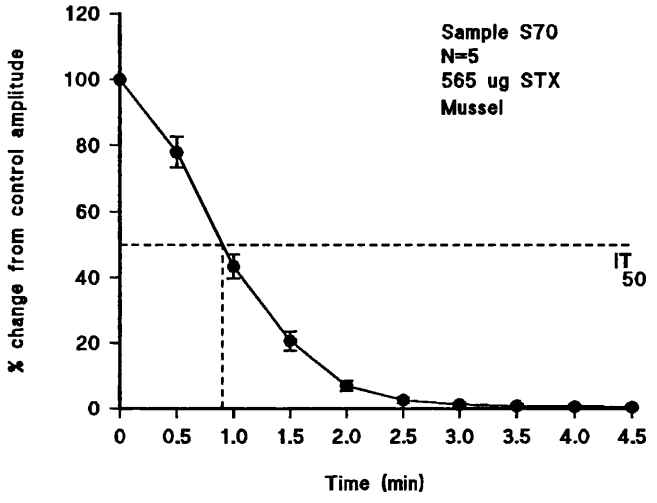

Sample S180
N=5
312 ug STX
Q. Scallop
% change from control amplitude
120
100
80
60
40
20
0
IT
50
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

Figure 5.11.

Inhibition curves for the two most potent PSP samples available from the Ring Trial. Data points are means ± s.e.m. Sample S70 (565 μg STX eq.) had an IT_{50} point of just under 1 min. Sample S49 (606.8 μg STX eq.) inhibited the CAP within 1.5 min, with an IT_{50} of around 0.5 min.


Sample S70
N=5
565 ug STX
Mussel
IT 50
% change from control amplitude
Time (min)
120
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

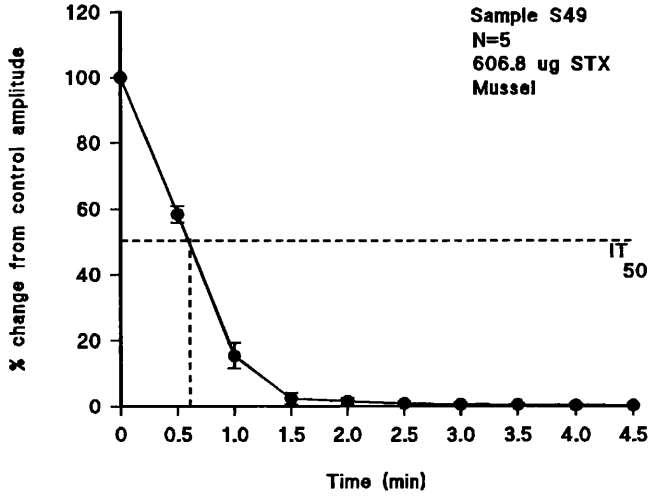

Sample S49
N=5
606.8 ug STX
Mussel
IT 50
% change from control amplitude
Time (min)
120
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

Figure 5.12.

The mean inhibitory values for each PSP sample plotted as individual data points.

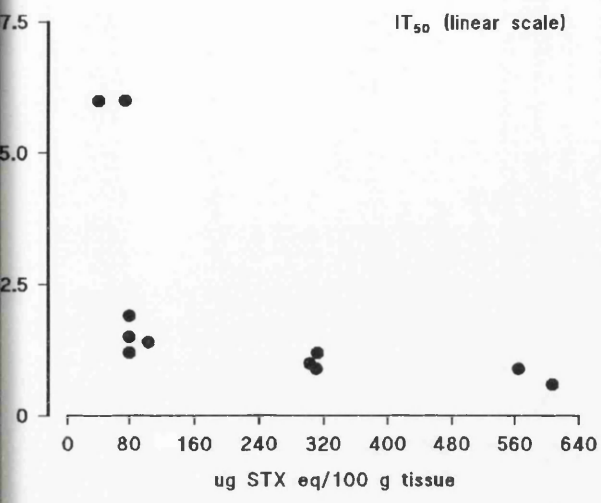
a) The IT_{50} time points plotted on a linear scale.

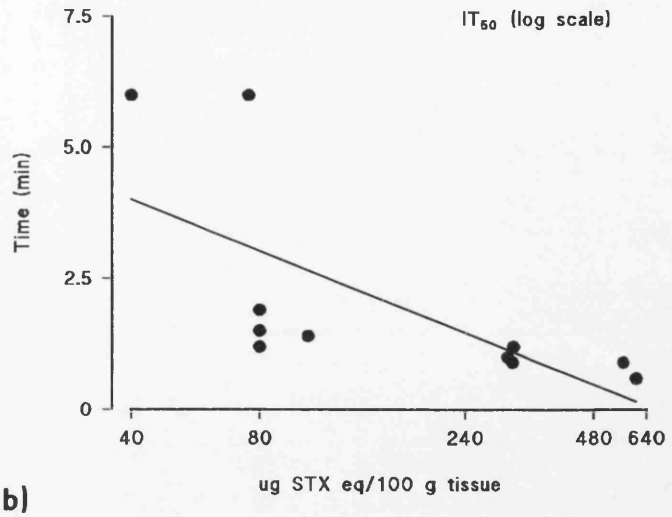
b) Logarithmic transformation of the IT_{50} times points.

c) IT_{90} time points plotted on a linear scale.

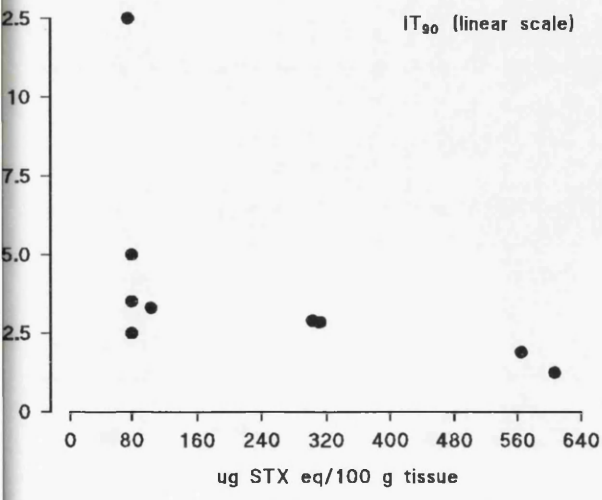
d) Logarithmic transformation of the IT_{90} times points.

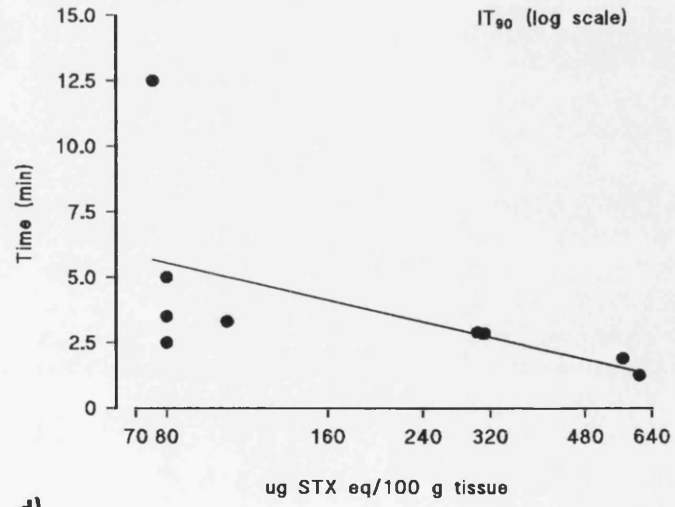
A linear regression line fitted to the mean data points (b) and (d) yeilded a correlation of $r^2 = 0.452$ for IT_{50} and $r^2 = 0.308$ for IT_{90}.


IT_{50} (linear scale)
7.5
5.0
2.5
0
0 80 160 240 320 400 480 560 640
ug STX eq/100 g tissue


IT_{50} (log scale)
Time (min)
7.5
5.0
2.5
0
40 80 240 480 640
ug STX eq/100 g tissue

b)


IT_{90} (linear scale)
12.5
10
7.5
5.0
2.5
0
0 80 160 240 320 400 480 560 640
ug STX eq/100 g tissue

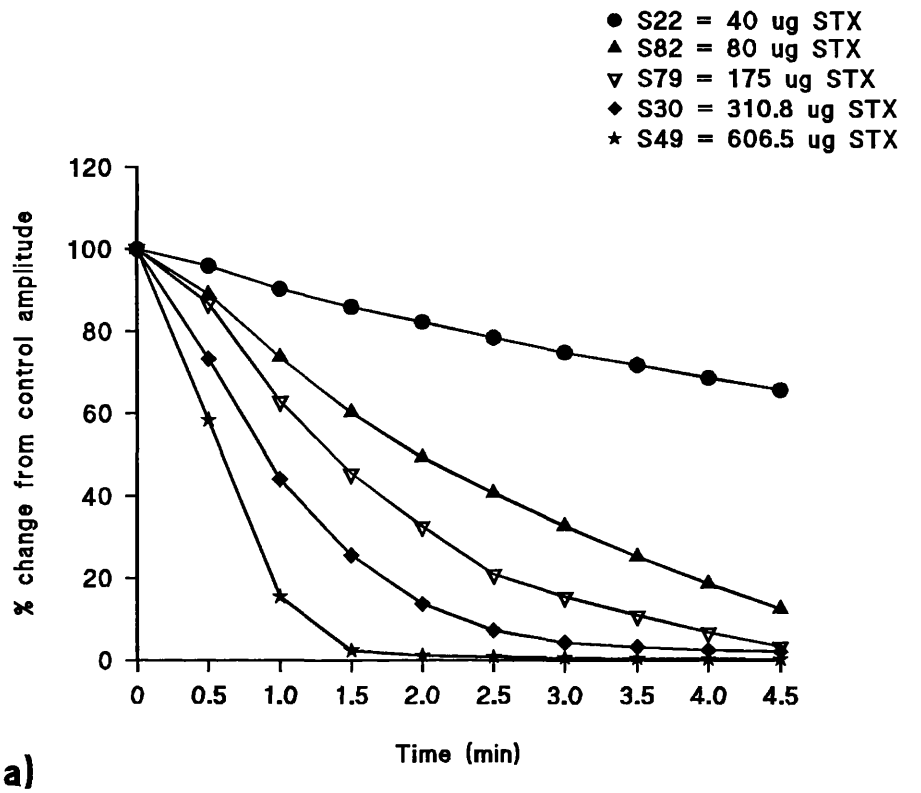

IT_{90} (log scale)
Time (min)
15.0
12.5
10.0
7.5
5.0
2.5
0
70 80 160 240 320 480 640
ug STX eq/100 g tissue

d)

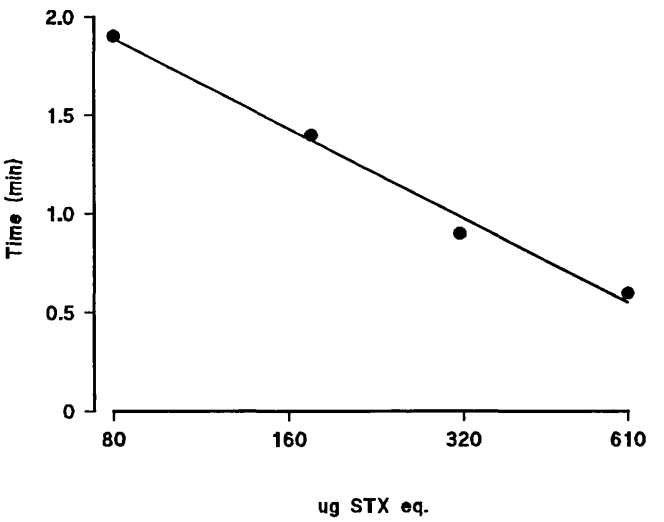
Figure 5.13.

a) A set of inhibition curves calculated from PSP samples of varying STX concentration which were all derived from contaminated mussels. The samples were applied in increasing toxicity beginning with the 40 μg sample. A wash-recovery period followed application of each sample. Each sample was chosen as being approximately twice the potency value of the previous sample applied.

b) The IT_{50} values for each sample equal to or above the AOAC safety limit of 80 μg. A linear regression is fitted to the IT_{50} values ($r^2 = 0.989$).


● S22 = 40 ug STX
▲ S82 = 80 ug STX
▽ S79 = 175 ug STX
◆ S30 = 310.8 ug STX
★ S49 = 606.5 ug STX
% change from control amplitude
120
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (min)

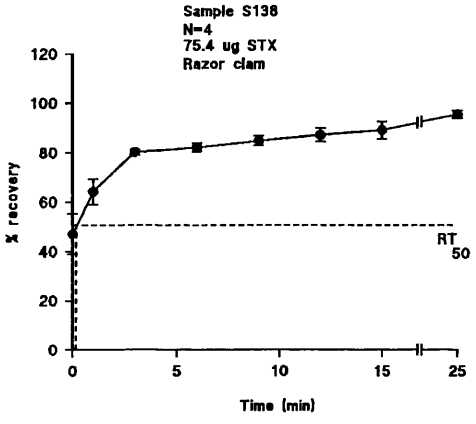
a)

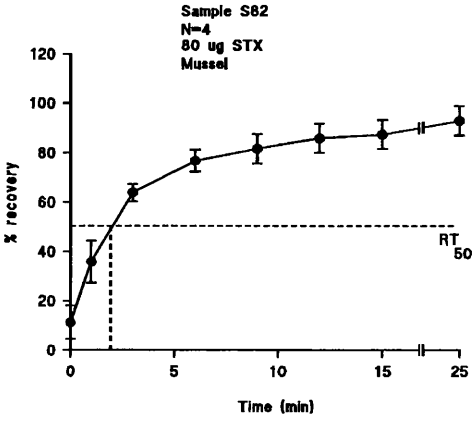

Time (min)
2.0
1.5
1.0
0.5
0
80
160
320
610
ug STX eq.

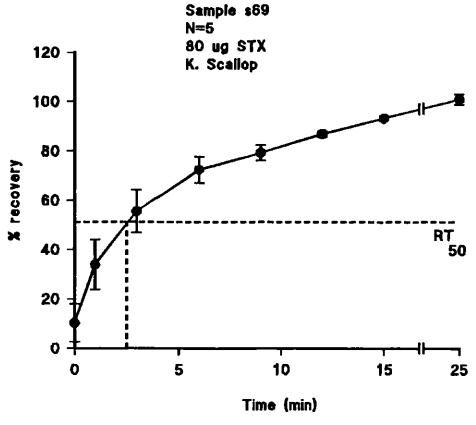
b)

Figure 5.14.

Recovery curves for PSP samples that contained approximately 80 μg STX eq. The rapid RT_{50} for S138 is attributed to the intial weak inhibitory effect on the CAP. Note the recovery curve for each sample slows down after 75-80% recovery of the CAP amplitude. Data points are means ± s.e.m.


Sample S138
N=4
75.4 ug STX
Razor clam
% recovery
Time (min)
RT
50


Sample S82
N=4
80 ug STX
Mussel
% recovery
Time (min)
RT
50


Sample s69
N=5
80 ug STX
K. Scallop
% recovery
Time (min)
RT
50

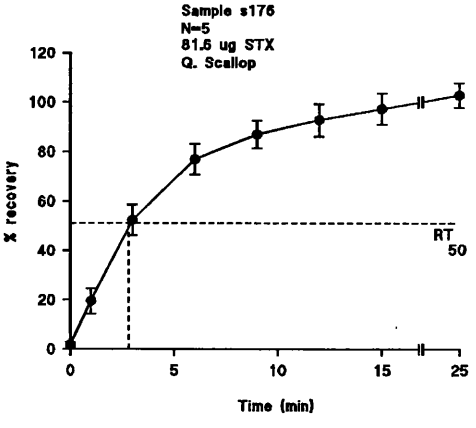
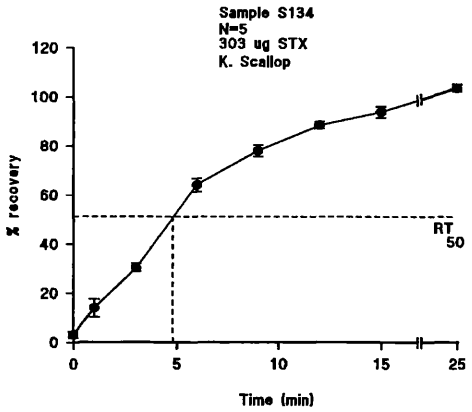
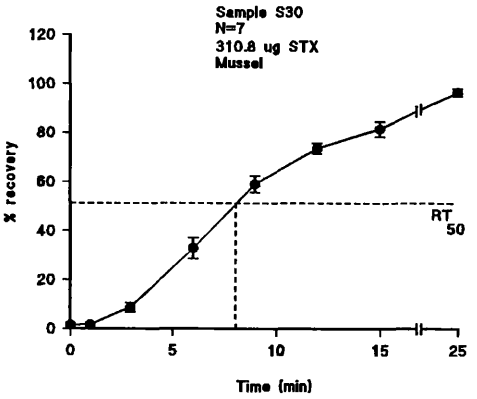
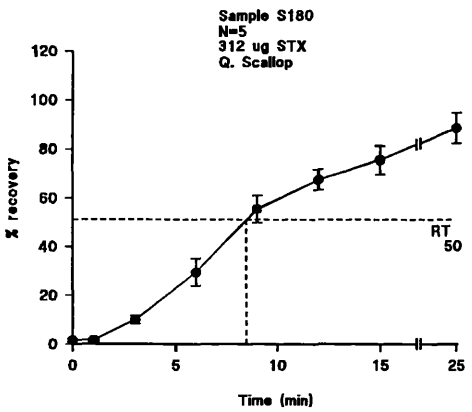

Sample s176
N=5
81.6 ug STX
Q. Scallop
% recovery
Time (min)
RT
50

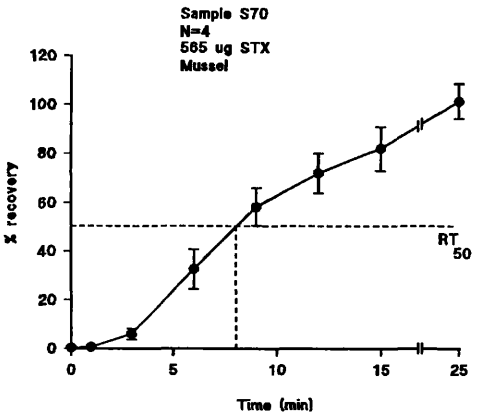
Figure 5.15.

Recovery curves for PSP samples containing 300 μg STX eq., or above. Data points are means ± s.e.m. Once the CAP had begun to recover, following complete inhibition, the curves followed by a fairly linear recovery line.


Sample S134
N=5
303 ug STX
K. Scallop
% recovery
Time (min)
RT
50


Sample S30
N=7
310.8 ug STX
Mussel
% recovery
Time (min)
RT
50


Sample S180
N=5
312 ug STX
Q. Scallop
% recovery
Time (min)
RT
50


Sample S70
N=4
565 ug STX
Mussel
% recovery
Time (min)
RT
50

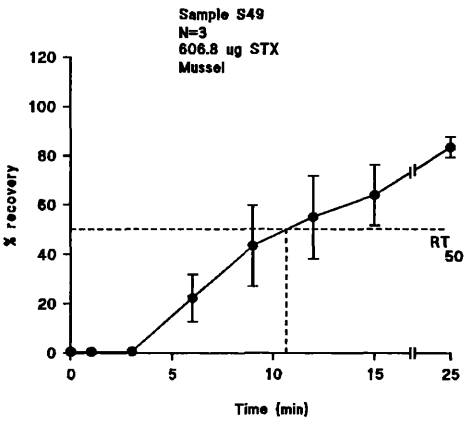
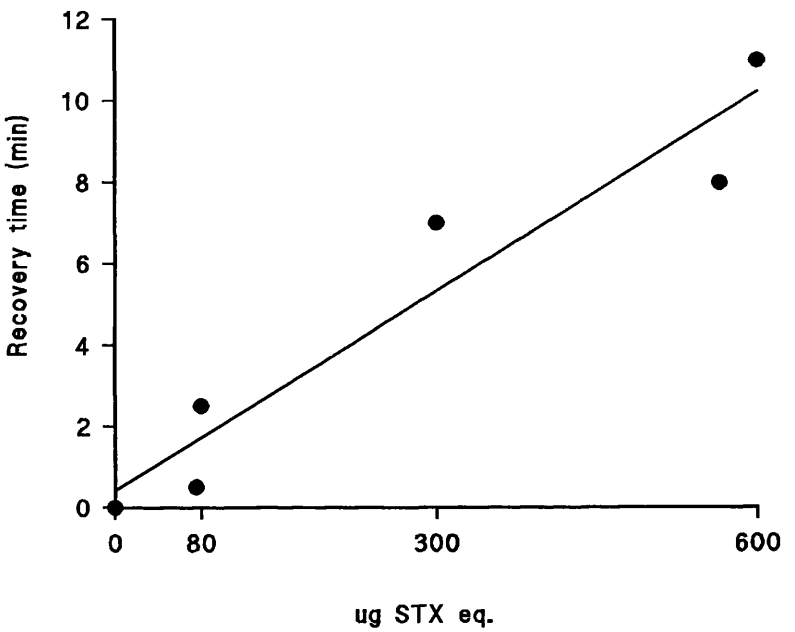

Sample S49
N=3
606.8 ug STX
Mussel
% recovery
Time (min)
RT
50

Figure 5.16.

Mean recovery values of the frog CAP. A linear regression is fitted to the mean RT_{50} values for the samples ($r^2 = 0.918$). Note recovery is dependent on the concentration of STX in a sample.


12
10
8
6
4
2
0
Recovery time (min)
0
80
300
600
ug STX eq.

Chapter 6

Neuro 2a Cell Line Membrane Properties Using Intracellular and Photographic Methods

6.1. INTRODUCTION

Use of tissue culture bioassays for the detection of PSP toxins has increased as ethical pressures have mounted to develop an alternative to the mouse bioassay. The cell line Neuro-2a (N2a) cloned from the C-1300 mouse neuroblastoma, which originated as a spontaneous tumour in the body cavity of an albino A/J mouse in the region of the spinal cord, has been used extensively (Kogure *et al.,* 1988; Jellet *et al.,* 1992; Gallacher and Birkbeck 1992; Manger *et al.,* 1993, 1994, 1995a, 1995b; Hamasaki *et al.,* 1996; Truman and Lake 1996).

The original tumour was isolated by A.M. Claudman in 1940, and was maintained for many years by sub-cutaneous transfer in other A/J mice. Establishment of functional clonal cell lines occurred around the late 1960's using an animal-culture alternate passage technique (Augusti-Tocco and Sato 1969; Schubert *et al.*, 1969). Cells maintained in suspension retained a spherical appearance similar to that found in the tumour *in vivo,* and divided rapidly. However, when placed in culture onto a surface to which they could adhere, the tumour cells underwent striking morphological changes. Many long processes (neurites) extended from the cell body, similar to those formed by nerve cells *in vivo*, which then formed loose networks between the cells.

6.1.1. Factors influencing differentiation processes

The phenomemon of neurite extension by these neuroblastomas has been shown to be inversely related to the number of dividing cells in the culture, which in turn is related to the concentration of serum in the culture media (Seeds *et al.*, 1970). Almost all cells incubated without serum extend neurites, and cell division decreases markedly. In the presence of 10% serum, few cells extend processes and after a short lag the culture begins to grow logarithmically. Higher serum concentrations (10-20%) induced neurite retraction. These observations suggest that neurite extension is linked to certain stages of the life cycle, rather than being dependent on *de novo* protein synthesis (Seeds *et al.*, 1970; Furmanski 1973).

Several other factors may also be critical (Schubert *et al.*, 1973). These include cell density (which induces differentiation through either the accumulation of metabolic secretions by the cells, or by increased cell-cell contact) and the interaction between the cell and the surface of the culture dish (which may control macromolecular synthesis).

The isolation of clones and subclones has followed these initial culture studies, and it has become evident that the various clones differ in their morphology, level of protein and enzyme synthesis and electrophysiological properties. Despite the presence of some genetic instability, most clones maintain and express specific neuronal properties for extended periods (Kimhi 1981). Various characteristics define differentiation, including a change in morphology, specific antigens appearing on the membrane, the appearance of specific weight glycoproteins, increased levels of neurotransmitter synthesizing enzymes or establishment of functional synapses (Augusti-Tocco *et al.*, 1973; Littauer *et al.*, 1979)

Furthermore, treatments with various chemical agents such as dibutyryl cyclic AMP (dbcAMP), dimethylsulfoxide (DMSO), hexamethylene bisacetamide (HMBA) prostaglandin E_1 (PGE_1), nerve growth factor (NFG) or isobutylmethylxanthine (IBMX), all induce morphological or electrical differentiation in various neuroblastoma cells. The degree of these effects depends on the clone or hybrid type (Kimhi *et al.*, 1976; Spector 1981; Docherty *et al.*, 1991; Quandt 1994).

6.1.2. Electrophysiological properties of C-1300 neuroblastoma and clones

The early studies established that cells cultured from the C-1300 tumour were of neural origin. They had the ability to produce key enzymes for the synthesis of neurotransmitters (Augusti-Tocco and Sato 1969; Schubert *et al.*, 1969) and to generate action potentials (Nelson *et al.*, 1969). Intracellular recordings from morphologically-differentiated cells showed that they could be stimulated electrically to generate action potentials with amplitudes of up to 85 mV (Schubert *et al.*, 1969).

Over the last two decades it has been shown that electrical excitability and its ionic dependence in neuroblastoma cells undergo development during differentiation or maturation. By inference, this reflects a change in the ion channels involved. During the differentiation of neuroblastoma cells, four classes of responses have been observed: passive responses, partial responses, delayed rectification and action potentials (Nelson *et al.*, 1969; Harris and Dennis 1970). Marked changes in spike amplitude and configuration, and maximal rates of change, are observed depending on culture conditions and state of growth (Schubert *et al.*, 1969).

Neuroblastoma cells in suspension, which are unable to differentiate, can exhibit active membrane responses including action potentials (Schubert *et al.,* 1973), although these are actually Ca^{2+}-dependent, not Na^{+}-dependent (Miyake 1978). Similarly, morphologically undifferentiated human neuroblastoma (SK.N.SH) cells are relatively inexcitable and are deficient in the Na^{+}-dependent component of the action potential generating mechanism (Kuramoto *et al.*, 1977).

During normal differentiation, Ca^{2+} currents appear first (Docherty *et al.*, 1991), and, as morphological differentiation continues, the cells exhibit action potentials with separate Na^{+}- and Ca^{2+}-dependent peaks. Delayed rectification also appears about this time (Spitzer 1979; Spector 1981). Culture conditions are important at this point: for example, over-confluency can lead to a loss in excitability (Docherty *et al.*, 1991).

This development of electrical excitability is reflected in an increase in the amplitude of the action potential and increased rates of change of both its depolarising and repolarising phases. The ability to generate repetitive discharges also appears, reflecting modifications in the Na^{+} and K^{+} conductances and in the expression of the underlying voltage-activated channels for Na^{+}, Ca^{2+}, and K^{+}; a Ca^{2+}-activated K^{+} channel is the last to appear. In contrast to marked changes in both the Na^{+} and K^{+} conductances, the Ca^{2+} conductance does not alter significantly in its kinetics during development (Moolenaar and Spector 1978).

Resting membrane potential (RMP) has also been cited as a characteristic of cell differentiation (Nelson *et al.*, 1971; Kimhi *et al.*, 1976; Kuramoto *et al.*, 1977; Miyake 1978), with reported measurements ranging from about -10 mV to -70 mV for various neuroblastoma and hybrid lines. Low RMPs (-10 mV to -30 mV) characterise exponentially growing cells, whilst cells in a stationary phase exhibit RMPs of -15 mV to -50 mV. Cells considered differentiated produce more hyperpolarised RMP values of -45 to -55 mV. Culture and growth conditions, including treatment by chemical agents such as DMSO (Kimhi *et al.*, 1976), also cause changes in RMP; these changes appear to be developmentally regulated (Spector 1981).

6.1.3. Development of TTX and STX sensitivity in electrically excitable cells

The mature Na^+ channel is a heterometric complex of three glycoprotein subunits, with the α and β2 subunits being linked by disulphide bonds (see **section 1.13**). The three subunits undergo several steps of post-translational processing before a structurally mature sodium channel is formed (Catterall *et al.*, 1986). Isotopic labelling methods of embryonic rat brain and of neonatal rat brain *in vivo*, revealed that two thirds of the α subunits are not linked to the β2 subunits by disulphide bonds. These free α subunits are full-sized, membrane-associated, and have the complex carbohydrate chains of mature α subunits, but are unable to bind STX (Catterall 1986). It is assumed that they form a reserve of Na^+ channels utilized during development (Schmidt *et al.*, 1985).

This theory is supported by findings that neurotoxin activation of voltage-dependent Na^+ channels provokes a rapid channel down-regulation in cultured immature rat brain neurones, resulting in a 50% to 70% decrease in [^{3}H] STX binding capacity. However no internalisation of Na^+ channels was observed with mature neurones (Dargent *et al.*, 1994). These findings support the theory of a shift during differentiation from Ca^{2+}-dependency to Na^+-Ca^{2+}-dependency, and finally to Na^+-dependency (Nelson *et al.*, 1969; Harris and Dennis 1970).

Induced differentiation causes SH-SY5Y cells, a clone of SK.N.SH neuroblastoma cells, to become extremely STX sensitive, with complete inhibition of the Na^+ current at a low (4 nM) STX concentration (Brown *et al.*, 1994). Action potentials could be elicited from the human neuroblastoma cell line LA-N-5 before the cells are induced to differentiate with retinoic acid. However, as reported for other neuroblastoma cells, the Na^+ current was composed of two components: a STX-resistant one and a STX-sensitive one. The STX-resistant component was activated at a more negative voltage. After differentiation the total Na^+ current density increased significantly, but the STX-resistant component did not. These results confirm the up-regulation of STX-sensitive channels during differentiation (Weiss and Sidell 1991).

The action potential generating mechanism in various neuroblastoma lines has been found to be largely, but not completely blocked by TTX (Nelson 1973). Intracellular recordings of cells from clones NIE-115 and N-18 showed that TTX inhibited a fast-rising action potential, but had almost no effect on a slower second peak, which could be eliminated by Co^{2+} (i.e. was Ca^{2+}-dependent) (Spector *et al.*, 1973). In the clonal nerve cell line PC12, derived from a rat pheochromcytoma, the Na^+ channels were relatively resistant to 5×10^{-6} M TTX when exposed within two days of plating. This is a 20-50 fold higher concentration than required to inactivate mature nerve cells (Stallcup 1979). A comparison of six different cell types of neuronal, cardiac or skeletal muscle origin for TTX sensitivity or resistance, suggested that there could be a sequential appearance of isoforms of Na^+ channels during ontogenisis and cell differentiation (Frelin *et al.*, 1986).

Similar changes during differentiation have been reported for chick muscle cells and amphibian neurones *in vivo* (Spitzer 1979). Cultured chick muscle cells differentiate through the established sequence from being initially relatively inexcitable, through Ca^{2+}- then Na^+-Ca^{2+}- then Na^+-based action potentials. TTX sensitivity also increases as the cells mature in culture. In cardiac cells the fast Na^+ channels are not functional at the early stage of development, possibly due to their

kinetics of inactivation being faster than those of activation (Renaud *et al.*, 1982), but maturation processes occur which lead to the inactivation kinetics becoming slower.

These results all indicate that developmental changes occur during maturation which involve several sequential and possibly independent processes. The expression of STX-sensitive channels early in development reflects either structural changes in the cell membrane or the expression of a new type of Na^+ channel. The development of new channels appears to be more likely, as biochemical analysis has shown a difference in stability between STX-sensitive and STX-insensitive channels, the latter type being metabolically unstable (Renaud *et al.*, 1982).

6.1.4. The use of VER and OUB as modifiers of electrical excitability

One of the basic principles of the *in vitro* neuroblastoma bioassay is to disrupt the electrical excitability and self-regulating ionic mechanisms of the cell membrane by exposing the cells to certain chemicals. These modify the membrane in such a manner as to allow a persistent influx of Na^+ ions into the cells, causing swelling and eventually death (e.g., Kogure *et al.*, 1988). Veratridine (VER) and ouabain (OUB) are the two agents most commonly used by the research groups involved in developing the cell bioassays.

The alkaloid VER causes persistent activation of Na^+ channels at the resting membrane potential (RMP) by blocking Na^+ channel inactivation and shifting the voltage dependence of channel activation to more negative membrane potentials (Catterall 1986). VER causes a change in RMP through an increase in passive Na^+ permeability at concentrations in the range of 50 to 200 μM. Depolarisations of up to 30 mV were induced in frog nerve (McKinney 1984), whilst a sixfold uptake of ^{22}Na in neuroblastoma and muscle cells was reported (Catterall 1981). TTX and STX inhibit VER action in a non-competitive manner. Increase in ^{22}Na uptake was inhibited completely by TTX, with a half-maximal inhibition at 10 nM (Catterall 1981).

The onset of VER action is rapid. Binding experiments have shown that the maximal effect of 100 μM VER is reached after 1 min of incubation (Lawrence and Catterall 1981). VER is a partial agonist, activating 8% of available Na^+ channels (West and Catterall 1979), and therefore allows the channel to close, such that its probability of being open is only 0.6 at the maximal dose (Catterall 1977). Channels modified by VER still open and close frequently with a time scale of between 1 and 10 s, and the channel can still spend a substantial fraction of its time in the closed state. Increasing the VER concentration enhances the probability of channel opening, primarily by increasing the rate constant for opening (Garber and Miller 1987).

Kinetic analyses of VER-action on frog muscle (Sutro 1986) and N18 neuroblastoma cells (Barnes and Hille 1988) confirm that Na^+ channels must be open before VER can bind to its receptor. It will not react with normal channels when they are resting or inactivated. The binding of VER to its receptor is therefore use-dependent. It binds to open Na^+ channels at an intracellular site during the depolarisation phase, and unbinds after repolarisation (Ulbricht 1972; Honerjager *et al.*, 1992). The binding complex between VER and an open channel has a typical voltage-dependent life of around 1 s (Hille 1992). The lifetime of the VER- Na^+ complex equates to the duration of the modified open state of the Na^+ channel (Zong *et al.*, 1992). VER-modified channels enter substates with conductances 1/3 to 2/3 that of the normal conductance and have a smaller current which is a more slowly-developing inward current generated by the sustained depolarisation (Barnes and Hille 1988; Corbett and Krueger 1989). However, after going through a typical VER-induced low-conductance modification, a channel does not enter a closed (inactivated) state, but is immediately available for another VER modification (Honerjager *et al.*, 1992).

The cardiac glycoside OUB, which specifically inhibits the Na^+/K^+ dependent ATPase, is used in conjunction with VER to modify cell electrical activity in the bioassay. OUB binds to the α subunit of the Na^+/K^+ ATPase at an intramembrane site, not extracellularly as previously thought (Arystarkhova *et al.*, 1992). The Na^+/K^+

ATPase, or Na^+-K^+ pump, is an electrogenic, or active transport mechanism which utilizes the cell's metabolic energy in the form of ATP to sustain a gradient across the membrane for both Na^+ and K^+ (Thomas 1969). These gradients are used as an energy source for generation of the membrane potential, and for the regulation of cell volume and for the secondary transport of numerous molecules (Skou and Esmann 1992). The transport ratio is 3 Na^+ to 2 K^+, and in the normal cell with its high permeability to Cl^- the pumping of Na^+ and K^+ adds only a few millivolts to the membrane potential.

Inhibition of the Na^+/K^+-ATPase results in an increase in intracellular Na^+ down its concentration gradient, which also leads to an enhanced Ca^{2+} influx through the Na^+-Ca^{2+} exchange carrier (Skou and Esmann 1992). Low concentrations of OUB can cause depolarisation (Clausen and Flatman 1977) as the normal Na^+-K^+ gradient is disrupted. Thus the increased passive influx of Na^+ by the action of VER is uncorrectable if the Na^+/K^+ ATPase is inhibited and their combined action results in intracellular hyperosmoticity with the consequent water uptake causing the cells to acquire the rounded morphology observed by Kogure *et al.* (1988). VER has also been reported to increase the rate of Ca^{2+} entry into cells (Jacques *et al.*, 1981) and combined with Na^+, the intracellular accumulation of Ca^{2+} would contribute to cell death.

6.1.5. Study objectives

The objective of the series of experiments described in this chapter and the next chapter was to extend our knowledge of the underlying electrical properties of the neuroblastoma N2a cell line during differentiation, by a combination of photographic observations and intracellular techniques, and to compare these results with previous studies on the original tumour cells (C-1300) and subsequent clones lines. In this chapter the basic membrane properties of the neuroblastoma N2a cell line are described in relation to the progress of differentiation, and to exposure to the neurotoxins VER and OUB, and also STX, under the assay conditions of Gallacher

and Birkbeck (1992). Experiments were performed in two separate series, in different laboratories.

6.2. MATERIALS AND METHODS

SERIES 1

6.2.1. Cell maintenance

Neuro-2a (N2a) cells were maintained in the Dept. of Microbiology, University of Glasgow, using the following procedure. Growth medium for the cells is detailed in Table 6.1. All medium components and culture flasks were supplied by Gibco (BRL, Life Technologies) with the exception of foetal calf serum (FCS), which was supplied by Flow Laboratories Ltd.

N2a cells from a frozen stock (Passage 190) and cells from a fresh passage (ATCC CCL131, Neuro-2a, Passage 184) obtained from Flow Laboratories were used. Upon arrival, or, retrieval of frozen stock from liquid nitrogen, cells were initially maintained in medium containing 10% FCS, in 80 cm^2 tissue culture flasks. Thereafter, once the cells had achieved sufficient confluency, this was reduced to 5% FCS. The cultures were maintained in an air incubator, relative humidity 100 %, at 37 °C.

Once established, cells were subcultured every 4 days. This, and all other cell procedures were performed under aseptic conditions in a Laminar flow hood. Medium in the flask was removed, 4 ml of sterile phosphate buffered saline (PBS) added per flask, swilled over the cells and then poured off. Trypsin-EDTA, 1 ml, (supplied as a 1x solution in modified Puck's saline, by Gibco) in 6 ml of growth medium was added to detach the cells from the base. Fresh medium was added to the cell suspension and the cells divided into 3 flasks. Each sub culturing day was counted as Day 0 for the subsequent new cell passage.

Maintenance procedure for N2a cells required for electrophysiological experiments was exactly the same as that for the cell assay. Seeding of cells onto

cover slips was normally done at the same time as preparing cells for the tissue culture assay. Cells were plated out onto sterile 17 mm glass cover slips (not into 96 well microtitre plates) at a density of approximately $1x10^4$ cells ml^{-1}. Cells were used from 2-3 h to 5 days after plating. between passages 185 to 204.

Carboxymethyl cellulose (CMC), supplied by BDH in a final concentration of 0.75% (w/v), was added to the maintenance medium and used during seeding of cells as a suspension medium, in an effort to prevent cell clumping and a obtain a more even monolayer distribution of cells for the assay microtitre plates and cover slips.

6.2.2. Incubation of cells with ouabain and veratridine

Intrinsic to the design of the N2a tissue culture assay (Gallacher and Birkbeck, 1992) is a 24 h incubation period of the cells, in a combination of ouabain (OUB) and veratridine (VER), together with either TTX or a test sample. In order to simulate these assay conditions, and also, to investigate the actions of each agent alone, on cell resting membrane potential, the following set of experimental conditions was used.

1) Cells were plated out and maintained in either in growth medium (5% FCS) alone or growth medium plus CMC, as controls. 2) Cells were maintained in 2% FCS medium after seeding 3) Cells were exposed to 0.05 mM VER alone. 4) Cells were exposed to 0.3-0.4 mM OUB alone. 5) Cells were treated with both OUB and VER.

However, the assay had been designed to create a situation whereby maximum cell death estimated at 60% would occur (Gallacher, S., PhD thesis 1992), due to the combined actions of VER and OUB at the above concentrations, unless a sodium channel blocking agent was present. This parameter posed problems when needing to use a number of live cells for the electrophysiology experiments. Therefore, VER and OUB were added for a shorter incubation time, of between 1 to 3 hours, or, were added to control cells whilst an experiment was running to observe acute exposure effects. The addition of tetrodotoxin (TTX) after the acute addition of VER or OUB was also tried.

6.2.3. Electrophysiological solutions, equipment and protocols

i) Solutions for intracellular recordings

The mammalian saline used as an extracellular bath solution was composed of the following constituents (in M): NaCl 2.5; KCl 1.0; $CaCl_2$ 1.0; $NaH_2PO_4.2H_2O$ 1.0; $NaHCO_3$ 1; $MgCl_2$ 0.1, made up to 1L with de ionised water. Glucose (1 gm) was added immediately prior to use. The solution was maintained at 34-36°C in a water bath adjacent to the electrical apparatus to simulate assay solution temperature conditions.

ii) Microelectrodes

Glass microelectrodes used to impale the cells were pulled from capillary glass, type GC100F-10 (Clark Electromedical Instruments) using a single stage Industrial Science Associates Inc. MI puller with tip resistances between 30 and 60 M Ω. Electrodes were filled with 1M KCl and mounted in a MEH-1FS, World Precision Instruments (WPI), microelectrode holder supported by a Narishige SM-20 micro-manipulator.

iii) Recording equipment

Membrane potentials were recorded on an S-7000A Modular Microprobe System, (WPI), comprising of a S-7071A Electrometer Module (EM) which could record in either current or voltage mode, and an S-7100A Control Module (CM) with an LCD digital meter, designed to display current/voltage readings and electrode resistance. A silver/silver chloride (Ag/AgCl) half cell connected to the ground terminal on the CM was placed in the bath to provide an electrochemical reference electrode. Compensation for the junction and/or tip potential between the microelectrode and the reference ground electrode was corrected for. Signals were filtered at 0.1 kHz.

During experiments the EM remained in voltage mode. When an electrode penetrated a cell membrane the digital meter instantly registered the change from

baseline (zero) to a negative value of millivolts. Membrane potential records obtained were digitised using a Cambridge Electronic Design (CED) 1401 and could be printed on line using a Gould (DSO) 1640 storage oscilloscope internal plotter. Records could also be stored on tape using a Racal Recorder [Store 4] or on computer disk for later analysis using Spike2 software by CED.

iv) Protocols and recordings

Cells were collected in culture dishes from Microbiology and maintained in the laboratory in a heated glass chamber inside a water bath which also warmed the extracellular solution. A cover slip of cells was placed into an open 35 cm culture dish mounted on the stage of a Wildovert inverted microscope. Some experiments were performed on a Leitz Fluovert FU inverted microscope.

Control cells (those not incubated in OUB or VER) were perfused with mammalian saline at a flow rate of 2.5 ml min^{-1}, controlled by a Gilson Minipuls-3 peristaltic pump. Bath volume was maintained at 2 ml Although the tubing was insulated, travel length and bath volume, made it difficult to maintain saline temperature at 37°C. Experiments were therefore performed around 26-27°C.

Cells incubated with OUB or VER were placed in the bath with the perfusion system off to delay wash off and dissociation of the toxins. The same volume of saline was added as the controls. After half an hour, cells were discarded and new ones used because of any dilution effect caused by removing the cells from the VER/OUB solution and placing them in saline alone.

Cells selected for intracellular experiments had to have a clean membrane, i.e., no pieces of other cell debris or growth media particles lying on the surface, which could block the electrode tip. Preferably they would be chosen from a monolayer rather than a clump to ensure the chosen cell was indeed the cell that was impaled.

All resting membrane potentials obtained were recorded so that the range of potentials within the cell population could be compared for size and age, and, controls versus cells incubated in VER and OUB. In experiments where VER and OUB were

applied to the bath, resting membrane potentials had to exceed –25 mV and the recording had to be stable for 1 min prior to adding any toxin. If the resting potential was less than –25 mV the complete effect of the toxin could be missed as both VER and OUB cause a large depolarisation of the cell membrane.

For all cells, the initial membrane potential from when the microelectrode impaled the cell was noted, as well as recorded on tape and/or oscilloscope. Every subsequent 5 sec, the potential was noted again. If a noticeable depolarisation drift in potential occurred during recording, the experiment was terminated. This shift indicated the electrode was either drifting out of the cell, or there could be damage to the cell membrane. Time course gave some indication of cell stability and robustness. After the electrode was withdrawn, baseline reading was checked again, both on the digital meter and oscilloscope. If the value on the meter was greater than 5% of the membrane potential the data was discounted as incurring too high an error margin. This was particularly important where cells had small resting potentials.

SERIES 2

6.2.4. Cell maintenance

A 75 ml flask of Neuro 2a cells were obtained from Flow as passage 186 and maintained in RPMI (5% FCS) in a 5% CO_2 incubator (Table 6.1). Cells were subcultured every 4 days by adding 2 ml of trypsin-EDTA (2%) until the cells began to loosen from the flask base. The suspension was poured into a 10 ml centrifuge tube containing 5 ml of RPMI medium and spun at 500-800 rpm for 3 min. The supernatant was poured off and the pellet resuspended in 1 ml of fresh medium.

A sample of the cell suspension (10 µl) was added to a Neubauer chamber and cell density was counted. An appropriate dilution factor was calculated to provide a cell count of about $1x10^5$ cells/ml when plated onto sterile coverslips. Once the required cell suspension volume for experiments was aliquoted off, the remaining volume was divided into flasks and maintained for future use.

6.2.5. Incubation of cells in HMBA

Cells plated on cover slips were incubated in medium containing N, N' Hexamethylene-bis-acetamide (HMBA). A 500 mM stock solution was prepared by adding 10 g of HMBA to 100 ml deionised water, 2 ml of which was added to 98 ml of maintenance medium to give a 10 mM solution. Use of HMBA as a differentiating agent had been investigated by S. Gallacher (*pers. comm*, and PhD Thesis) in an effort to increase cell sensitivity for the bioassay and was used here at her suggestion. Cells were used between 2 and 3 days after plating.

6.2.6. Electrophysiological solutions, equipment and protocols

i) Solutions for intracellular recordings

Immediately before recording, cells were washed, then maintained in a mammalian Ringer solution of the following composition (in M): $NaHCO_3$ 1; KCl 1; $MgCl_2$ 0.1; Na_2HPO_4 0.1; NaH_2PO_4 0.1; NaCl 3; HCl 1; $CaCl_2$ 0.1; glucose. The solution was bubbled with 5% CO_2 and 95% O_2 for a few minutes before the $CaCl_2$ was added to prevent precipitation. The Ringer solution was gassed throughout the experiments to maintain a pH of 7.4. Flow rate was 2 ml min^{-1}. Microelectrodes were filled with 1M KCl.

ii) Intracellular microelectrodes

The electrodes were pulled from glass supplied by Hilgenburg (Germany) with an outside diameter (O.D.) of 1.5 mm and an inner diameter (I.D.) of 0.87 mm, using a multicycle programme on a P-87 Sutter Instrument and Co puller. Tip resistances measured between 60 and 80 MΩ in the bath.

iii) Intracellular recording equipment

A coverslip of cells was clamped into a customised bath then mounted on the converted stage of a Leitz Fluovert FS inverted microscope. The bath reference electrode was an agar bridge filled with 3% agar in 3 M KCl. An MVM 1503

(Softronic, Innsbruck) was used as the recording amplifier. A Servogor 120 (BBC) chart recorder was used to plot membrane potential traces. Scale was 2.5 cm/ 10 mV with a speed of 3 cm/min.

iv) Intracellular protocol

A variety of cell sizes and morphologies could be chosen as there was no problem with space clamping or series resistance errors. Electrode tip and junction potentials were corrected for and a baseline established. Impalement of a cell was observed as a deflection from the baseline and was a measure of a cell membrane potential. All membrane potentials were recorded. Only cells with a potential more negative than –20 mV, with a stable baseline (± 2 mV) for at least 1.5 min were exposed to the toxins. As both VER and OUB were dissolved in ethanol at stock concentrations, it was used as a control variable.

The various toxin combinations for application were: 1) VER alone, 2) OUB alone, 3) VER and OUB together, 4) VER and STX together, 5) OUB and STX, 6) VER and OUB and STX.

Following toxin application, changes in baseline were monitored until no further depolarisation effect was observed. The perfusion system, switched off when a stable, control membrane potential baseline had been achieved, was restarted and the toxin washed off. If the baseline returned to within 1-2 mV of original control position, a second set of toxins from the combinations above was added and the same procedure repeated. If the toxins washed off successfully, a third combination could be added. After three sets no further toxins would be applied and a new dish of cells would be set up. Results were accepted when the electrode was removed from the cell but not the Ringer and the trace returned to ±2 mV of the original electrode baseline. This suggested no potential error had crept in during the experiment.

6.2.7. Photographic growth study

N2a cells used in this study were cultured from the same stock as those used for intracellular experiments. Maintenance conditions were as described in 6.2.1. An early study by Augusti-Tocco and Sato (1969) using C1300 cells, the original tumour line from which N2a were cloned, suggested culture conditions stimulate the immature neuroblasts present in the tumour to complete (or at least to proceed further in) their maturation. Schubert *et al.* (1969) suggested that C1300 tumour cells under tissue culture conditions can be induced to differentiate into neurones.

A Leitz Fluovert microscope fitted with a camera was used at X200 and X300 magnification to observe and photograph cells. The cells remained in growth medium to avoid the possibility of morphological changes, such as, rounding up, frequently seen when cells are placed in an experimental solution. The volume of the medium was reduced to make observations and photography clearer.

Cells were photographed over a 6 day period following plating. Particular emphasis was placed on membrane surface changes and development of contacts between cells (neural networks).

6.2.8. Scanning electron microscopy study

Kogure *et al.* (1988) used changes in the morphological shape of cells exposed to VER and OUB as the criteria for their cell bioassay, as viewed under a light microscope. This study, examined more closely the changes in cell structure and morphology caused to cells incubated in VER and OUB through the use of a scanning electron microscope (SEM).

As before, N2a cells were maintained under assay conditions. 24 hr prior to requirement, cells were plated at a density of $1x10^5$ cells/ml (as per assay), onto 10 mm cover slips. A set of cells were retained as controls. The remaining cover slips were divided into 3 sets. Two sets were treated with either 0.05 mM VER or 0.3 mM OUB only. Another set was exposed to 0.05 mM VER plus 0.3 mM OUB. The final

set was treated with VER, OUB and 125 nM STX. All sets were incubated at 37°C for 24 hr.

The cover slips were then prepared using either Freeze Drying or Critical Point Drying methods. Treated cells were then coated with a fine layer of gold and photographed using a Philips SEM 500 scanning electron microscope.

Another set of N2a cells ranging from 0 to 4 days which had not been exposed to toxins was also prepared. As with the previous study, morphological changes over the 4 day period were photographed.

6.3. RESULTS

6.3.1. Intracellular observations

Data were obtained from a total of 208 cells from the two experimental series. In normal Ringer solution, resting membrane potentials (RMP) from cells ranging in age from 0 days (plated less than 24 h) to 8 days after plating on cover slips, varied from –5 mV to –69 mV (mean –21.92 ± 3.02 mV). Collection of data concentrated on cells between 0-4 days, and more specifically between 1-2 days (see *n* numbers in Figure 6.1.a), to correlate with cell age (after plating) used in the tissue culture assay of Gallacher and Birkbeck (1992). Cell RMP is plotted against the age of the cell in Figure 6.1.a, which clearly shows that many cells had quite a low RMP. When the respective mean values for each age group were plotted (Figure 6.1.b) there was a general trend for the mean RMP to be below –25 mV, agreeing with the overall mean value of –21.9 mV. This was especially evident in the age groups considered pertinent for the assay. Calculation of the number of cell RMPs, in each 10 mV increment, showed that for both series 1 and 2 (Figure 6.2.a and 6.2.b, respectively), the largest proportion fell in the –11 to –20 mV range.

6.3.2. Effects of veratridine, ouabain and STX on resting potential

(i) Acute addition of neurotoxic agents

Experiments in which the neurotoxic agents were added to the experimental chamber after a steady impalement was achieved, produced similar results in both series 1 and 2. In each set of experiments the different neurotoxic agents were first tested separately and then in different combinations, including addition of STX and ethanol. VER and OUB were used at concentrations found to give the optimal results (i.e., maximal cell death) in the cell culture assay of Gallacher & Birkbeck (1992).

A set of experiments in series 1 compared post drug application, following acute exposure, with control RMP values. The matched control potentials showed that when applied acutely, 0.4 mM OUB caused a significantly greater depolarisation ($p = 0.012$) than VER (Figure 6.3.a). Exposure to OUB caused an average depolarisation of 20.62 mV (mean RMP –12.60 mV, ± 3.37 mV) from a mean RMP value of–33.22 mV (±3.37 mV). Exposure to 0.05 mM VER resulted in a mean change of 14.70 mV (mean RMP value –21.15 mV, ±7.51 mV) from a mean control RMP value of –35.85 mV, ±10.84 mV.

In all acute drug application experiments (series 1 and 2) both OUB and VER depolarised the membrane within 3 min of application (Figure 6.4). OUB produced a rapid depolarisation (Figure 6.4.a), whilst the effect of VER was slower and less pronounced (Figure 6.4.b). In a set of series 2 experiments in which 50 nM STX was applied in combination with either OUB, VER, or OUB + VER, a maximum reduction of about 35% was found (from 8 mV to 5 mV change), with the average inhibition being around 10% (i.e., 1.0 mV). A representative trace from one such experiment is shown in Figure 6.4.c.

Results from a set of experiments from series 2 in which changes in RMP values from single drug and combinations of drugs applications (Figure 6.3.b) were compared with a sub-set of control cells (mean –24.39 mV), were quite varied. None of the agents applied caused a significant change from the control set of RMP values. VER uncharacteristically showed almost no change in mean RMP to controls.

Ethanol, the vehicle for VER, also did not appear to affect the RMP (mean –24.23, ±2.02 mV).

When the changes in membrane potential relative to the mean control values of their respective experiments (plotted as the baseline, 0 mV) were calculated (Figure 6.3.c), OUB and VER when applied together had a greater effect than did either alone, producing depolarisations ranging from 6.5 mV to 15.0 mV (mean 9.30 mV, ±3.46 mV). STX (50 nM) appeared to have just less than a 50% inhibitory effect on the VER action (VER mean change 4.83 mV; VER + STX 2.62 mV), but had no effect on the depolarisation action of OUB (not shown, n=2).

(ii) Incubation experiments

Cells were incubated in either 0.05 mM VER, 0.4 mM OUB, or both VER and OUB. Initial experiments in which cells were incubated in these agents for the same time period as required for the tissue culture assay (24 h) resulted in a great proportion of cells dying before intracellular experiments could be performed. Therefore, VER and/or OUB were added only 1-4 h prior to testing, but even with this relatively short incubation period, many cells appeared swollen when viewed under the light microscope (**section 6.3.3**). Cells with this morphological appearance frequently produced extremely low RMP values, implying that these cells possessed damaged or leaky membranes.

Results from cells giving steady impalements are shown in Figure 6.3.d. All values are from 1 day old cells, the control values being obtained from cells on coverslips not exposed to the drugs. The control cells had a mean RMP of –27.42 mV, ±5.52 mV, n=12). Incubation with VER and OUB incubation resulted in a depolarisation: VER caused a depolarisation to a mean value of –13.44 mV, ±3.86 mV, whilst OUB caused a slightly smaller change to a mean value of –15.86 mV, ±3.87 mV. Cells that had been incubated with both VER and OUB produced not only the greatest depolarisation, and with some cells an actual reversal of the RMP to 11.97

mV, ±11.91 mV, but also the greatest range of RMP values (–23.7 mV to 49.7 mV, n=6).

6.3.3. General morphological changes during a 4 day growth period

Photographs were taken of cells that had been plated out and maintained in culture medium with 5% FCS, for between 0 and 4 days. During this time period morphological differences, such as, shape, membrane appearance, neurite outgrowth, became evident, as illustrated in Plate 6.1 (a-d). Cell growth and morphological differentiation in N2a neuroblastoma cells appeared to be influenced by proximity to other cells and size of the incubation culture dishes, as well as percentage concentration of FCS in the culture medium (as observed later during the study, **section 7.2.1**, when FCS was varied between 10% and 1%, cell division decreased and neuritic process extension increased as the concentration of FCS was lowered).

Within a few hours of plating many cells had adhered to the base of the culture dish with a tendency to form aggregates of 5 or more cells (Plate 6.1.a). Cells that had attached singly or in groups of two or three tended to extend processes more rapidly than those in the larger aggregates. These neurites sometimes extended for several millimetres (Plate 6.1.b) within 24-48 hr. Within 2-3 days many of the cell bodies had become denser in appearance with an enlarged nucleus (Plate 6.1.c). By 4 days dense neural networks had developed between many groups of cells. However, it also became obvious that a mixture of morphologies existed varying from cells that had remained small and round, to others that were bipolar, and others that had a greatly increased soma size and become flattened (Plate 6.1.d).

It also became increasingly evident over the course of the whole study that as the passage number became greater, i.e., the number of times the cells were split increased, changes occurred in cell shape and membrane morphology. In many cells membrane appearance became very grey and granular with blebbing occurring. The cells either became large, very flat and vacuolated, or remained small and undifferentiated.

For the purposes of the intracellular experiments a selection of morphologies were used, although the larger, denser cells gave more stable recordings (Plate 6.2.b,c,d). For patch clamp experiments (**section 7.3**) the rounder, smaller cells with no neurite extensions where possible were chosen, as this reduced space clamp error caused by action potentials that could be produced by longer neurites during voltage clamping of the cell membrane. In Plate 6.2.a, a glass patch pipette can be seen positioned on a typically suitable small cell.

6.3.4. Effects of VER and OUB on cell membrane morphology

Plate 6.3 is representative of a population of assay control cells and clearly illustrates that the N2a cell line is not a homogeneous population but consists of cells with a variety of morphologies including: bipolar, triangular, spherical and elongated cell bodies. Each cell type appears to extend neurites specific for the morphology of the cell. Although some of the more fragile neurites have been damaged by the CPD process in Plate 6.3, it is possible to make out that extensive networks interlinked the clusters of cells in the central area of the photograph.

Networks between cells are better shown in Plate 6.4. between 1 day old (top photograph) and a denser network in 2 day old cells (bottom photograph). These images clearly show the profusion of small spines that cover the entire surface of the cell body, some of which have extended to interconnect with spines protruding from its neighbour. The most central cell in a loose aggregate frequently extended the largest number of neurites which linked with the surrounding cells. Cells that had extended 2 or 3 long, thick, less branched neurites had flatter cell somas compared to the rounded shape of cells extending many thin, multibranched processes.

The following sets of photographs (Plates 6.5, 6.6 and 6.7) compare survival rates as well as morphological changes of cells exposed to OUB alone, VER alone and a combination of OUB, VER and STX (125 nM). All the experiments were set up at the same time.

The top photograph of Plate 6.5 shows a set of control cells with typical mixed morphologies as seen in Plate 6.3. The lower photograph of Plate 6.5 is of cells exposed to OUB. The most striking morphological feature is the extreme roundness of all the cells and their swollen appearance compared to the control set. Many neural networks still seem to be intact despite the morphological changes and very few cell deaths were observed.

Very few cells however, survived exposure to VER alone as the top photograph in Plate 6.6 shows, where only two cells, lying close together, were alive in this section. Both are very rounded in appearance and neither are extending neurites. The lower photograph of Plate 6.6 shows a typical proportion of cells that had been protected from the depolarising actions of VER and OUB by STX. Again, no cells are extending neurites and all are very rounded in appearance. Much cell debris is also evident.

The final two photographs (Plate 6.7) taken at a greater magnification show more clearly external changes caused by exposure to either OUB or VER. In the top photograph, exposure to OUB has caused one cell to retract its smaller spines that were evident on cells in Plate 6.4. but the larger neurites remain extended. However, VER (lower photograph) caused complete retraction of all neurites and spines.

6.4. DISCUSSION

6.4.1. Resting membrane potentials

The findings of these experiments agree closely with studies on other neuroblastoma cell lines (Spector 1981). The mean low RMP value of -21.92 mV agrees with observations by Kuramoto *et al.* (1977) for undifferentiated SK.N.SH cells and exponentially growing N18 cells (Miyake 1978). Unless microelectrode impalements were of a continuous poor quality (Schubert *et al.*, 1973), which seems unlikely since no large RMP values were even transiently observed during impalements, the results suggest the majority of cells being used in the Gallacher and Birkbeck (1992) assay are still undifferentiated. Attempts to differentiate the cells

further for increased sensitivity of the assay (S. Gallacher *pers. comm.*) have therefore not yet been achieved under the present assay culture conditions and differentiation regime.

The overall range of RMP values obtained -5 mV to -69 mV encompasses values expressed by the N2a cell line grown for a period of 5 days under assay culture conditions. The higher values (> -35 mV) probably reflect differentiated cells (Harris and Dennis 1970; Moolenaar and Spector 1977). This agrees with previous findings (Kimhi 1981) that a regular culture of neuroblastoma cells represents a random distribution of cells in different stages of their life cycle. However the average RMP value of -21.92 mV for all age groups found in this study suggests that 5% serum is too high to arrest cell division or to promote differentiation (Schubert *et al.*, 1971).

6.4.2. Acute experiments

These experiments examined the acute effects of VER on the Na^+ channels in resting cells, as they would be under normal assay conditions, save for the long incubation period (see Table 6.2). They provide a useful comparison for the more detailed voltage clamp study reported in Chapter 7. The results showed that VER and OUB act separately. Acute exposure to VER produced a slower and smaller spontaneous depolarisation than that of OUB (shown in Figure 6.4), which supports the theory that VER binds to open channels, and under normal culture conditions only a small population of Na^+ channels will be open in any one cell (West and Catterall 1979). The small changes in membrane potential is also consistent with the theory that VER action is voltage dependent (Hille 1992). It seems probable that the changes in RMP reflected the immediate effect of these compounds on Na^+-leakage channels, as well as on the small percentage of available open Na^+ channels.

The rather slow onset of about 2-3 min for the depolarising current/potential to develop compared to 0.2 s, or less, for frog single nerve fibres (Ulbricht 1972) may be partly to do with i) the equilibration-time of VER in the bath; ii) the diffusion-time of the toxin through the cell membrane; iii) that no electrically stimulated depolarising

impulses had been applied to the membrane, or iv) no other chemical-depolarising treatment, such as the addition of OUB, had been undertaken. Other studies have however reported similar latencies to this study. VER depolarisation of cultured heart cells was observed after 1-4 min (Sperelakis and Pappano 1969) and more recently, Honerjager *et al.*, (1992); reported depolarisations within 1-2 min for isolated heart cells. Similarly, membrane depolarisation of frog satorius muscle in varying $[K^+]$ solutions was observed over a time period of 1-3 min (McKinney 1984), and a 5 min equilibrium time was reported for NIE-115 neuroblastoma cells during ^{22}Na uptake experiments (Jacques *et al.*, 1978).

The depolarising effect of VER on the RMP in N2a cells was reversible, occurring within 5-10 min during experiments where a washout of the compound was tried (Figure 6.4.b,c). This was faster than the 10-15 min required for full reversibility of RMP reported for NIE-115 cells (Catterall 1975), although experimental procedures were different.

The rate of onset for OUB depolarisation of the membrane followed a similar time course for that of VER. However, once the depolarising current appeared a larger, semi-steady state depolarisation appeared almost twice as quickly as that for VER. The depolarising effect of OUB was also rapidly reversible.

The small inhibitory effect by STX on the combined actions of VER and OUB was rather disappointing. Binding studies (Catterall 1975; Tamkun and Catterall 1981) give a K_D for TTX of 11 nM and a K_i of between 1 -10 nM for STX and therefore a larger inhibition than observed here was expected for 50 nM STX.

The action by VER-OUB is a chemical effect that works by modifying the basic membrane processes, which in the case of the cell bioassay occurs under passive conditions. As such, it can only serve as a model for the events that occur in the human nervous system when PSP contaminated food is ingested. Under these conditions the PSP toxins interact with, and affect electrically excitable cells that are "spiking"; complete nerves that are propagating action potentials, not quiescent as the cells in the neuroblastoma bioassay

One way to produce such an effect would be to raise the extracellular K^+ concentration enough to cause a large depolarisation similar to an action potential process before applying VER (McKinney 1984). Another method would be to provide an electrical input to mimic the depolarising effect of an action potential. This is where the patch clamp technique with its high time resolution and provision for membrane voltage control is able to provide a very strong and rigorous investigative method.

6.4.3. Incubation experiments

In this set of experiments the RMP was measured from cells under assay conditions and consequently showed the long term effects of OUB and VER, both together and alone. Of the cells that survived the incubation period, those exposed to OUB alone had a smaller change in their RMP. This suggests that OUB initially immobilizes the electrogenic Na^+/K^+-dependent ATPase, allowing an influx of Na^+ down its concentration gradient, the extent of the Na^+ influx being dependent on the external Na^+ concentration (Jacques *et al.*, 1978). Once an equilibrium is reached there would be little further influx, as the voltage-gated Na^+ channels are not modified by the actions of compounds such asOUB. Catterall (1975) reported a logarithmic influx of passive Na^+ with 0.5 mM OUB, the same OUB concentration giving a maximal stimulating effect in similar Na^+ uptake experiments (Jacques *et al.*, 1978).

The depolarizing action of VER would be slower (as in the acute application of VER) but more prolonged, and as VER binds to the small population of open Na^+ channels there would be a correspondingly small influx of Na^+. This small depolarisation would be sufficient to open a set of closed Na^+ channels (but not those channels which are in an inactive state) causing further depolarisation of the membrane. As VER dissociates from its binding site, these channels can remain in a state able to bind another VER molecule (Catterall 1977). Thus over time, as during the cell bioassay incubation period of 24 h, a large sustained depolarisation develops.

This VER-stimulated depolarisation is also enhanced with increasing external Na^+ concentration (Jacques *et al.*, 1978).

The combined actions of OUB and VER co-operatively elevate cell permeability. OUB evokes a rapid depolarisation, and simultaneously these channels are being held open by the binding effect of VER. As the pump is immobilized there would be no K^+ rectification to repolarise the membrane potential. Thus the OUB effect stimulates a larger VER-initiated depolarisation. In response to the influx of Na^+ the depolarisation increases, more Na^+ channels open, VER binds to these channels and the depolarisation increases further.

Thus when both VER and OUB are used together, RMP's ranging up to positive values are recorded, indicating that the equilibrium, or reversal potential, for Na^+, normally around +40 to +60 mV, depending on the external Na^+ concentration, has been reached. At such a point, under a constant depolarisation, many of the Na^+ channels are either inactivated or held in a steady open state. The long term effect of this condition is to produce intracellular hyperosmoticity, with a consequential water uptake causing the cells to acquire the rounded morphology observed by Kogure *et al.* (1988). Combined with the intracellular accumulation of Na^+ and Ca^{2+} (Jacques *et al.*, 1981) these effects cause cell death. An increase in cytosolic Ca^{2+} leads to disruption of intracellular Ca^{2+} homeostasis and closely regulated biochemical mechanisms. These lead to cytoskeletal modification, plasma membrane blebbing and activation of Ca^{2+}-dependent proteases and lipases (Choi 1988; Bertolino and Llinas 1992; Orrenius *et al.*, 1992). The concomitant addition of STX or PSP samples with VER and OUB, at the start of the incubation period should prevent a proportion of cell deaths (Gallacher and Birkbeck 1992), dependent upon the potency of Na^+ channel blocking toxins.

6.4.4. Morphological features

The changes in morphological features observed in the N2a cells over a four to five day period agree with those from previous studies on neuroblastoma cells

(Augusti-Tocco and Sato 1969; Schubert *et al.,* 1969) Immediately following trypsinization the cells exhibited a rounded morphology. When transferred to a surface to which they could adhere, such as the culture dishes or cover slips, most remained fairly rounded for the first 12-24 h with few processes, the production of which then increased over the 4-5 day period.

Unfortunately in this part of the study it was not possible to confirm the findings of Seeds *et al.*, (1970) who reported that the percentage concentration of serum in the medium influenced neurite outgrowth, a characteristic frequently regarded as one expression of cell differentiation. Thus it was also not possible to observe whether RMP attained a more hyperpolarised value along with the increase in neurite production. However the results from **section 6.3.1** (and Figure 6.2.a) suggest that in 5% FCS no significant change in RMP occurred even though morphological changes were evident.

The typical rounded characteristics in cell morphology described by Kogure *et al.* (1988) after exposure to VER and OUB where clearly evident in the SEM study. The detail of the photographs highlighted the extent of cell damage caused by the action of the two toxins which disrupt the intracellular physiological and biochemical mechanisms (**section 6.4.3**) and also show the complete retraction of all neurite processes, not described before. Even with cells protected by the inclusion of STX in the media, there was evidence of neurite retraction and swelling of the cell soma, suggesting the cells cellular processes were still affected by the action of VER and OUB.

6.4.5. Conclusion

To date, the initial incubation period in microwells for the cells in the neuroblastoma assay systems for PSP detection is approximately 24 h in a high (10%) FCS content media (Table 6.2). Only Gallacher and Birkbeck (1992) and Manger *et al.* (1993, 1994, 1995a,b) have reduced this to 5% in an effort to improve the stability of the assay. This period is followed by a further 24 h incubation period; following

addition of VER, OUB, and STX or PSP sample (Gallacher and Birkbeck, 1992: Jellet *et al.*, 1992; Hamasaki *et al.*, 1996 and Truman and Lake 1996). Manger *et al.*, (1995,b) have reduced this period to under 1 h. Two comments can be made here. Firstly, previous binding experiments (Catterall 1975, 1977; Jacques et al., 1978) and the results of the acute experiments in this study suggest that a large VER-OUB depolarisation occurs rapidly. It should therefore be possible, as Manager *et al.* (1995,b) have achieved, to reduce this step to a much shorter time period (although Gallacher (PhD thesis, 1992) did examine incubation times and concluded 24 h was necessary for a high enough percentage of "cell deaths" to occur for the assay to work successfully). Secondly, as Kimhi (1981) states, a regular culture of neuroblastoma represents a random distribution of cells at different stages of the life cycle. The results from this study suggest that when used at 24-48 h after plating, the majority of cells in the assay remain undifferentiated in the growth phase of the cell cycle. It would therefore be of great value to assess how closely this population reflects the ion-channel behaviour of fully differentiated cells and whether Na^+ current expression is of a consistent nature. This will be examined using the patch clamp technique in a further series of experiments with N2a and other cell lines in Chapter 7, as this feature is undoubtedly a key characteristic to the success of the assay.

Finally, whilst it is apparent that the cell bioassay is a tool to measure the toxicity of PSP in contaminated shellfish, a point to remember is that the use of tumour cells and the chemical depolarisation of the membrane is an artificial situation. The cells are therefore in a distressed, unnatural state, not truly reflecting normal nerve tissue. A set of experiments will be performed (**section 7.3.5**) using dissociated ganglia cells from rats which in theory represents an example of mammalian nervous tissue that more closely depicts normal nerve tissue.

Table 6.1. Composition of maintainance media for the cell types used in both intracellular and patch clamp experiments. Constituents were added to sterile water to make a total volume of 500 ml (except where main salt solution was a normal (1x) concentration). pH was adjusted to between 7.3 to 7.45 using NaOH.

MEDIA	CELL TYPE					
Constituents ml	Neuro-2a (6.2.1)	Neuro 2a (6.2.4)	Neuro 2a (7.2.1)	NG108-15 (7.2.2)	DRG (7.2.3)	SCG (7.2.3)
RPMI-1640* (1X)	460	-	-	-	-	-
RPMI-1640** (10X)	-	50	50	-	50	-
MEME (10x)	-	-	-	50	-	-
DMEM (1x)	-	-	-	-	-	453.9
SATO-MIX.†	-	-	-	-	-	11.1
Foetal calf serum	25	25	25	50	50	-
Glutamax I (100x)		5	5	-	5	-
Glutamine	-	-	-	-	-	10
HAT (50X)	-	-	-	10	-	-
Pen/strep ‡	10	5	3	3	5	10
Fungizone (Amp B) 250 µg/ml	5	-	-	-	-	-
$NaHCO_3$	-	6	7	6	-	-
Insulin	-	-	-	-	-	10
Transferrin	-	-	-	-	-	5

RPMI 1640* with 25 mM HEPES and L-Glutamine
RPMI 1640** without HEPES or L-Glutamine
Pen/strep ‡ contained penicillin (200 IU/ml) and streptomycin (200 µg/ml).

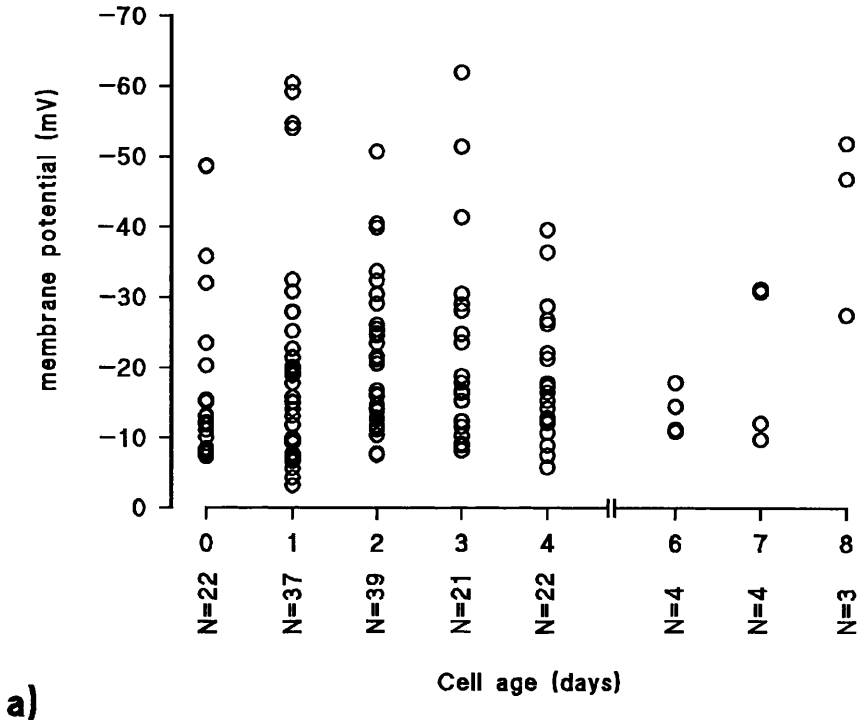
Table 6.2. Cell bioassays using N2a neuroblastoma cells. Concentrations of serum, VER and OUB for each assay are given. Incubation periods prior to exposure to toxins (period 1), following exposure (period 2), and the total period cells are plated before results are recorded (period 3) are compared.

Assay	Incubation period 1	% Serum in media	VER mM	OUB mM	Incubation period 2	Total incubation
Kogure *et al.*, 1988	24-48 h	13.5	0.05	1.0	8 h	32-56 h
Gallacher & Birkbeck 1992	24 h	5	0.1	0.5	24 h	48 h
Jellet *et al.*, 1992	24 h	10	0.05	0.025-0.5	24 h	48 h
Manger *et al.*, 1993, 94, 95	24 h	10	0.05	0.5	24 -48 h	48-72 h
Hamasaki *et al.*, 1996	24-48 h	10	0.05	1.0	24	48-72 h

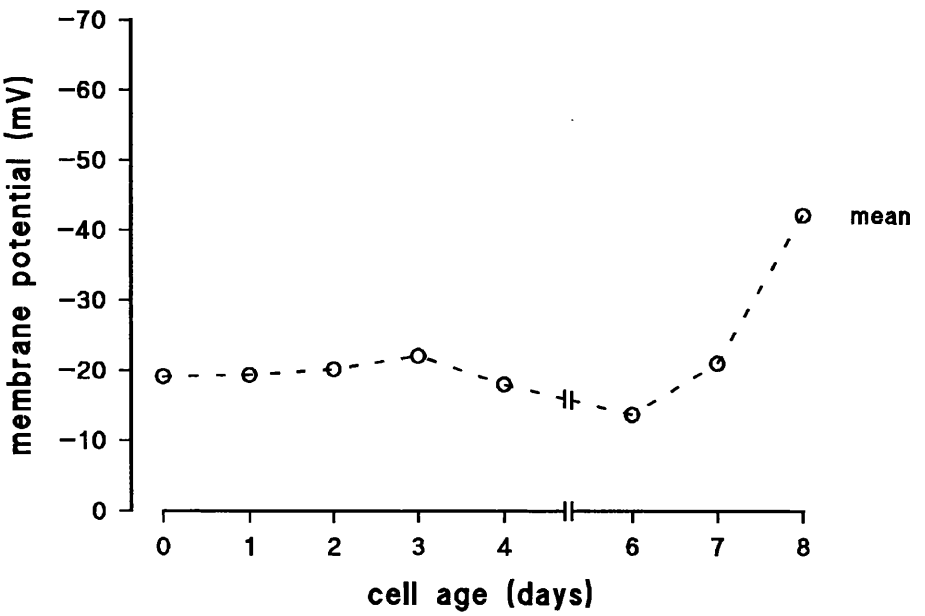
Figure 6.1.

a) Resting membrane potential (RMP) values for N2a neuroblastoma cells between 0 - 8 days after plating onto coverslips. The number of RMP values per age is given under the respective cell age on the ordinate axis. (total $n = 151$).

b) The same data as above are plotted as the mean value for each age. A fairly uniform potential of around -20 mV is apparent. An upward trend in RMP is only seen within the two oldest age groups (7-8 days), although cell numbers were smaller.


membrane potential (mV)
−70
−60
−50
−40
−30
−20
−10
0
0
1
2
3
4
6
7
8
N=22
N=37
N=39
N=21
N=22
N=4
N=4
N=3
Cell age (days)

a)

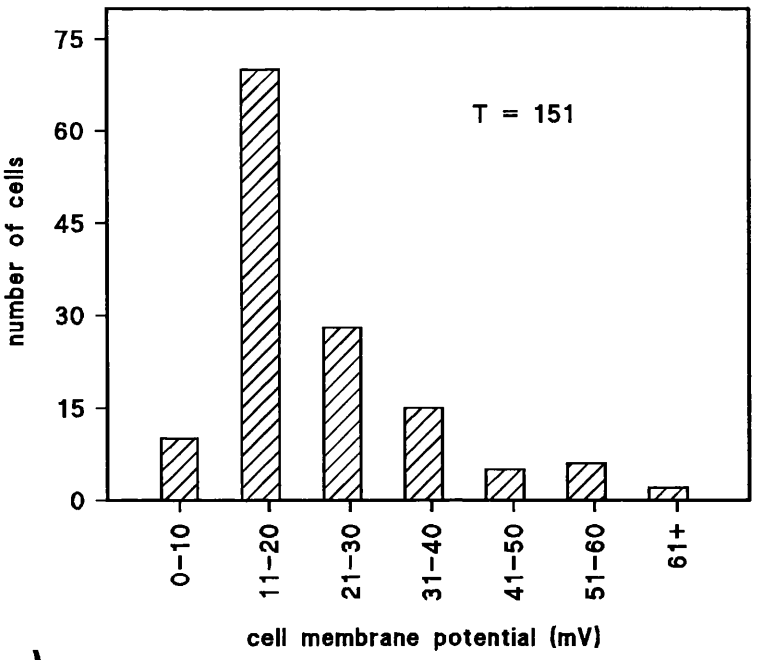

membrane potential (mV)
−70
−60
−50
−40
−30
−20
−10
0
mean
0
1
2
3
4
6
7
8
cell age (days)

b)

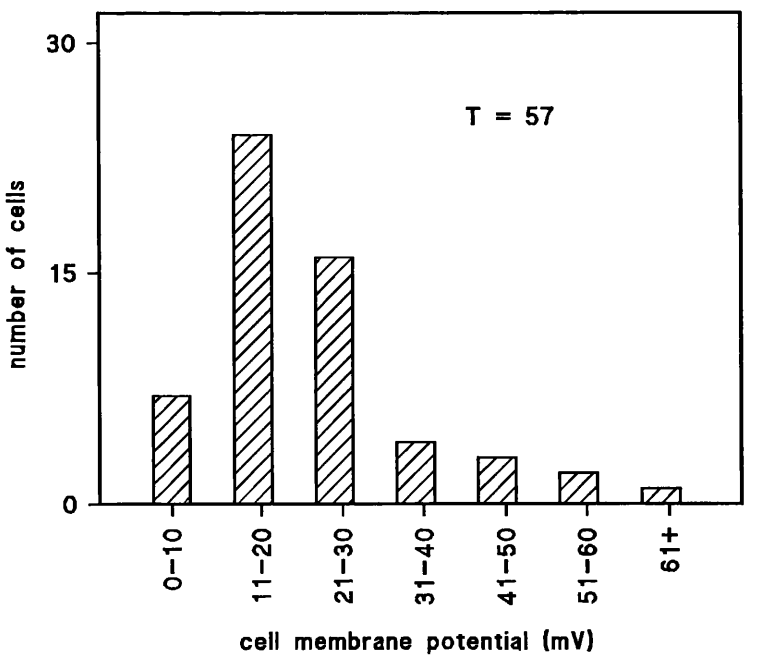
Figure 6.2.

a) RMP values plotted in 10 mV increments showing the number of cells, regardless of culture age, per group (Series 1 data). The number of cells exhibiting an RMP in the -11 to -20 mV range was significantly greater ($p = > 0.5$) than that for any other group.

b) Comparative data collected from a subset of N2a cells (Series 2) shows a similar trend for the greatest proportion of cells to possess RMP values between -11 to -20 mV.


75
60
45
30
15
0
number of cells
T = 151
0–10
11–20
21–30
31–40
41–50
51–60
61+
cell membrane potential (mV)

a)


30
15
0
number of cells
T = 57
0–10
11–20
21–30
31–40
41–50
51–60
61+
cell membrane potential (mV)

b)

Figure 6.3.

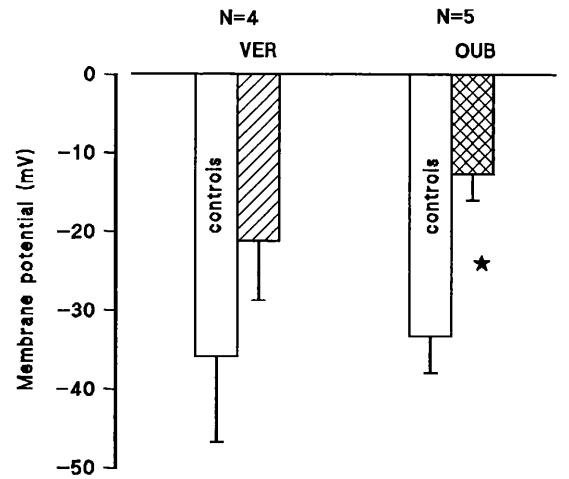
The effect of VER, OUB, STX and ethanol (ETH) on the RMP of N2a neuroblastoma cells.

a) Cell RMP was measured before (open bars) and then following application (hatched bars) of either VER or OUB. Values represent the mean ± s.e.m, of 4 or 5 cells for VER and OUB, respectively.

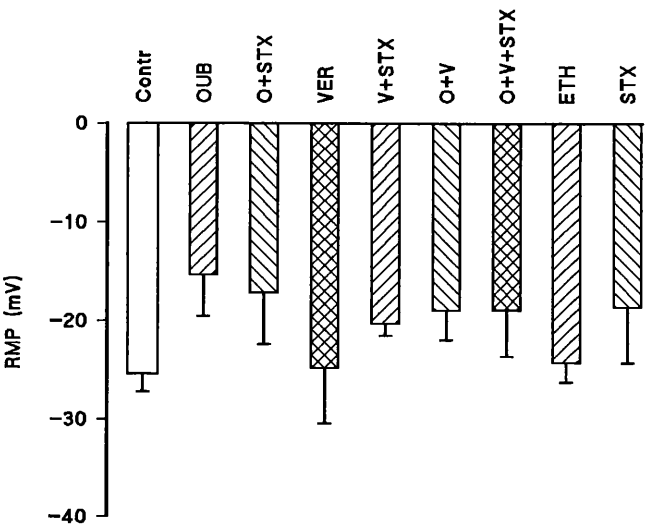
b) Mean changes in RMP values for compounds applied either alone or in combination, compared to a mean control (open bar).

c) Effects of VER, OUB and STX, shown as relative changes in membrane potential from the resting value (zero). All effects are depolarising. Error bars represent ± s.e.m.

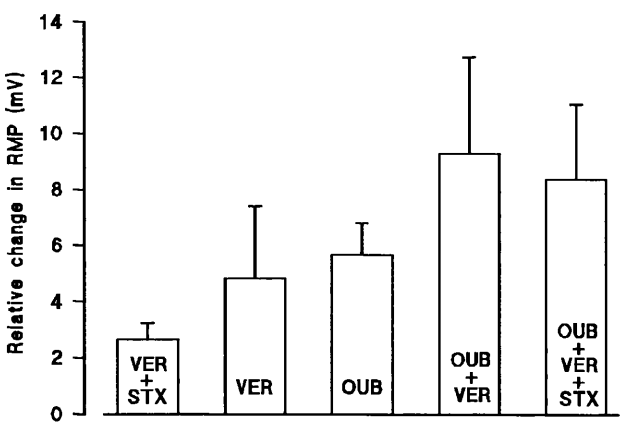
d) Cell RMP values measured after an incubation period of 1-4 h. Control RMP values from cells not exposed to the toxins are shown in the plain bar. Error bars represent ± s.e.m.


N=4
VER
N=5
OUB
0
−10
−20
−30
−40
−50
Membrane potential (mV)
controls
controls
★

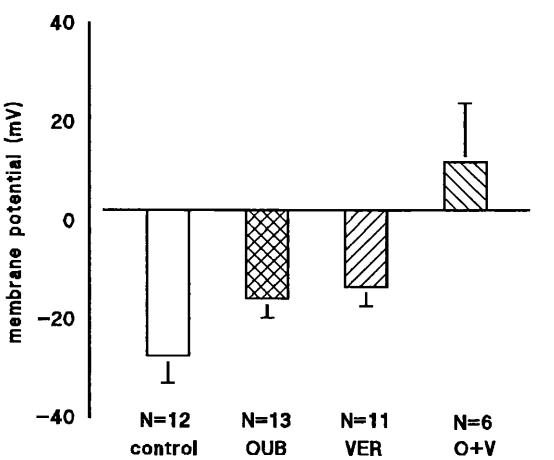
a)


Contr
OUB
O+STX
VER
V+STX
O+V
O+V+STX
ETH
STX
0
−10
−20
−30
−40
RMP (mV)

b)


14
12
10
8
6
4
2
0
Relative change in RMP (mV)
VER + STX
VER
OUB
OUB + VER
OUB + VER + STX

c)


40
20
0
−20
−40
membrane potential (mV)
N=12
control
N=13
OUB
N=11
VER
N=6
O+V

d)

Figure 6.4.

a) Representative trace of an intracellular recording from a N2a cell showing the time latency before the rapid depolarisation of the membrane following external application of 0.4 mM OUB.

b) Recording from a N2a cell showing a small passive depolarisation of the membrane by 0.05 mM VER after a time lag of 3 min following application of the compound.

c) Intracellular recording from a N2a cell in normal Ringer. OUB (**O**, 0.4 mM), VER (**V**, 0.1 mM) and **STX** (50 nM) are applied in various combinations. The thick black arrow indicates penetration of cell (RMP -27 mV).

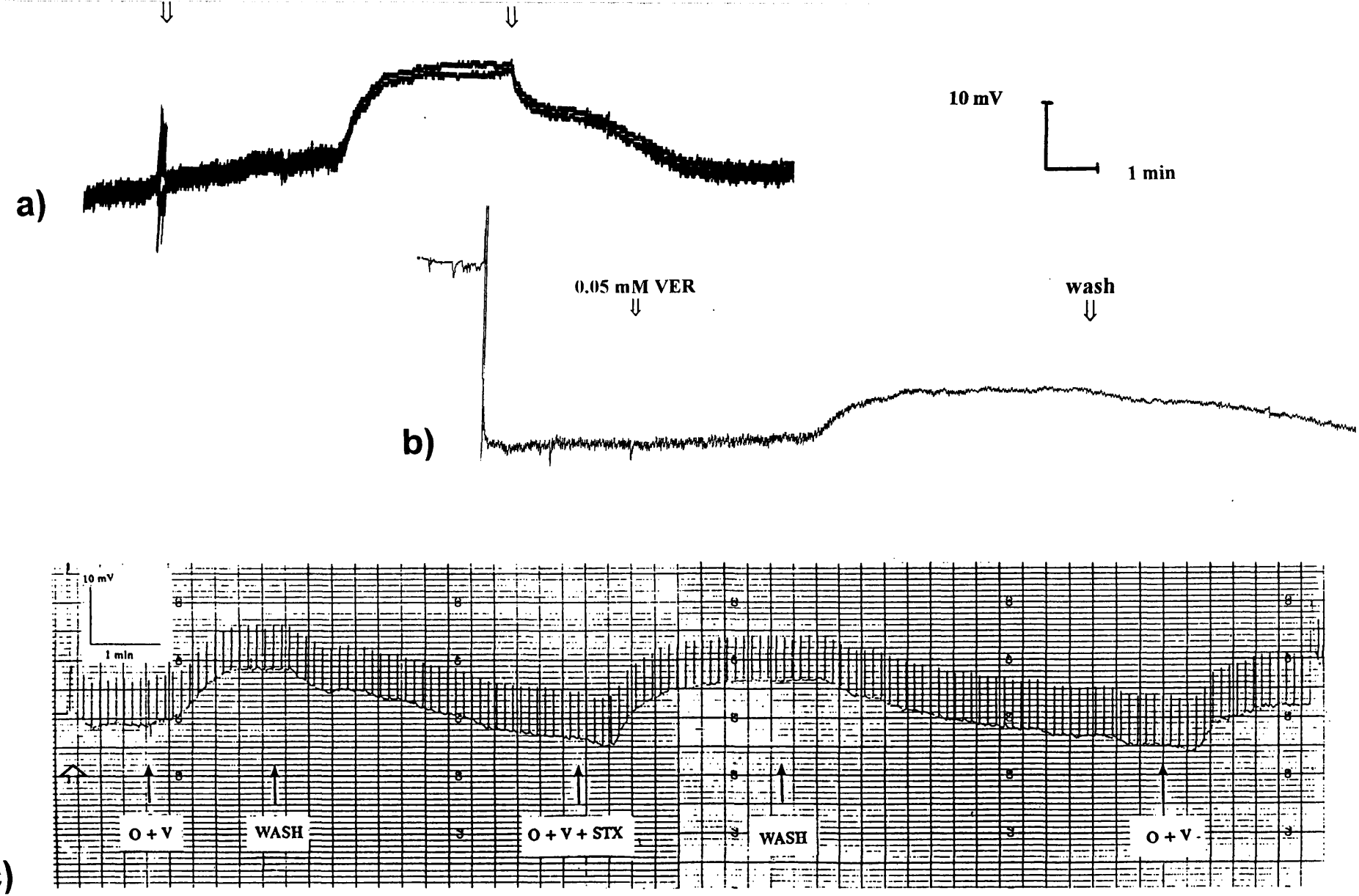

a)
10 mV
1 min
b)
0.05 mM VER
wash
c)
10 mV
1 min
O + V
WASH
O + V + STX
WASH
O + V

Plate 6.1.

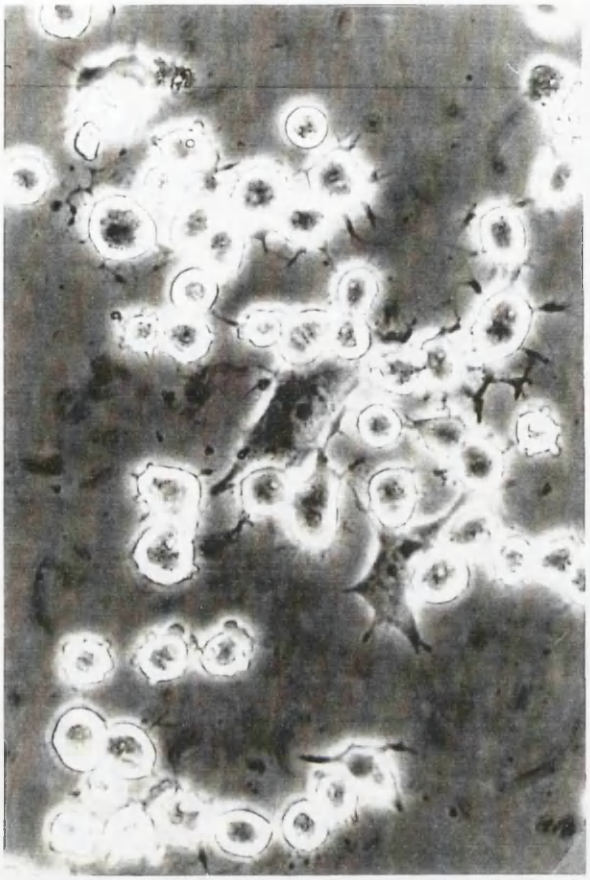
Morphological changes in N2a neuroblastoma cells between 0 and 4 days. The cells are from a representative population of N2a neuroblastoma used in the cell bioassay of Gallacher and Birkbeck (1992).

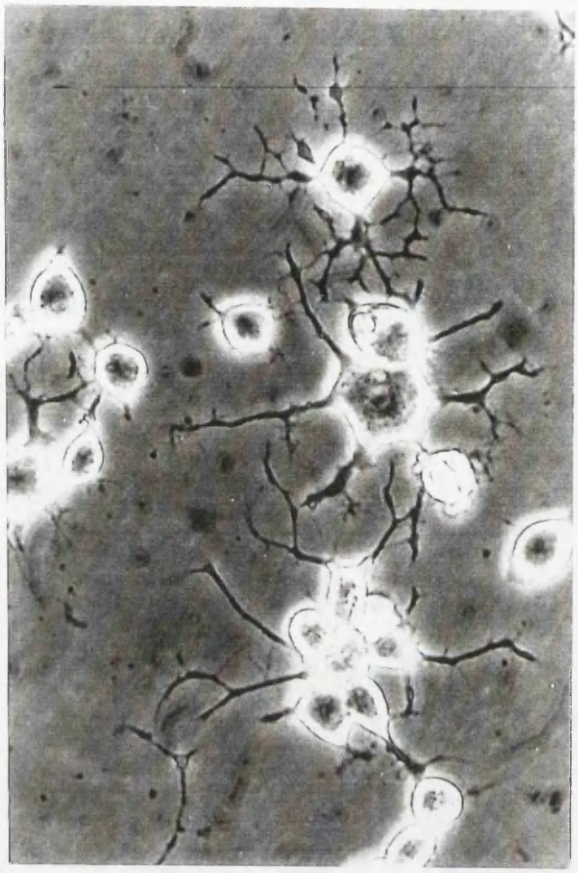
Top left - N2a cells plated for less than 12 h.

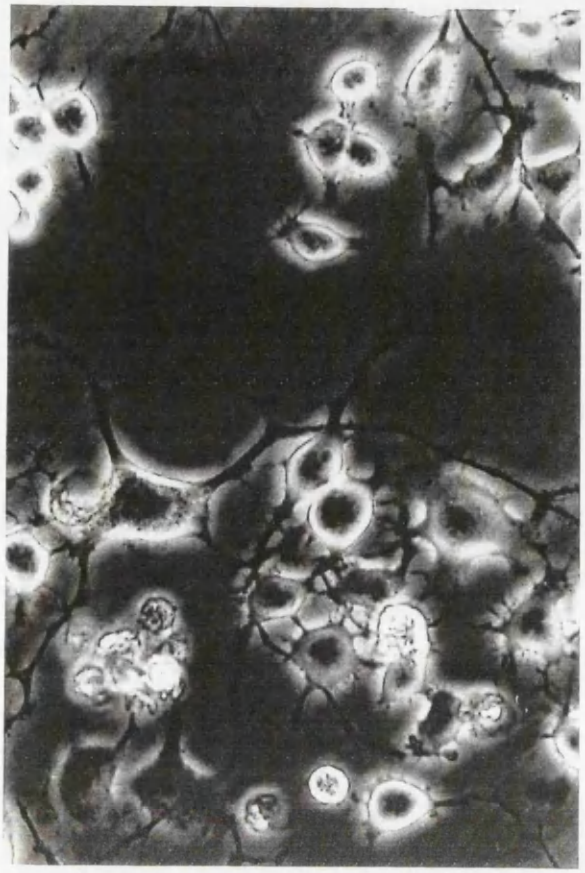
Top right - Cells plated for approximately 24-36 h.

Bottom left - Cells plated for approximately 3 days.

Bottom right - Cells plated for approximately 4 days.







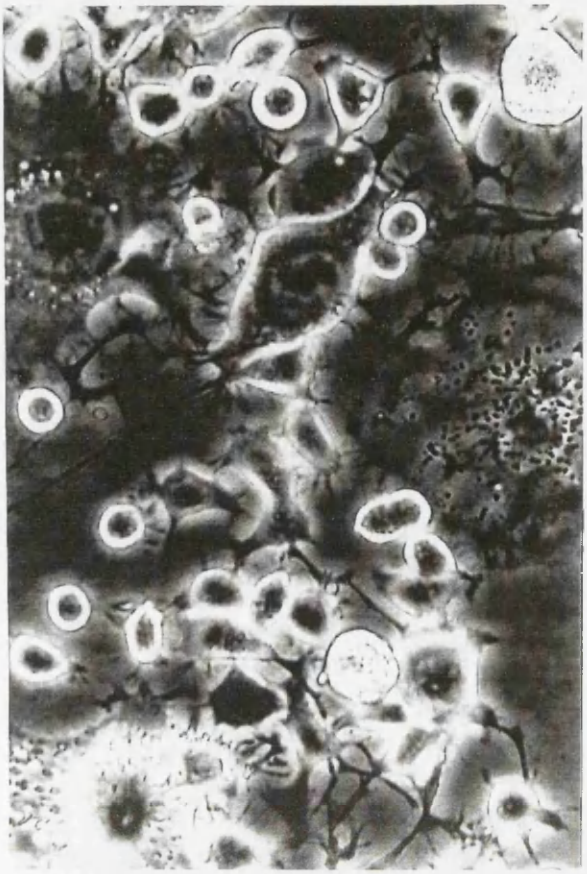
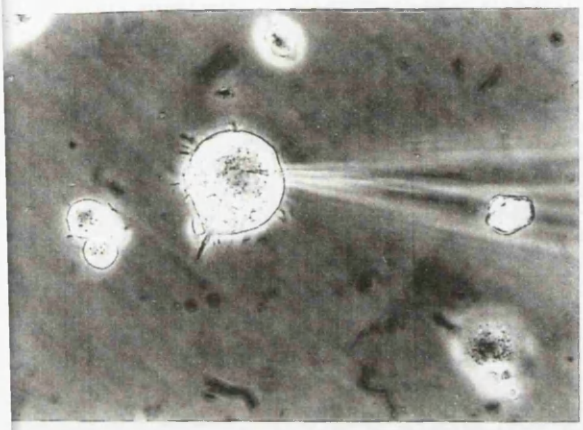
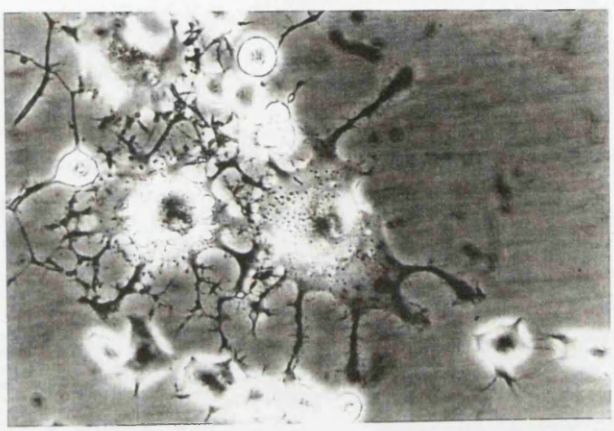


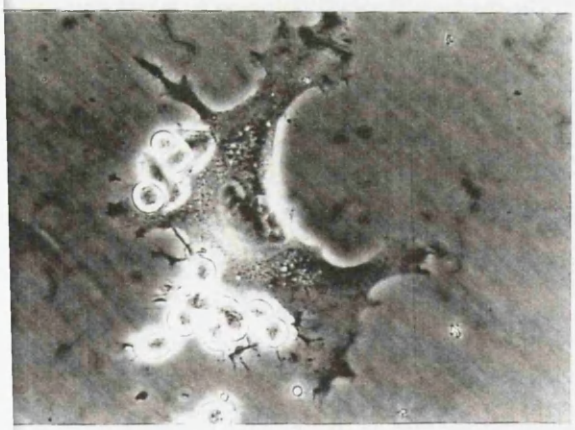
Plate 6.2.

Typical morphologies of N2a cells, illustrating the range of cell differentiation seen within a normal population of cells grown in 5% FCS and used in the PSP cell bioassay.

The cell morphology shown in the top left micrograph is typical of that chosen for patch clamp experiments to avoid space clamp error that can arise from action potentials arising in the neurite extensions. A glass microelectrode can be seen being aimed towards the centre cell.







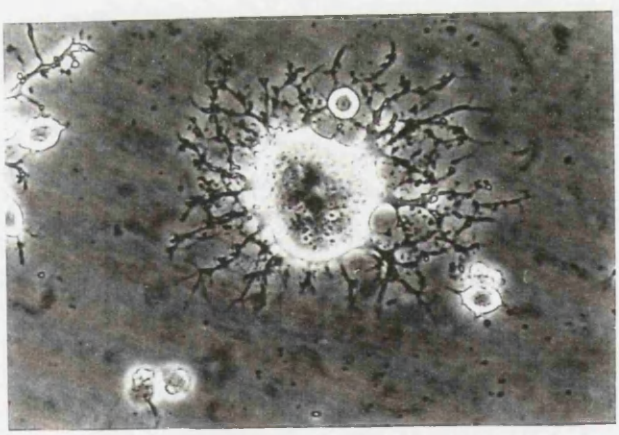


Plate 6.3.

A scanning electron micrograph (S.E.M.) of a representative population of N2a cells plated for 24 hours under assay conditions, showing the diversity of cell morphologies. Scale bar = 10μm.

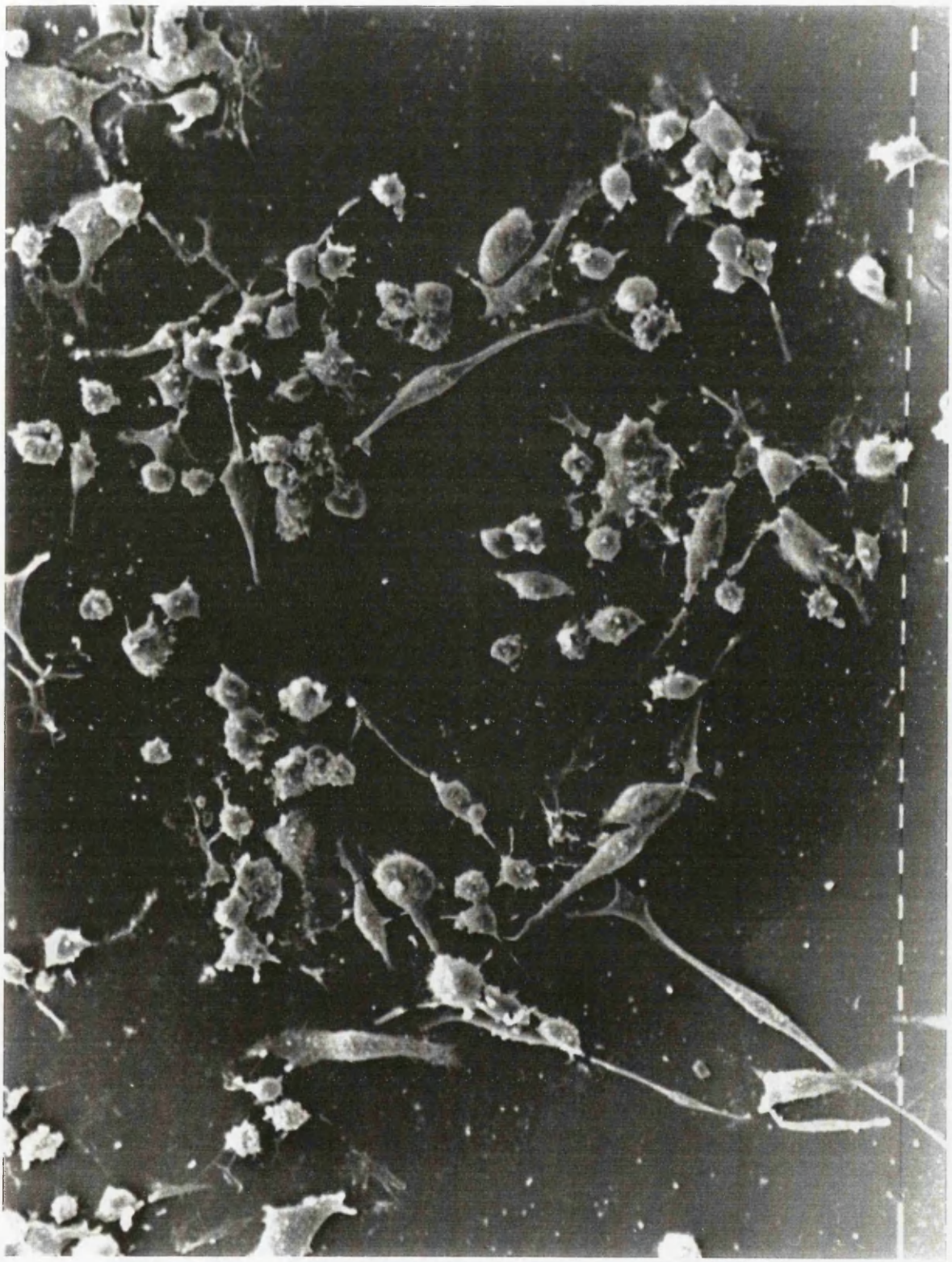
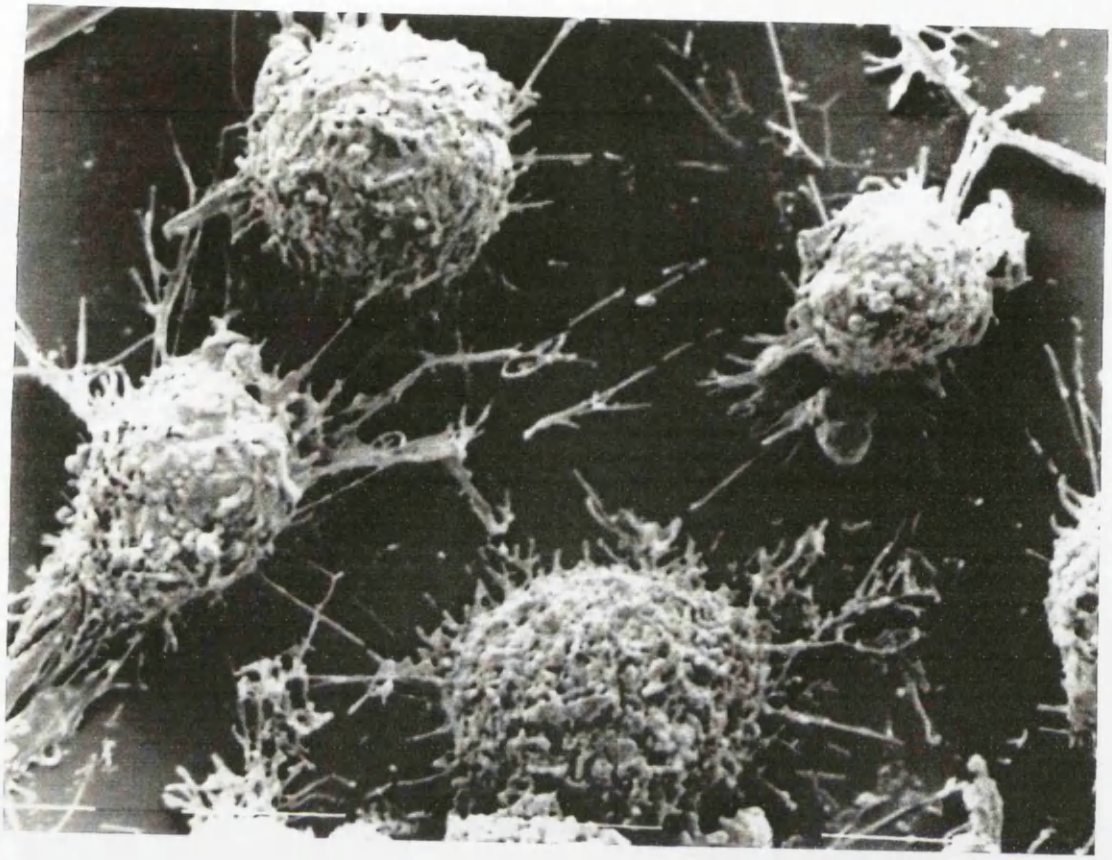


Plate 6.4.

Two S.E.M. micrographs of representative 1 day old (top) and 2 day old (lower) N2a cells grown under assay conditions showing the growth in neuritic processes that occurs during a 24 h period. Scale bar = 10 μm.



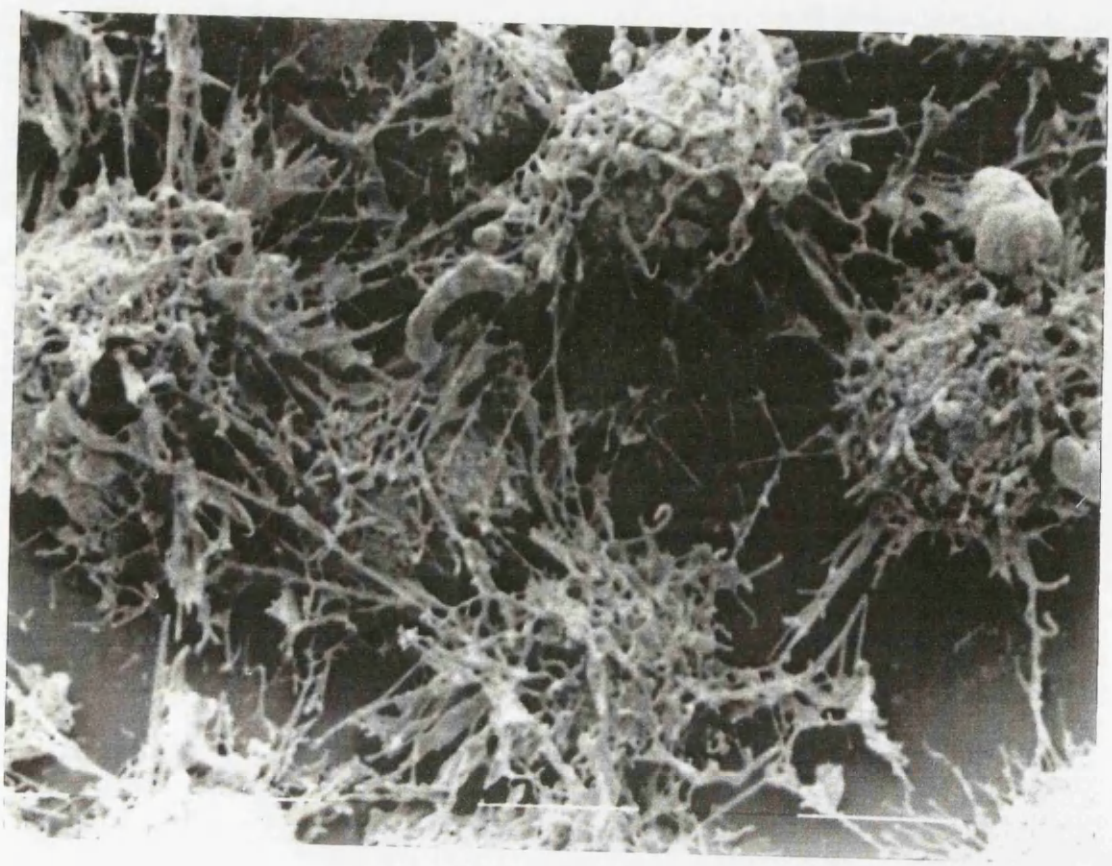
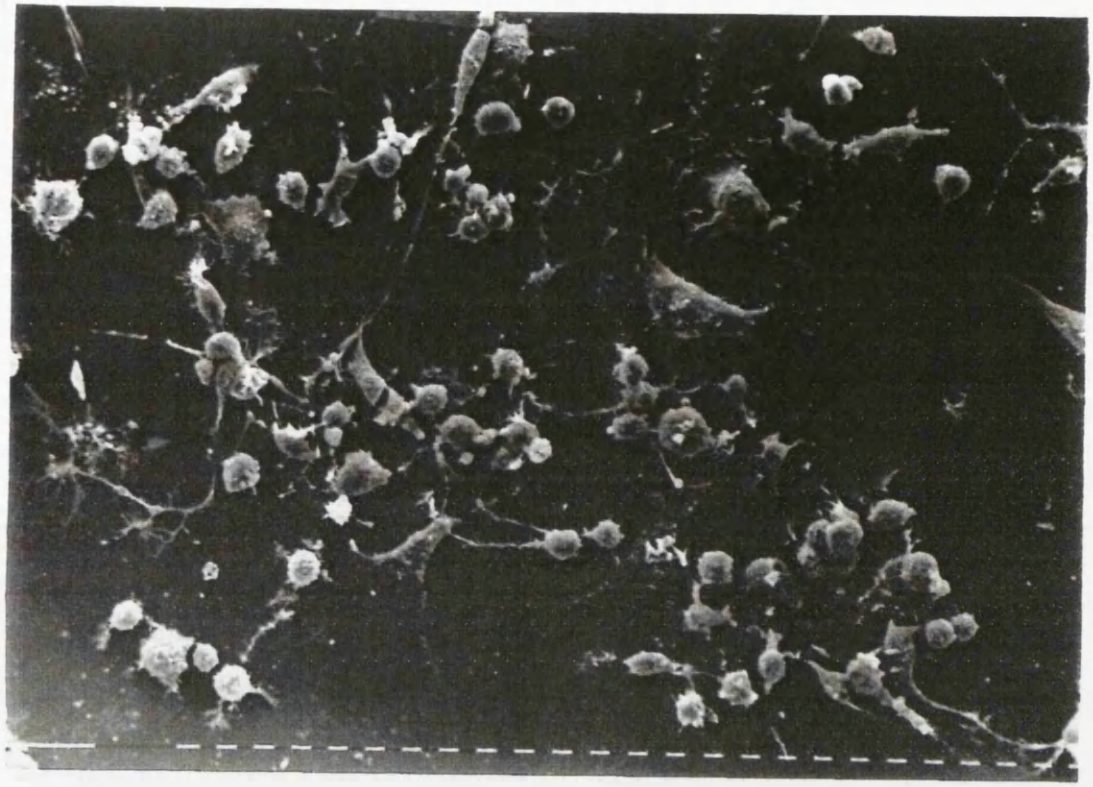


Plate 6.5.

N2a cells plated and maintained under assay conditions. The cells are at the same age and stage of development as those used at the time of an assay. Scale bar = 10 μm.

Top - Control cells.

Bottom - Cells exposed to 0.3 mM OUB for 24 h.



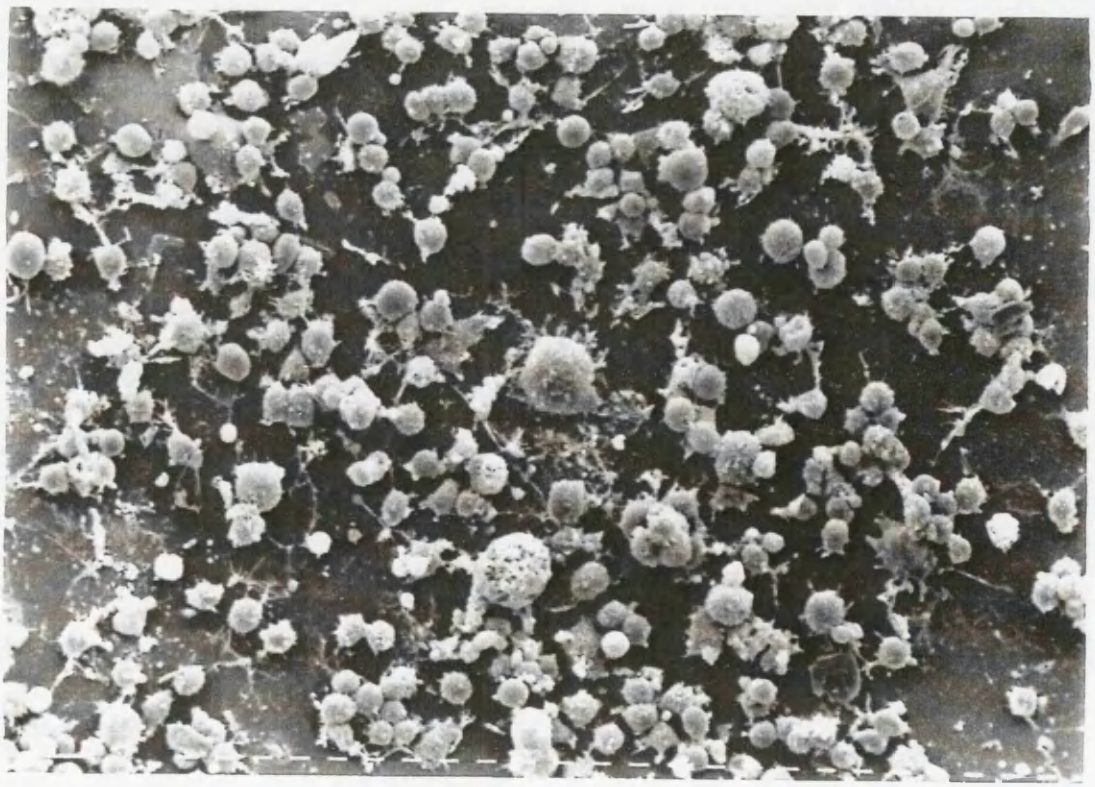
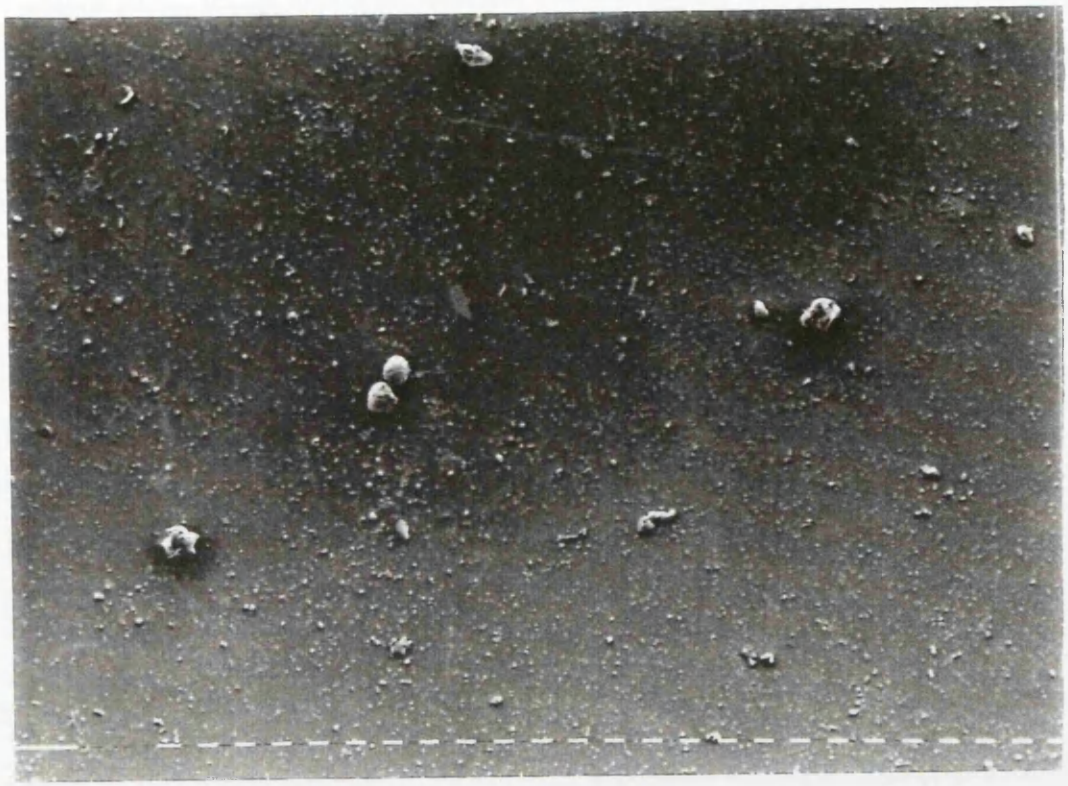


Plate 6.6.

N2a cells plated and maintained under assay conditions. The cells are at the same age and stage of development as those used at the time of an assay. Scale bar = 10 μm.

Top - Cells exposed to 0.05 mM VER for 24 h.

Bottom - Cells incubated in a combination of 0.05 mM VER, 0.3 mM OUB and 125 nM STX for 24 h.



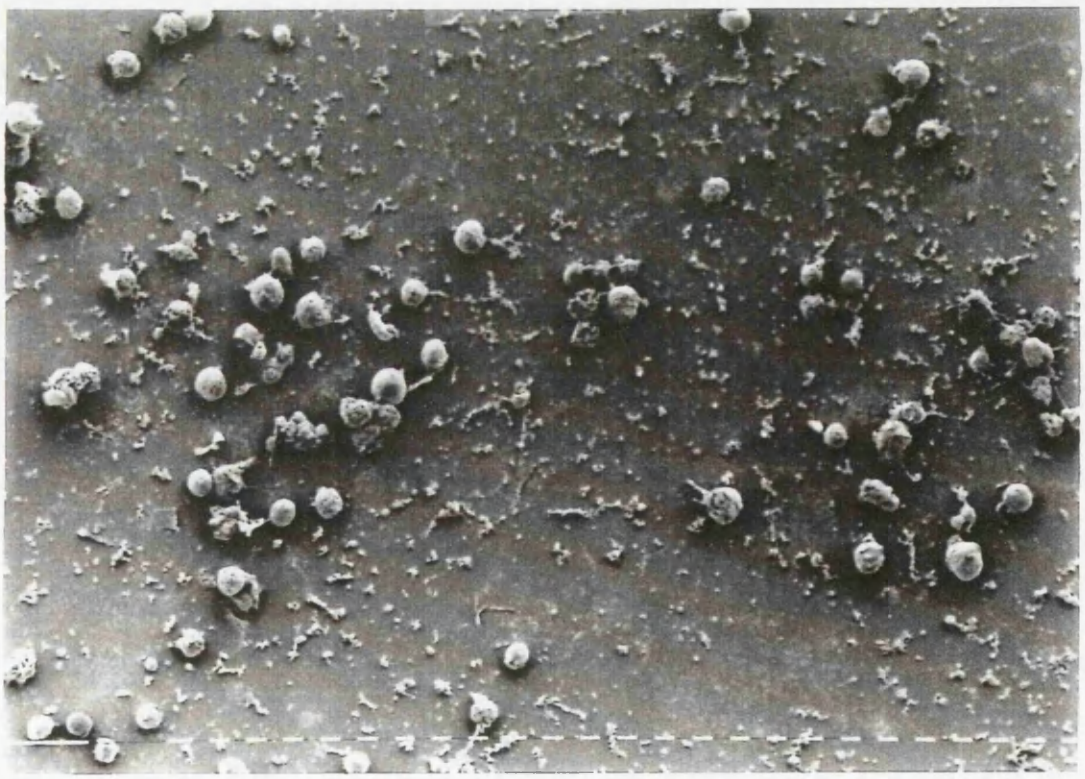
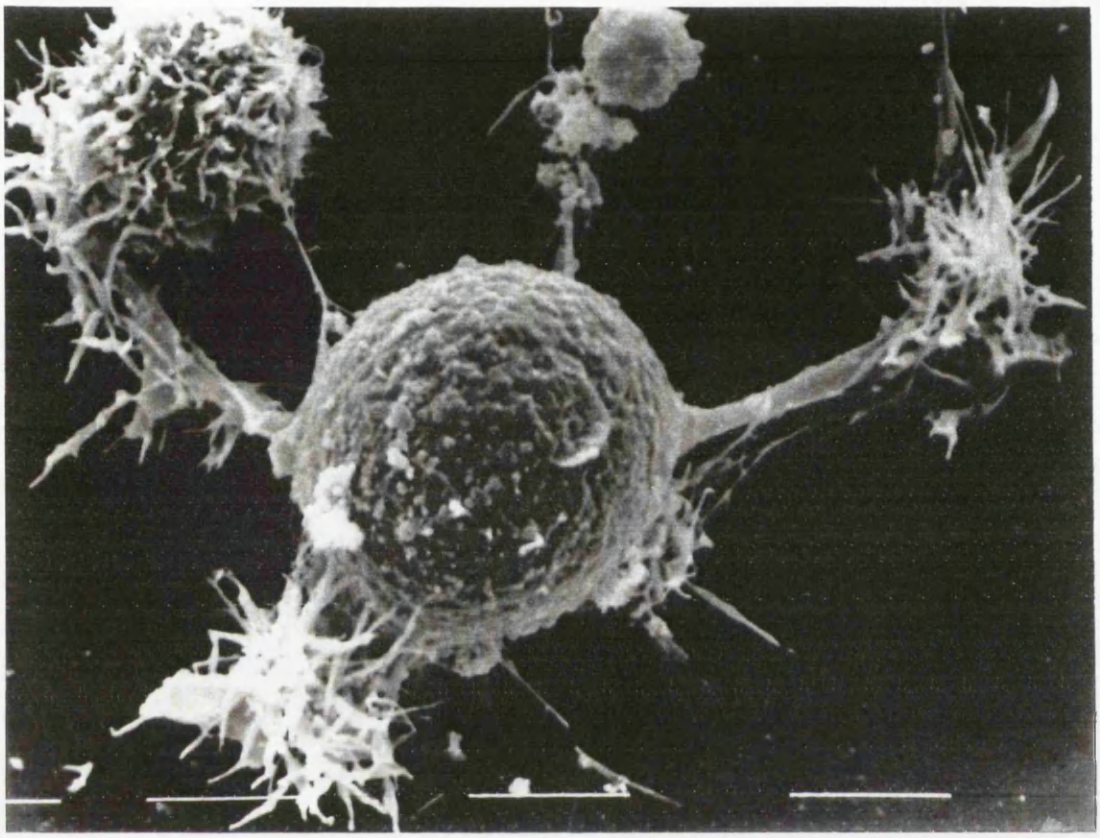
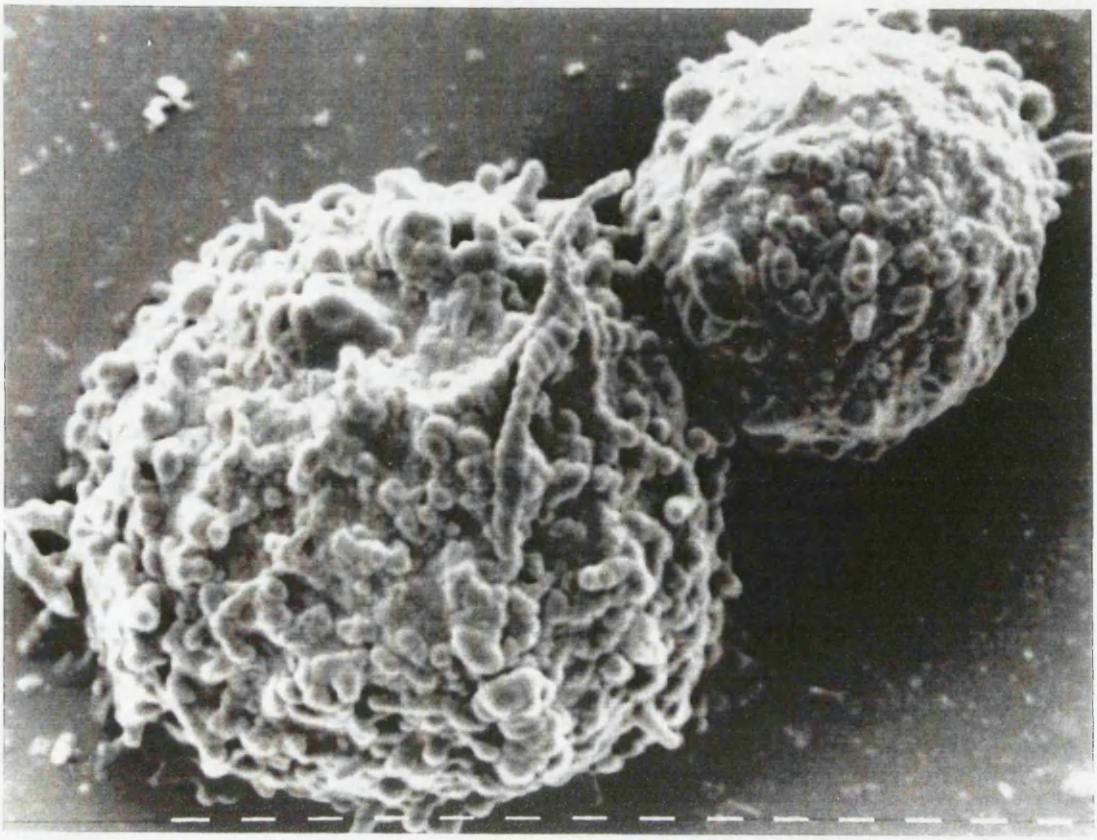


Plate 6.7.

Magnified S.E.M. of typical N2a cell membrane morphologies following a 24 h incubation period in 0.3 mM OUB (top photograph) and 0.05 mM VER (lower photograph). Scale bars = 10 μm and 1 μm respectively.





Chapter 7

Patch Clamp Techniques and MTT Cytotoxicity Study: Four Cell Types Assessed for Cell Bioassay Robustness using STX and PSP Samples

7.1 INTRODUCTION

The introduction of the patch clamp method (Neher *et al.*, 1978; Hamill *et al.*, 1981) has proved to be a powerful tool for the characterisation of voltage-dependent currents in electrically-excitable cells. Additionally, this electrophysiological approach, with its advantage of high time resolution, has provided information on the mechanisms by which toxins bind, activate, modify, or block the transmembrane activity of cells. Cultured neuroblastoma cells provide a convenient experimental system in which to study the actions of such neurotoxins using patch clamp techniques (Narahashi 1988; Kiss and Nagy 1988; Kao and Hu 1989; Caulfield *et al.*, 1992; Quandt 1994).

7.1.1. Na^+ channel properties of neuroblastoma cells

Various neuroblastoma cell lines and clones have had their ionic conductances characterised both in their undifferentiated states and after differentiation, as described in detail in **section 6.1.3**. Prior to differentiation, inward currents are often composed of two components (one calcium and one sodium) and can be STX-resistant (STX_R). After differentiation, a greater density of Na^+ channels is observed, the kinetics change and the inward current normally becomes STX-sensitive (STX_S).

Differentiation can also significantly increase the TTX-sensitive (TTX_S) current in cells expressing TTX-resistant (TTX_R) currents when undifferentiated (Weiss and Sidell 1991). Some neuroblastoma cell lines, including clones of the C-1300 line, express action potentials that cannot be completely blocked by TTX (Nelson *et al.*, 1971; Moolenaar and Spector 1977, 1978; Stallop 1979), and persist in a Ca^{2+}-free medium, suggesting that they depend on a TTX_R Na^+ current. However, other cell lines, such as NG108 cells, show a TTX_R fraction in the inward current, but this can be completely eliminated by removing Ca^{2+} from the extracellular medium, suggesting that there is no TTX_R Na^+ current involved (Quandt and Narahashi 1984; Docherty *et al.*, 1991).

7.1.2. Na^+ channel agonists

The Na^+ channels of electrically-excitable neuroblastoma cells are also affected by neurotoxins such as BTX, ATX-II, and VER that cause persistent activation of the Na^+ channels. These toxins dramatically increase the passive Na^+ permeability of the cell membrane and are therefore highly toxic to the cells. There are however some variant neuroblastoma clones that appear to lack voltage-sensitive Na^+ channels and remain resistant to the effects of these neurotoxins (West and Catterall 1979). Conversely, some Na^+ channels are more sensitive to these neurotoxins. VER, which is a partial agonist, was found to have greater intrinsic activity in synaptosomal preparations, activating 54% of sodium channels at saturation, compared to 8% in neuroblastoma cells (Tamkun and Catterall 1981). Therefore the choice of cell line or preparation is of critical importance.

7.1.3. Neuroblastoma cell bioassay

One of the main underlying principles of the neuroblastoma cell bioassay for the detection of PSP toxins is the agonistic effect of VER on the Na^+ channels in the cell membrane. During the assay a steady or sustained depolarisation results from the slow modification of channels by VER, and the concomitant increase in resting Na^+ permeability causes the channels to remain open at rest and at more depolarised potentials (Strichartz *et al.*, 1987). The addition of STX or related sodium channel blocking toxins can reduce or prevent VER-induced depolarisation by binding to the Na^+ channel and blocking the passage of Na^+ ions through the membrane; the integrity of the cell is thus preserved. The bioassay involves titrating a standard amount of VER (and OUB) with STX or its analogues and using a net measurable effect (e.g. cell survival rate) as a quantitative determination of the concentration of STX or its analogues (Jellet *et al.*, 1992).

Currently, at least five research groups are using the N2a clone of the C1300 neuroblastoma cell line in a cell bioassay as a quantitative detection method for the absolute potency of PSP toxins in shellfish extracts. If present in the sample, the PSP

toxins inhibit the cytotoxic effect of the Na^+ channel activator, VER. All the assays are automated; two, Jellet *et al.* (1992, 1995) and Gallacher and Birkbeck (1992) with crystal violet and neutral red endpoints respectively; the other three (Manger *et al.*, 1993, 1994, 1995a,b; Hamasaki *et al.*, 1996; Truman and Lake 1996) use a modified MTT tetrazolium reagent end point. Details of the assays have been described in **section 2.4.7.**

7.1.4. Instability problems of the cell bioassay

Progress continues to be made in the application of cell-based assays for detection and quantification of total toxicity in shellfish samples, in terms of reducing both incubation and detection time (Manger *et al.*, 1995b; Hamasaki *et al.*, 1995), and in the evaluation of a series of kits ranging from fully quantitative to qualitative versions (Jellet *et al.*, 1995). However one difficulty of the cell bioassay is the maintenance of cells in a stage of consistent expression of sodium channels. "Instability" of the cells was cited as the main reason for terminating a parallel mouse bioassay/cell bioassay study of PSP in shellfish, which involved 3 participating laboratories in Britain during the 1995 CSL Ring Trial. (S. Gallacher and K. Way, 1994, *pers. comm.*, and subsequently, 1996, personal communication to J. Jellet, cited in Quilliam 1997).

7.1.5. Study objectives

The initial objective of this study was to examine Na^+ currents in the N2a cell line before and after treatment with VER, OUB and STX. This cell line is currently being used extensively as an assay for PSP quantification. Moreover, a comparison of the pharmacological and functional effects of STX, and of PSP samples of unknown toxicity and composition, on the amplitude and kinetics was also attempted. Previous studies of PSP toxins have concentrated on the purified components, and this is the first study using electrophysiological techniques to observe the effects of AOAC

shellfish extracts on the Na^+ membrane currents of the cells currently used in the PSP bioassays.

As the choice of cell line or cell preparation is of critical importance with regard to its sensitivity and robustness, an extended series of patch clamp experiments was performed to assess their suitability. Two were neuroblastoma cell lines: i) Neuro-2a (N2a), ii) NG108-15 (NG108) which is a hybrid cell line formed from the fusion of a rat glioma and a mouse neuroblastoma, and two were primary cell types: enzymically-isolated, rat pup, dorsal root ganglia (DRG) and superior cervical ganglia (SCG). Initial experiments to test for STX sensitivity in a human neuroblastoma cell line (SK.N.SH) were also performed.

Unknown compounds in the PSP extracts (Sato *et al.*, 1988) may cause cytotoxic damage to cell membranes that could pass undetected because of the already cytotoxic effect of incubation in VER and OUB. An MTT assay was therefore performed to detect whether any PSP samples could by themselves cause cell death.

7.2. MATERIALS AND METHODS

7.2.1. Maintenance of Neuro-2a cells

N2a cells were supplied from the same stock as that used for cell assay by the Department of Microbiology, University of Glasgow. Initially cells were grown in Eagle's Minimum Essential Medium (MEME) with Earle's Salts, without L-Glutamine and sodium bicarbonate, supplemented with 5 % FCS. (With 10% FCS, cell division was too rapid, and cells had to be split every second day). After approximately 20 subcultures, cell appearance, viability and experimental performance began to deteriorate. This occurred with each fresh passage retrieved from frozen stock. Dr. P. Munro (Department of Microbiology, University of Glasgow, *pers. comm.*) and Mr. K. Way (MAFF, Plymouth, *pers. comm.*) observed that their N2a cells behaved in a similar manner.

Initially a 2% non-essential amino acid (NEAA) concentrate (Gibco), as recommended by ETCC (Porton Down) and Flow, was added to the maintenance

medium. However since cell viability was low the medium base was changed to RPMI 1640 (Table 6.1), NEAA was omitted, and FCS was reduced to 3%. Thereafter cell viability improved, with sub-culturing every 4 days. Ingredients for growth medium that required freezing were aliquoted into volumes that needed freeze-thawing only twice. This reduced the risk of deterioration in the products. Cells could then be passaged at least 30 times before instability was detected.

Sub-culturing methods involved aspirating off the old medium and adding 5 ml of fresh maintenance RPMI to the flask. Cells were removed mechanically from the flask base. A uniform suspension was obtained by mild tituration using a 10 ml pipette. One ml of the suspension was then pipetted into each 80 cm^2 flask required and 19 ml of growth medium was added to give a total of 20 ml per flask. Cells to be used for electrophysiological recordings were seeded onto 13 mm cover slips with $2x10^4$ cells/slip, in 24 well plates and maintained in 3% FCS.

7.2.2. Maintenance of NG108-15 cells

NG108-15, a hybrid cell line formed by a Sendai virus-induced fusion of a mouse neuroblastoma clone N18TG-2 and a rat glioma clone C6 BV-1 (Klee and Nirenburg, 1974, ECACC, 1994) which had been obtained from the European Tissue Culture Collection (Porton Down) as passage 36 were kindly supplied by Dr. E.G. Rowan, Department of Physiology and Pharmacology, University of Strathclyde. This cell line is also known as 108CC15 (Moolenaar and Spector 1978)

This cell line was cultured along with N2a cells in order to compare their electrophysiological responses to various differentiation treatments used to enhance sodium channel density and thus to assess their suitability for toxicity monitoring.

Culture of NG108 cells was as described for N2a cells, in MEME growth medium detailed in Table 6.1. Thymidine (HAT) was an essential constituent to prevent the cells reverting to glioma cells only. Subculture procedures followed those in **section 7.2.1**, using a 2% Trypsin-EDTA solution. Cells required for recordings were seeded at a density of 2×10^4 cells/slip and maintained as above.

7.2.3. Preparation of primary cell types

Two primary neuronal cell types, dorsal root ganglia and superior cervical ganglia, dissociated from 1-5 day old Sprague-Dawley rats were prepared for comparative studies of voltage-gated channels with those of the tissue culture cells.

Dorsal root ganglia cells (DRG) were dissected and prepared through acute enzymatic dissociation, essentially as described by Lindsay *et al.* (1991) after the rat pups had been killed by cervical dislocation. Dissected ganglionic tissue was incubated at 37°C, in 2.5 mg/2 ml collagenase, Ca^{2+}, Mg^{2+} -free HBSS solution for 15 min. This solution was then discarded, the cells were washed twice in HBSS and incubated in 2 ml of a 0.25% trypsin Ca^{2+}, Mg^{2+} -free HBSS solution for 5 min. Cells were then dissociated by gentle tituration and centrifuged for 5 min at 50 g. The supernatant was removed and the cells were resuspended, then plated onto 13 mm cover slips, pre-treated with one of the substrates described below. Cells were maintained in an RPMI-1640 medium described in Table 6.1 in a humidified atmosphere in 5% CO_2-95% O_2 at 37°C.

As soon as cells appeared to have adhered to the substratum they could be used for recordings (Plate 7.1). Most cells were used between 12 and 36 h after plating, whilst the cell bodies were still almost spherical. Healthy cells possessed bright, smooth membranes, and well-defined cell bodies with small neurite extensions. Cells with small granular markings were avoided, as were those which exhibited large leak conductance after a seal was obtained.

Superior cervical ganglia cells (SCG) were dissected by a method similar to that of Stansfeld and Mathie (1993) from the same rat pups used to obtain the DRG cells. Treatment and maintenance for SCG cells essentially followed that for DRG cells, except that dissected ganglia cells were chopped into 4-5 small pieces before placing them in a solution of collagenase for 1 h and in trypsin for 15 min. Maintenance medium is described in Table 6.1. Cells used for recordings followed the criteria for DRG cells.

For each day that rat pups increased in age, both enzymic treatments were increased by 5 min in order to allow for the increase in the thickness of the sheath encapsulating the cells.

7.2.4. Preparation and use of differentiating agents and substrata

Techniques employed to investigate the enhancement of sodium channel density involved the use of (*i*) low FCS medium, (*ii*) chemical agents added to low serum maintenance media, (*iii*) substrate modifications, (*iv*) combinations of all three.

(*i*) *Low FCS medium*

FCS was reduced to a final concentration of 2% in the maintenance medium for N2a cells and 1% for NG108 cells. Low FCS medium was used 24 h after plating.

(*ii*) *Chemical agents*

Low-serum media were supplemented with 10 μM prostaglandin E_1 (PGE1) and 50 μM 3-isobutyl-1-methylxanthine (IBMX) (Docherty *et al.*, 1991; Spector 1981). The chemicals were obtained from Sigma. A 1 mM stock solution of PGE1 was prepared by dissolving 1 mg PGE1 in 2.82 ml distilled water, which was filter sterilised (0.45 μm Whatman filter), aliquoted into 125 μl volumes and stored at -70°C until required. A further 1/100 dilution of stock PGE1 in low FCS media gave a final well concentration of 10 μM. IBMX was prepared as a 1 mM stock solution by adding 2.22 mg to 10 ml distilled water. This was filter sterilised, aliquoted into volumes of 625 μl and stored at -20°C. A final well dilution of 50 μM was obtained by a 1/20 dilution of IBMX : low-serum media.

Media supplemented with both PGE1 and IBMX were added to N2a and NG108 cells one day after plating. Initially, cells were treated just once, and after 48 h in PGE1/IBMX they were returned to a low-serum only medium. Later, the cells were retained in PGE1/IBMX until used for recordings.

A second chemical agent used to induce differentiation was dibutyryl cyclic AMP (dbcAMP) (Chalazonitis and Greene, 1974; Kiss and Nagy 1988). A stock solution of 100 mM was prepared by adding 100 mg dbcAMP to 2.03 ml of de-ionised water which was then filter sterilised. A further 1/100 dilution of stock dbcAMP in low FCS medium gave a final well concentration of 1 mM. This was added to both tissue culture lines one day after plating. Originally, cells were treated only once with dbcAMP, which was then replaced with just low FCS medium. This was later changed to continuous treatment, with fresh dbcAMP every 2 days until cells were used.

(iii) Preparation of substrata

Originally, tissue culture cells were plated onto 13 mm glass cover slips (Chance Propper Ltd) with no additional modifications. Plastic cover slips (13 mm, Thermanox, Nunclon, Intermed) with no additional treatment were also tried. In addition, four types of substrata were tried, to enhance differentiation. Poly-L-lysine (Sigma) a synthetic polycationic macromolecule, and three types of collagen: (adhesive glycoproteins); calf skin (Sigma), rat-tail (ICN) and vitrogen (Celtrix) were prepared for coating glass coverslips. Substrata-treated coverslips were either prepared in advance and stored in HBSS in tissue culture dishes, or prepared on slips already placed in individual wells of multiwell plates. Both methods could then be U-V treated overnight to ensure sterility.

Poly-L-lysine-(hydrobromide), mol. wt. 70,000-150,000, was prepared as a stock of 5 mg /ml distilled water, then diluted 1: 250 parts. This was dispensed onto cover slips and left to dry overnight under U-V conditions. Any mixture not set by the following morning was removed. The slips were washed in HBSS before plated with cells. Poly-L-lysine was also used in conjunction with 1:30 ICN rat-tail collagen, as a trial substrate specifically for SCG cells.

Calf skin Type 1 collagen was prepared as a 0.1 % solution in HBSS. This gave a 1:10 ratio matrix.

Rat tail Type 1 collagen was prepared as a series of 3 %, 4%, 5% and 10 % solutions equivalent to 1:30, 1:40, 1:50 and 1:100 ratio consistency.

Vitrogen 100 was prepared as a 1:20 parts mixture using 0.4 ml vitrogen : 8 ml distilled water, to which were added 1 ml of 10x buffered saline, 1 ml NaOH, and 0.005 ml phenol red. After application onto the cover slips, the mixture was left for 6 h, then any excess was poured off. The slips were then left to dry overnight under U-V conditions. Prior to use slips were washed in HBSS.

Cells were either seeded onto one of the substrata described above and incubated in normal growth medium, or were incubated in one of the defined media containing differentiating agent.

7.2.5. Dilution of PSP shellfish extracts

Two AOAC shellfish extracts, S70 and S1, were tested to determine their effect on the Na^+ channels in three cell types. The samples were diluted 1:1000 and 1: 10,000 parts firstly as a direct comparison to the ELISA assay, and secondly as a cautionary action to minimize possible cell membrane damage whilst recording Na^+ currents, since lytic effects have been previously reported (Jellet *et al.*, 1992).

7.2.6. Electrophysiological solutions, equipment and protocols

i) Solutions for general current indentification for clonal cells

Prior to recording, cells were transferred to an external solution adapted from Barnes and Hille (1988) with the following ionic composition (in mM): NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, Na_2HPO_4 0.06, glucose 5, HEPES 10, buffered to pH 7.3 with NaOH. The pipette solution was composed of (in mM): NaCl 5, KCl 140, $MgCl_2$ 1, glucose 10, HEPES 10, ethylene glycol-bis (β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) 1, adjusted to pH 7.3 with KOH.

The osmotic strength of these and the following solutions were measured using an Osmette S (Precision Systems, Inc.) osmometer. The solutions had measured osmolarities of 310 mOsm external (bath) : 297 mOsm internal (pipette), and were

acceptable according to the criteria of Stansfeld and Mathie (1993). These and the solutions below were all filtered using a 0.45 μm filter (Millipore) for external, and 0.2 μm filter for internal solutions.

ii) Solutions for sodium current indentification

The external solution was of the following composition (in mM), NaCl 140, TEA-Cl 20, $MgCl_2$ 1.2, $CaCl_2$ 2.5 $CoCl_2$ 2.0, $CsCl_2$ 5.0, HEPES 10, glucose 10, Na_2HPO_4 0.06, with pH adjusted to 7.3 with CsOH. Osmolarity was 330 mOsm. The pipette solution contained (in mM), NaCl 10, TEA-Cl 25, $CsCl_2$ 120, $MgCl_2$ 2, HEPES 10, EGTA 10, adjusted to pH 7.3 with CsOH. Osmolarity was 310 mOsm. As before all solutions were filtered before use.

iii) Solutions for general current indentification for primary cells

The same solutions for general voltage-gated current indentification used with clonal cells were also suitable for use with primary cells with the modification that choline chloride replaced approximately half the external Na^+ ions. Thus the composition was 60 mM choline Cl and 80 mM NaCl. This substitution normally reduced the size of the Na^+ current without altering the kinetics, thus preventing constant saturation of the amplifier.

iv) Solutions for sodium current isolation for primary cells

Several combinations of choline: Na^+ were tried. The following composition was considered to give best overall results for cell stability and near optimum working size of current. The extracellular solution contained (in mM) NaCl 80, choline-Cl 60, $CoCl_2$ 2, TEA-Cl 25, $CaCl_2$ 0.1, $MgCl_2$ 5, $CsCl_2$ 5, glucose 10, HEPES 5, adjusted to pH 7.3 with CsOH. Sucrose was used to adjust osmolarity to around 330 mOsm.

v) Recording pipettes

Patch pipettes were pulled from borosilicate glass capillaries, type GC150F-15, 0.2 O.D. x 0.69 I.D. (Clarke Elec. Instr.) on a Sutter P-87 (Brown Flaming) horizontal puller. Pipettes were fire-polished using a Narishige micro forge MF-83. During early experiments pipette tips were coated with melted beeswax. This was later substituted by Sigmacote (Sigma). Pipette resistance was between 0.3 and 4 MΩ when filled with internal solution, measured against a Ag/AgCl half cell reference electrode connected to the signal ground of the EPC-7 Controller via the probe cable. Junction potentials were normally very small (< 4 mV) and were compensated so that there was zero current flow before a seal was established. The EPC-7 headstage was attached to a stage-mounted mechanical micro manipulator (Narishige). Once the pipette was positioned at the optimal angle, the manipulator was clamped rigidly to prevent drift, and the final positioning was made using an oil-hydraulic manipulator (Narishige).

vi) Electrophysiological recording

Experiments were performed at room temperature with the cells in a custom-built perspex bath mounted into the stage of a Nikon TMS inverted microscope at X400 magnification. Changes of the extracellular solution were achieved by total replacement of the bath medium using a simple gravity-driven perfusion system with a flow rate of 2 ml min^{-1}. A constant well volume of 2 ml was maintained by an adjustable valve attached to the bath outflow tubing which itself led to two carboys, set up as a pump driven (35/B, Medcalf Bros Ltd.) vacuum system.

To change from bath to experimental solutions a set of 50 ml syringes individually attached to separate three-way valves, based on a Marriot-type pressure-driven system, was used. These syringes, plus the carboy containing the standard superfusate, were connected to a common tubing source leading to the bath. Each valve could interrupt the flow of the main superfusate whilst regulating the flow of the experimental solution into the bath.

As before, the whole-cell patch-clamp technique (Hamill *et al*, 1981) was employed to measure ionic currents. A List model EPC-7 was used as the recording amplifier with a 1 GΩ headstage. Membrane currents were filtered at 3 kHz. Capacitative currents were minimised using the negative capacity compensation of the patch clamp amplifier. The series resistance, R_s, for N2a and NG108 cells was uncompensated to avoid oscillations of the amplifier which damaged the cells. For DRG and SCG, compensation to 70 % was used on larger cells.

A Farnell PG 101 pulse generator was used to assist the measurement of pipette resistance and to monitor the increase in resistance as a seal was established, visualised on a Hameg HM 205-3 storage oscilloscope. Voltage command protocols were generated by a Viglen 4DX 33 computer and applied through a LAB-PC+ interface board (National Instruments UK) using Dempster WCP software, kindly supplied by Dr. J. Dempster, University of Strathclyde. Data were digitised at a 75 kHz sampling rate and stored on disk for off-line analysis using the WCP programme.

Membrane area was estimated from whole-cell capacitance (read from the dial of the EPC-7) assuming a value of 1 $\mu F/cm^2$. Loss of voltage control due to R_s and space-clamp errors sometimes occurred when membrane area and peak Na^+ current became larger following induced differentiation of some of the clonal cells. This was also a problem with some of the primary cells.

vii) Current to voltage measurement protocol

A protocol similar to that used previously to measure current-voltage relationships was employed. A set of 15 depolarising test pulses of 30 ms, applied at 1 Hz, incrementing by 10 mV from a holding potential of -80 mV was used to elicit voltage-dependent currents. Each test pulse was followed by a matching P-P/4 leak subtraction protocol as described by Bezanilla and Armstrong (1977). Essentially, each test pulse was followed by four repetitive hyperpolarising pulses (i.e., P-P/4) with an amplitude ¼ of the test pulse. These records were not subtracted automatically on-line, but were saved in a separate file, so that original records could

be examined prior to analysis. The digital sampling interval was 0.05 ms, with 1024 samples per record, and the total record duration was 51.2 ms.

viii) Repetitive step protocol

A protocol was designed to monitor the stability of the evoked current amplitude by using a single 100 ms repetitive depolarising test pulse of 75 mV from a holding potential of -80 mV. Under normal conditions this would evoke a maximal Na current. The pulse was repeated every 5 sec for the length of the experiment. Each test pulse was interleaved with a P-P/1 leak subtraction hyperpolarising pulse ¼ of the test pulse. The sampling interval was 0.05 min, with 1024 sample points per record.

7.2.7. MTT assay preparation.

The tetrazolium salt 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used in a simple colormetric method to asses cytotoxicity. MTT is an indicator of living cells. The tetrazolium salt is cleaved by dehydrogenase enzymes in active mitochondria to produce a dark blue formazan product (Mosmann, 1983).

N2a and NG108 cells were seeded into 96-well micro plates at a density of 1x 10^{-4} or 1x10^{-5} cells/ml in 100 µl of growth medium per well. The outside row at each end of the plate received only growth medium (blank controls). Prior to further processing, cultures were incubated for approximately 24 h.

Cells were exposed to a PSP extract (1:60 dilution), or VER 0.05 mM, OUB 0.4 mM, STX 100 nM, alone or in various combinations, or, increased $[Ca^{2+}]_o$ of 3, 4, 5, 6 or 10 mM, all final well concentrations. Cultures received 90 µl of test compound(s) and growth medium. Each compound was tested in replicate (4-5 wells). Blank wells and control cells received 90 µl of growth medium to make up for the volume difference. Cultures were then incubated again for either 2, 18 or 24 h.

MTT (Sigma) was prepared as a 5 mg/ml filtered stock solution in either RPMI (N2a cells) or MEME (NG108 cells). Following incubation with test

compounds, 20 µl MTT (1:10 dilution) was added to all wells with no removal of the overlying medium. Cultures were incubated for approximately 2 h at 37°C or until a suitable reduced dark formazan deposit was observed. The overlying medium was then removed, without a wash step, and 100 µl of DMSO (Sigma) was added to each well. The plates were read immediately on a Dynatech 5000 automated multiwell scanning spectrophotometer using a test wavelength of 570 nm.

7.2.8. Statistical analysis of data

Values for changes in amplitude or RMP are expressed as a percent of the mean ± SEM. Comparisons between data were performed using a Mann-Whitney U test (Minitab). A value of $p < 0.05$ (two tailed) was taken as indicating statistical significance. The slope of the linear regressions ($y = a*x + b$) was fitted using an automated analysis data transformation (FIGP 6.0 Biosoft). A Student t-test was performed to test for statistical significance of *r* between inhibition of peak amplitude and STX concentration (**section 7.3.5.iv**). The critical value of $t_{0.025}$ (df3) = 3.182.

7.3. RESULTS

7.3.1. General Na^+ current characteristics of Neuro 2a cells

Undifferentiated cells - Using a 15 step depolarising protocol (**section 7.2.6, vii**), from a holding potential of -80 mV (Figure 7.2.a), cells exhibited macroscopic currents as shown in Figure 7.2.b. The upward traces represent outward K^+ currents, whilst the downward traces are typical of inward Na^+ currents. Measurements of the peak Na^+ current amplitude from $n = 40$ cells resulted in a mean value of 1310.52, ± 157.83 pA (Figure 7.4.a. N2a plain bar). Many of the whole-cell patches obtained were unstable, frequently lasting only 10 min, and the ability to obtain high resistance seals and measurable macroscopic currents diminished as the number of culture passages increased above 20.

***Differentiated* cells** - Cells were subsequently cultured using a variety of chemical differentiating agents and substrata (**section 7.2.4.**). There was a noticeable change in the size of the Na^+ currents (Figure 7.2.c) and the mean peak Na^+ current increased to 2992.3, ± 413.04 pA, n= 25 (Figure 7.4.a. N2a striped bar), reflecting a significant (p=0.0002) increase in Na^+ channel density. A comparison of cell size between undifferentiated and differentiated cells revealed a mean value of 27.2 pF ±3.58, and 23.1 pF ± 2.33, respectively. Statistical analysis showed that there was no significant difference (p = 0.176) between cell size before and after differentiation. The stability of the patches increased to 15 - 30 min, but the problem with general cell condition still existed after about 20 passages.

A typical current-voltage (*I-V*) relationship for the Na^+ current recorded from a holding potential of -80 mV shows an activation peak at about -25 mV (Figure 7.5.a). The E_{Na} is estimated to be +60 mV, which agrees with the value derived from the Nernst equation for $[Na^+]$ in the external solution and the predicted concentration of Na^+ in the cell.

Resting membrane potentials were measured under current clamp conditions. A mean RMP value of -28.5, ± 2.38 mV was obtained from n = 19 cells (Figure 7.4.b. N2a striped bar). RMP values obtained from undifferentiated N2a cells from the previous intracellular experiments (**section 6.3.1.**) had a mean value of -21.9, ± 3.01 mV (Figure 7.4.b. N2a plain bar). Statistical analysis showed that there was no significant difference (p = >0.05) in RMP between undifferentiated and differentiated cells. However the values were obtained under different measurement conditions.

7.3.2. General Na^+ current characteristics of NG108-15 cells

Undifferentiated cells - The suitability of the hybrid cell line NG108-15 was also examined as a possible alternative assay system. Under similar culture conditions to those for N2a assay cells only very small Na^+ currents were elicited (Figure 7.3.a). The mean peak amplitude measured from n = 6 cells was 429.04, ± 107.59 pA (Figure 7.4.a. NG108 plain bar).

Differentiated cells - Cells were subsequently cultured using the differentiating agents IBMX, or, PGE_1 + cAMP, as well as being plated on a variety of substrata (**section 7.2.4**). A typical family of ensemble currents recorded from an NG108 cell after a short incubation period in one of the differentiating culture media is shown in Figure 7.3.b. The transient Na^+ current had increased in amplitude, with a faster activation time course. Some cells (not shown) exhibited a faster rising K^+ component. These culture conditions resulted in cells exhibiting a significantly larger (p=0.0003) peak Na^+ current; mean 3933.6, ± 383.09 pA, n = 27 (Figure 7.4.a. NG108 striped bar). This was approximately a ten fold increase in Na^+ current size. A comparison of cell size between undifferentiated and differentiated cells revealed a mean value of 30.8 pF ±3.58, and 24.8 pF ± 2.43, respectively. Statistical analysis showed that there was no significant difference (p = 0.107) between cell size before and after differentiation.

A typical *I-V* relationship for a differentiated NG108 cell is shown in Figure 7.5.b, with peak activation at -25 mV. The E_{Na} is estimated to be +60 to +65 mV, similar to that for differentiated N2a cells. No RMP values were recorded from undifferentiated cells, but those measured from differentiated NG108 cells (n = 29) had a mean value of -40.04, ± 1.75 mV (Figure 7.4.b). This was a significantly more negative value (p = 0.001) than that for N2a cells (-28.5, ± 2.38 mV) cultured under the same conditions.

Morphological changes in NG108 cells exposed to cAMP are illustrated in Plate 7.2. Under control conditions (Plate 7.2. top) the cells had extended few interconnecting processes, but by 1.5 h (Plate 7.2. middle) a relatively extensive neurite network was observed. Two days after exposure greater cell body differentiation was observed (Plate 7.2. bottom) and a slightly denser interconnecting neurite network was seen, with some processes being very dark and thick.

7.3.3. General Na^+ current characteristics for dorsal root ganglia (DRG) and superior cervical ganglia (SCG)

DRG cells - Voltage-dependent macroscopic currents elicited by the same depolarising protocol as before (**section 7.2.6.*vii***) are shown in Figure 7.3.c and d. The transient inward currents illustrated in Figure 7.3.c, have a faster rise time and peak at a more negative potential than those seen in Figure 7.3.d. These faster inward currents were found to be sensitive to STX (STX_S) and TTX (TTX_S). The inward currents in Figure 7.3.d. are typical of currents subsequently found to be STX resistant (STX_R) (refer to **section 7.3.5.*v***). Quantitative measurements of Na^+ currents from these DRG primary cells (Figure 7.4.a) gave a mean peak amplitude of 6697.68, ± 557.32 pA (n = 27). The mean RMP value was -61.94, ± 4.4 mV (Figure 7.4.b) a value in good agreement with Dolphin *et al.* (1986) for such cells.

SCG cells - A typical set of currents elicited from SCG cells, previously shown to possess voltage-dependent Na^+ channels (Stansfeld and Mathie 1993), are illustrated in Figure 7.3.e. The mean peak amplitude measured for SCG cells was 7238.3, ± 890.30 pA (n = 5).

I-V curves for both DRG and SCG cells (Figures 7.5.c and d, respectively), show that the Na^+ currents of these primary cell types peak at about -25 mV from a holding potential of -80 mV.

7.3.4. General Na^+ current characteristics of SK.N.SH neuroblastoma cells

A human neuroblastoma cell line, SK.N.SH, being used in a separate study was subsequently cultured and differentiated with 2% DMSO, under the same conditions as the N2a and NG108 cells. Following differentiation, both the transient inward and the outward K^+ currents increased in amplitude. A typical set of currents from such a cell is shown in Figure 7.3.f, and a representative *I-V* relationship is illustrated in Figure 7.5.e. The inward current peaked at around -15 mV, with E_{Na} at +50 mV. SK.N.SH cells had a mean peak amplitude of 3530.9, ± 288.30 pA, n = 49,

(Figure 7.4.a). The mean RMP measured under current clamp conditions for the differentiated cells was -49.26,± 2.67 mV, n = 21, (Figure 7.4.b). This was a very similar RMP value to that of NG108 cells (-40.04, ± 1.75 mV) and a significantly more negative value (p = 0.001) than that for N2a cells (-28.5, ± 2.38 mV) cultured under the same conditions.

The presence of Na^+ currents was confirmed in each cell type by exposure to an external solution in which the NaCl had been replaced with choline and osmotically balanced. Representative *I-V* curves from two of the cell types, an NG108 and a DRG cell are shown in Figure 7.6.a. and b. In the absence of Na^+ no inward current was recorded.

7.3.5. Block of inward currents by STX or TTX.

The *I-V* curves show that 100 nM STX completely blocked the inward current in a N2a cell (Figure 7.7.a) and a NG108 cell (Figure 7.7.b) respectively, and that 50 nM STX inhibited almost 90% of the current in a typical SK.N.SH cell (Figure 7.7.c).

Before further assessment of the dose-response effects of STX on the two predominant clonal lines (N2a and NG108 cells), a set of time-matched control experiments (n = 5 and 8 respectively) was performed using the single step protocol (**section 7.2.6.viii**). The change in peak amplitude as a percentage of control values was measured over a 10 min period. The mean results from these experiments are plotted in Figure 7.8.a (N2a cells) and Figure 7.8.b (NG108 cells). At 2 min, the time for complete exchange of bath solution to occur, a mean 10% decrease in peak amplitude was observed in N2a cells, further decreasing to 40% by 10 min (p = 0.055). With the NG108 cells over the same time period there was a slight increase of 1-2% after 2 min and a mean 20% decrease at 10 min (p > 0.05), which was half that observed in the N2a cells.

A typical time-matched control experiment is plotted together with an example of the decrease in peak amplitude following exposure to 100 nM STX for both N2a cells (Figure 7.9.a) and NG108 cells (Figure 7.9.b). The control amplitude

persisted with only a slow decline (Figure 7.9.a) or no change (Figure 7.9.b); by comparison the decrease caused by STX was extremely rapid and large.

7.3.5.i) N2a cells assessment - At 1 nM STX, changes in peak amplitude were difficult to measure accurately, but no definite blocking trend could be determined. The results plotted in Figure 7.10.a show a maximum STX blocking effect of 50% in one cell at 2 min, but in other cells exposure for up to 10 min resulted in decreases that were no greater than the rundown in control experiments. With a concentration of 10 nM STX almost half the cells tested showed a 50% reduction in the peak amplitude by 2 min, and by 3-4 min this became a 90% reduction in several cells (Figure 7.10.b). When the concentration was increased to 100 nM, STX caused a 70-80% blocking effect by 2 min in all but one of the cells (Figure 7.10.c). The amplitude of all cells (n = 8) was significantly reduced ($p = < 0.05$). At 300 nM STX an almost complete block (*ca.* 98%) occurred within 2 min (not illustrated).

7.3.5.ii) Comparison of STX sensitivity between undifferentiated and differentiated cells - No significant difference ($p = 0.766$) in sensitivity to STX was found between undifferentiated and differentiated cells despite the increase in Na^+ current size. At the 2-4 min time point 100 nM STX caused a mean reduction of 88.3% ± 5.16 (undifferentiated cells) and 88.5% ± 6.75 (differentiated cells), $n = 5$.

7.3.5.iii) NG108 cells assessment - When 1 nM STX was applied (Figure 7.11.a) a slight increase in peak amplitude was observed in two cells at 2-4 min, whilst in a third cell a decrease of about 40% was recorded. Similarly, in the cells exposed to 10 nM STX (Figure 7.11.a) a decrease of about 32% was observed at 2 min. A greater number of experiments was performed using concentrations of 100 and 300 nM STX on this cell line. Application of 100 nM STX (Figure 7.11.b) caused at least a 70% reduction of peak amplitude by 2 min. The amplitude of all cells (n = 8) was

significantly reduced ($p = < 0.05$). At 300 nM STX significantly reduced ($p = < 0.05$) current amplitude by 80% within 1 min (Figure 7.11.c).

7.3.5.iv) IC_{50} values - The mean values for each STX concentration (derived from inhibition values recorded at either 2 or 4 min from each individual experiment) were plotted and linear regression lines were then fitted for both the N2a and NG108 results (Figure 7.12). The degree of cluster of the points around the lines shows that there is a strong correlation between the percentage inhibition of the peak amplitude and the concentration of STX, with r values of $r = 0.968$ for the N2a experiments and $r = 0.989$ for the NG108 experiments. To test the significance of r, a Student t-test was performed. With 3 df for a two tailed test at $\alpha = 0.05$, $t = 5.508$ for N2a, and $t = 9.615$ for NG108 experiments, these results show that there is a significant dependency of amplitude upon STX concentration.

From this graph (Figure 7.12) STX IC_{50} values for the two cell lines were calculated as 8 nM STX for N2a cells and 15.5 nM STX for NG108 cells.

7.3.5.v) DRG assessment - Using the same experimental protocols as those performed on the neuroblastoma cell lines, the responses of the primary DRG cells to STX exposure were assessed. The two main findings were that the size of the Na^+ currents before chemical modification was normally twice that of those from the differentiated neuroblastoma cells (which made them difficult to work with), and that a proportion of the DRG cells expressed STX_R properties.

Previous studies (Caffrey *et al.*, 1992; Elliot and Elliot 1993) have defined STX_S Na^+ currents in DRG cells as those which are suppressed by at least 97% by external application of 100 nM STX/TTX. The data obtained from acutely dissociated cells from neonatal 1 - 6 day old pups, and within 1 - 5 days of plating, show that from $n = 18$ usable cells, 82% expressed Na^+ currents that were reduced by less than 50% (mean = 18%) and were therefore classed as STX_R Na^+ currents. The remaining 18% possessed Na^+ currents that were suppressed by greater than 98% in the presence

of 100 nM STX or TTX, and were therefore classed as STX_S currents. None were inhibited between 50 - 98%.

According to Elliot and Elliot (1993), STX_S and STX_R currents are distinguishable by their different time courses of both activation and inactivation. Examples of families of STX_S and STX_R currents are shown in Figure 7.3.c. and d. The main differences were: 1) The threshold for activation of STX_S currents was around 10 mV more negative than that for STX_R currents. 2) The maximum peak of the STX_R current occurred at a more depolarised potential than the peak for a STX_S current. 3) STX_S currents exhibited a much faster time course of activation and inactivation than STX_R currents (Figure 7.13.a).

STX_S currents (Figure 7.13.b) were suppressed within 1 min of application of 100 nM STX, with total inhibition by 3 min. STX_R currents were little affected by STX application; for example, in Figure 7.13.c the effect of 100 nM STX on the peak current amplitude after 3 min was a suppression of only 12.3%. In another cell exhibiting STX_R currents, 300 nM STX reduced the peak amplitude by only 20% after 5 min of exposure. Pooled data obtained from cells exposed to either STX or TTX from both quantitative and qualitative experiments (Figure 7.14.a.b.c) clearly show a marked division of inhibition properties between the STX_S and STX_R currents.

7.3.6. The effect of PSP extracts on the transient Na^+ current

A PSP sample with a high STX content, S70 (565 µg STX eq.), was applied to three cell types: N2a, NG108 and DRG cells at either 1:1000 or 1:10,000 parts dilution. The concentration of 1:10,000 parts is equivalent to that used in ELISA assays. Prior to this, control experiments with 0.1 M HCl, the main vehicle for the PSP samples and STX, showed that at the concentrations used, these acids did not significantly affect the peak inward current (data not shown).

At 1:1000 parts the peak Na^+ current was reduced in all three cell types (Figure 7.15.a) during the 5 min test period. N2a cells (n=3) appeared to be the most

sensitive, with a maximum inhibitory response of 91% in one experiment. The Na^+ current in NG108 cells (n=4) was inhibited by between 41 and 69%, while a reduction of only about 24% was observed with a DRG cell. This smaller response is consistent with the presence of an STX_R current.

As N2a cells are the most commonly used neuroblastoma cell line for the PSP tissue culture bioassay, the second concentration of 1:10,000 parts (Figure 7.15.b) was tested on this cell line (n=3). The range of inhibition was very wide (6.78-74.46%) at this dilution, with a slower onset of the blocking effect (by almost 1 min), than with the previous concentration of 1:1000 parts. As shown in Figure 7.15.c, the 1:1000 parts concentration had a mean blocking effect of 61.4% compared to 41.7% by the 1:10,000 parts concentration. The small data set did not allow further analysis.

To verify that the reduction in the current amplitude was due to the blocking of Na^+ channels rather than a cytotoxic effect of the sample itself, a number of cells were perfused with 100 nM STX, underwent a wash-recovery period and were then perfused with S70. Examples of these experiments are shown in Figure 7.16. The smallest inhibitory effect again occurred with a DRG cell.

Earlier results from the CSL Ring Trial had indicated that a small number of negative (0 µg STX eq.) samples according to the mouse bioassay or HPLC, had given a positive blocking effect in the tissue culture assay. To test this finding, one of these samples (S1) which contained 0 µg STX eq by mouse bioassay, was applied to NG108 cells. In n=2 trials the sample inhibited the peak Na^+ current amplitude by around 40%, as shown in Figure 7.17. This reduction in the peak amplitude was equivalent to exposure to 6 nM STX, and suggests that the sample contained a component that has a depressive effect on Na^+ channel conductance.

Application of sample S1 to the external bath solution also resulted in an apparent lytic or cytotoxic-like effect on many of the cells on the coverslip, which immediately prior to addition of the sample had been healthy in appearance.

7.3.7. Cytotoxic effects of PSP samples using an MTT assay

In a series of viability tests, for a 2 h incubation period N2a cells exposed to the internal controls (STX, VER, OUB) showed a range of survival rates compared to the control value (Figure 7.18.a). Cells incubated in STX showed no change. Incubation in OUB alone appeared to enhance cell proliferation during this time period. A small increase in cell number was also seen with VER alone. Together, VER+OUB caused a small cytotoxic effect. The most significant cytotoxic effect ($p = 0.03$) was caused by a raised Ca^{2+} (6 mM CaCl) concentration in the incubation medium. None of the five PSP shellfish samples, with STX eq. values ranging from 0 μg (S99) to 651.6 μg STX eq (S32), showed any significant cytotoxic effect ($p = >0.05$) on the N2a cells. With one sample, S185 (118.4 μg STX eq) an increase in cell numbers was actually detected.

A further set of N2a cells left to incubate for 24 h (Figure 7.18.b) showed markedly different responses. All the internal controls, including STX, significantly reduced ($p = <0.05$) the cell survival rate. The raised Ca^{2+} concentration had the greatest cytotoxic effect. Decreases in cell survival rates were also observed with all five PSP samples tested. Sample S58 (90 μg STX eq) containing an STX level within the toxicity range found to have cytotoxic effects in some samples by Jellet *et al.* (1992) produced a significant cytotoxic effect ($p = 0.01$), but the 0 μg STX content sample (S99), which had produced a "positive" result in the cell bioassay, but not in the mouse bioassay during the MAFF 1994 Ring Trial, showed no significant effect ($p = 0.09$) on cell viability. The two most potent samples (S32 and S70) had little or no effect on depressing cell survival rates.

The cell assay response of NG108 cells after an 18 h incubation are shown in Figure 7.18.c. VER and VER+OUB caused a significant reduction ($p = 0.03$) of around 50% in cell survival rate. All three Ca^{2+} concentrations, 3, 4 and 5 mM, caused small reductions. The five PSP samples chosen had varied effects. One of the two negative samples, S2, had a small cytotoxic effect whilst S4 had no effect at all. Sample S184, just exceeding the AOAC safety limit, also had a cytotoxic effect, but

this was not as great as that previously seen in the N2a cells with S58 of a similar STX value.

7.3.8. Effect of VER on fast inward and tail Na^+ currents

An important step within the cell bioassay is the application of VER to artificially depolarise the membrane, thus modifying the characteristics of normal Na^+ channel behaviour. In this study, extracellularly applied VER (0.05 or 0.1 mM), reduced the transient inward Na^+ peak of all four cell types: N2a, NG108, DRG and SCG. There was also evidence of a non-inactivating current, indicated by a change in the transient current rate of inactivation and incomplete return to the control baseline. After the voltage pulse there was frequent evidence of a standing inward tail current. At a concentration of 0.05 mM the VER-induced modifications developed after 3-4 min, but a faster modification, within 1-2 min, was observed with 0.1 mM VER.

Typical control Na^+ currents elicited by using the single step protocol (**section 7.2.6**) from a holding potential of -80 mV in a N2a and also a NG108 cell are shown in Figure 7.19.a. and b, respectively. After application of VER, a reduction in the transient current was seen, together with a change in the inactivating portion of the current, and the growth of the standing tail current. This is more obvious in Figure 7.19.a. for a N2a cell following application of 0.1 mM VER.

With repeated pulsing at 0.2 Hz, the transient peak was progressively reduced and the non-inactivating part of the current and the standing tail current increased. An example is shown for a SCG cell in Figure 7.20.a. The shift to the left of the inward current may be the result of more adequate or stable voltage clamping of the membrane, as the size of the large Na^+ current is reduced by VER modification in this primary cell type.

VER-modified currents could normally be blocked by STX or TTX, the exception being the presence of STX_R currents in the DRG cells. In such a cell (Figure 7.20.b), the application of STX reduced the VER-modified current, but was

not able to completely inhibit the inward current. Complete inhibition of the VER-modified currents by STX is shown in Figure 7.21 for a typical N2a cell.

The reduction in peak amplitude caused by VER is summarised in Figure 7.22.a. and b. Although the higher concentration of VER (0.1 mM) caused a more rapid modification of the current components than did 0.05 mM VER, there appeared to be little difference in the size of reduction of peak amplitude between the two concentrations in the experiments performed (n=10). Small but progressive reductions occurred over a 1-10 min time period, and the maximum reduction observed was 46.9%. Any further modification of the current was not observed because of the limited number of experiments.

7.4. DISCUSSION

The examination of macroscopic Na^+ currents of five electrically excitable, neuronal cell types, three of neuroblastoma origin and two primary cell types, highlights the importance of using electrophysiological techniques to investigate the suitability of a cell type before it is used routinely use in a cell assay procedure.

Following initial whole-cell patch clamp experiments on N2a cells grown under the normal assay conditions of Gallacher and Birkbeck (1992), and in view of the report of anomalous cell bioassay results from the CSL Ring Trial, 1994 (CSL Report FD 94/161, 1995), the following questions can be posed. Is the N2a cell line an appropriate model for all PSP toxicity studies? Could another cell line be used to improve the robustness and reliability of the PSP bioassay? Do these (neuroblastoma) cell lines behave in a manner similar to primary neuronal cells, or vice versa? Are some of the unknown samples cytotoxic, and could this lead to problems with the reliability of the PSP cell bioassay ?

7.4.1. Appropriateness, robustness and sensitivity of the clonal cell lines

The appropriateness of the N2a cell line was examined after it was observed that there was instability in the cell line under original tissue culture conditions and in

the patch clamp experiments. This was highlighted by the restricted number of times this cell line could be split before there was a deterioration in cell survival rates in culture and when plated, by the general morphological deterioration, and by the poor quality of patch clamp seals together with very small, or occasionally, absent, macroscopic Na^+ currents (**section 7.3.1**). Similar findings of very small or absent Na^+ currents have been reported for other undifferentiated neuroblastoma cells (Nelson *et al.*, 1969; Kuramoto *et al.*, 1977; Ginsborg *et al.*, 1991).

NG108 and SK.N.SH cell lines maintained under similar culture conditions also elicited tiny macroscopic Na^+ currents resembling those from N2a cells, but these cell lines exhibited greater stability with higher passage numbers. This greater robustness was also observed following chemically-induced differentiation of the cells. All three neuroblastoma lines responded to differentiation. Macroscopic Na^+ currents had significantly increased amplitudes, especially with NG108 and SK.N.SH cells, and were more consistently elicited under voltage clamp conditions, implying the maturation of channel proteins and/or an increase in channel density.

Similar results have been obtained in previous studies on neuroblastoma cell lines that possess sufficient Na^+ channels to show some electrical excitability before differentiation (Kiss and Nagy 1988; Docherty *et al.*, 1991; Brown *et al.,* 1994; Quandt 1994). These showed that differentiation reduced variability in the results, and that maximum Na^+ currents were present in a larger proportion of cells without any change in channel functions.

According to Jellet *et al.* (1992), failure to use actively growing cells will result in an increase in the detection threshold and a decrease in the precision of the results obtained. Conversely, N2a cells routinely grown for a cell bioassay in the CSL laboratory, Weymouth, have frequently shown too great a sensitivity in PSP assays and have failed to yield a consistent detection limit value for STX (K. Way, *pers. comm.*). A comparison of sensitivity between undifferentiated (i.e., growing cells) and differentiated N2a cells, performed in this study (**section 7.3.5**) found no significant

difference in their inhibitory response to 100 nM STX, a result also seen with human neuroblastoma LA-N-5 cells (Weiss and Sidell 1991).

The reduced variability in Na^+ currents elicited in differentiated cells indicates that these cells would provide greater reliability with no loss of precision, as a greater number of cells would be at the same stage of development. A further indication of differentiation is the cell RMP (see **section 6.4.1.**). The significant change in the RMP values of the NG108 and SK.N.SH cells suggests these cell lines have undergone differentiation and are at a stable stage. The smaller change in the RMP of the N2a cells may reflect a shift from a logarithmic growth phase to a stationary phase, with some cells differentiated but others incompletely so (Spector 1981). If so, this cell line would be more difficult to assess for reliability and sensitivity.

Previous studies (Nelson *et al.*, 1971; Peacock *et al.*, 1972; Miyake 1978; Spitzer 1979) have shown that the inward current in undifferentiated neuroblastoma cells is carried initially by Ca^{2+}, then by both Ca^{2+} and Na^+, with few cells expressing only fully functional Na^+ channels. Thus, actively growing cells as used in the cell bioassays, grown in 10% FCS (e.g., Kogure *et al.*, 1988; Jellet *et al.*, 1992, 1995; Manger *et al.*, 1993, 1994, 1995a,b) probably represent an undifferentiated cell population, although adherence to the surface of the microwells may have promoted some cell differentiation (Schubert *et al.*, 1971; Kimhi 1981). The N2a cells in this study exhibited great variability in their Na^+ currents when plated under similar conditions, although the change in the percentage of FCS from 10% to 5%, as also introduced by Manger *et al.* (in a communication to J.L. Leftley in 1995), apparently improved cell bioassay reliability.

The greater consistency in current density shown by differentiated N2a and NG108 cells permitted the construction of a concentration-effect relationship for the inhibition of Na^+ currents by STX. The EC_{50} values show that N2a cells were slightly more sensitive than NG108 cells to the same concentration of STX. Taking into account that no correction has been made for differing experimental temperatures and cationic compositions between the physiological and binding experiments, the EC_{50}

of 7.8 nM for N2a cells is of the same order of magnitude as the EC_{50} of 4 nM for SH-SY5Y cells and the dissociation constant (K_d) of 4.6 and 7.8 nM reported for respectively, undifferentiated and differentiated LA-N-5 cells (Weiss and Siddel 1991; Brown *et al.*, 1994), 50% inhibition at 3.5 nM STX for N18 cells (Catterall and Morrow 1978) and a $K_{0.5}$ of 5-6 nM STX for rat brain synaptosomes (Tamkun and Catterall, 1981; Krueger *et al.*, 1983).

Similarly, the EC_{50} for NG108 cells of 15.5 nM compares well with other binding, ion flux and purified sodium channel preparation studies, which reported half maximal inhibitions of 8.5 nM or 11 nM TTX for N18 cells (Catterall and Nirenburg 1973; Catterall 1975), 14 nM TTX for purified rat brain Na^+ channels (Catterall *et al.*, 1986) and 30 nM TTX for NIE115 cells incubated in ATX II and VER (Jacques *et al.*, 1981). Thus across the wide variation of Na^+ channel sources and experimental situations, the 50% inhibitory values all fall within a narrow nanomolar range.

EC_{50} values obtained from the PSP neuroblastoma cell bioassays are slightly higher than those obtained in this study, which may relate to the culture methods of using actively growing, logarithmic phase cells. The water-soluble tetrazolium (WST-1) assay (Hamasaki *et al.*, 1996) yielded the closest EC_{50} values to those obtained in this study, with a half-maximal inhibition by TTX at 12.9 nM. An equivalent EC_{50}, described as 50% protection (Kogure *et al.*, 1988; Gallacher and Birkbeck 1992) required a TTX concentration of 50-55 nM, although the detection ranges were 10 nM-1.56 µM (Kogure *et al.*, 1988) and 10 nM-500 nM (Gallacher and Birkbeck 1992).

The rationale behind using actively growing cells (Jellet *et al.*, 1992, 1995) is to yield a steeper regression slope, thus increasing the consistency of the assay while accepting a decrease in the detection sensitivity. No EC_{50} values are given, but the routinely-derived lower detection value of 0.1 ng/10 µl, corresponds to 33.4 nM STX, or, 2.0 µg/100g shellfish tissue, well within the AOAC safety toxicity margin. The use of non-actively growing cells, for which no culture conditions (e.g., % serum, age, differentiating agents) are described, gave a lower detection limit of 0.02 ng/10 µl,

which converts to 6.7 nM. This is very close to the EC_{50} value obtained for differentiated N2a cells in this study.

Truman and Lake (1996) and Manger *et al.* (1993, 1994, 1995a,b) report very similar detection values to Jellet *et al.* (1992) for N2a cells used in MTT bioassays. However, the enhancement of sensitivity (0.1 to 0.02 ng/10 µl) reported by Manger *et al.* (1995a,b) was obtained by extending the MTT incubation period from 15 to 45 min. This appears to be a very short extension of time for these cells to attain such a degree of electrical excitability, and such results warrant further investigation by electrophysiological methods. Whilst Jellet and Manger appear to have optimized a suitable growth regime to provide reliable quantification of PSP detection, both Gallacher (1992, Ph.D. thesis) and K. Way (*pers. comm.*) expressed the need for greater robustness in their assays. Trials involving several differentiation methods, or the use of alternative cell lines, such as the NG108 or SK.N.SH cells studied here, may provide the basis for improving assay robustness.

7.4.2. Characteristics and response of Na^+ currents in primary cells

An important finding was that under the experimental conditions of this study, DRG cells did not respond to the Na^+ channel blocking toxins STX or TTX in a similar, consistent, dose-dependent manner, to that of the clonal cell lines (N2a and NG108, Figure 7.13) with which they were compared. The DRG cells exhibited some degree of suppression of the inward current after exposure to either STX or TTX, but the range of inhibition recorded was very wide (between 5.26% and 98.6%).

A number of studies involving intracellular and patch clamp techniques (Ogata and Tatebatashi 1992; Elliot and Elliot 1993; Caffrey *et al.*, 1992; Roy and Narahashi 1994) have indicated the existence of at least two, and possibly three, kinetically and pharmacologically distinct Na^+ channels in dissociated DRG cells. Similar findings were obtained in this study. The largest proportion of DRG cells tested (82%) exhibited currents that were only partially suppressed by either STX or TTX (0.1-0.3 µM). Although the experimental criteria of this study were primarily to test for

specificity and consistency in the inhibitory responses to either STX or TTX (and thus PSP samples), three current types were identified : (i) a fast activating and inactivating STX_S current (Figures 7.7.c. and 7.15.a); (ii) a slow activating and inactivating STX_R current (Figure 7.3.d); and, (iii) an intermediate type (Figure 7.15.a) with activation and inactivation kinetics between types (i) and (iii), which was also STX_R. The voltage-dependence, kinetics, and relative distribution of these current types as functions of cell size and age have already been well characterized (e.g., Matsuda *et al.*, 1978; Omri and Meiri 1990; Schwartz *et al.*, 1990; Caffrey *et al.*, 1992; Elliot and Elliot 1993). Further analysis of current kinetics was not attempted, as the objective of the study was to establish the consistent response to STX.

Electrophysiological and ion flux methods (Lawrence and Catterall 1981; Rogart 1986; Guo *et al.*, 1987) comparing TTX_S and TTX_R channel current kinetics and the binding properties of different nerve cell types conclude that the structure, functional homology and mechanism of action of these Na^+ channel subtypes is unchanged. They differ only in their binding affinity properties, having a slower association rate which affects the rate at which the toxin-receptor complex becomes stable, combined with a faster dissociation rate.

7.4.3. Cytotoxic effects observed with PSP samples

The few previous electrophysiological studies of the effects of PSP toxins on Na^+ channel mechanisms using cultured cells have used purified compounds of either STX, or its numerous analogues applied separately (Kao and Hu 1989; Narahashi 1988; Hall *et al.*, 1990; Strichartz *et al.*, 1995). Hall *et al.* (1990) have tested prepared mackerel liver extracts to provide qualitative data on the toxins present, but despite their routine use in tissue culture assays, raw shellfish extracts have never previously been tested electrophysiologically, as this study has done. The PSP sample S70 at the two dilutions chosen showed a blocking action similar to, but less inhibitory than, 100 nM STX, with no change in channel kinetics. A comparison of the blocking effects of

the two main neuroblastoma lines showed that a 1:1000 sample dilution on NG108 cells resulted in a reduction in amplitude that almost matched that of a 1:10,000 dilution on N2a cells. This may indicate that the NG108 cell line maintained under the same conditions as the N2a cells could provide a better system for toxin testing with a greater stability but a lower absolute sensitivity.

Evidence of potentially cytotoxic components in shellfish samples extracted under AOAC guideline procedures was observed with PSP sample S1 (Figure 7.17), and similar effects have been reported elsewhere (Jellet *et al.*, 1992; Manger *et al.*, 1993), although at much lower dilutions (1:5 or 1:4 respectively). Sample S1 was found to contain no detectable STX by HPLC and the mouse bioassay, yet caused a considerable reduction in peak Na^+ current amplitude of around 40%. This is similar to non-toxic PSP samples spiked with a known amount of STX, which were up to 30% less toxic than STX alone (Sato *et al.*, 1988; Jellet *et al.*, 1992; Truman and Lake 1996). This suggests that some co-extracted components in the sample matrix can suppress (or possibly absorb) STX toxicity, especially as the lytic and the toxicity-depressive effects remain present after a 10 kd filtration. However both can be resolved using a SPE-C18 clean up procedure (Jellet *et al.*, 1992).

Truman and Lake (1996) found no evidence for cytotoxicity in any of the shellfish samples they tested. Furthermore they suggest that apparent "false-positive" results, i.e., low levels of STX detected by the neuroblastoma assay, are in fact real, but that these levels are below the detection limit of the mouse bioassay. This may account for the anomalous CSL Ring Trial results and those found here with sample S1, although HPLC should have been sensitive enough to detect low STX levels. The shellfish samples previously tested on the CBC (**section 4**, n=12) and frog sciatic nerve (**section 5**, n=13) preparations had no apparent lytic or cytotoxic effect on these tissue types at a dilution of 1:100. However in these preparations intact nerves composed of many axons and associated support tissues were involved, which perhaps made them more robust than the cell assay.

The preliminary study of the potential cytotoxicity of samples conducted through a small number of MTT assays showed that at the level of dilution chosen (1:60), there was some evidence for cytotoxic effects. The cytotoxic effect was not immediately apparent (when tested on a large number of cells), as very little change in cell survival rate was seen after 2 h compared to values between 25% and 75% after 24 h in the N2a cell assays. All the samples had some cytotoxic effect on these cells, with the greatest lytic effect occurring with S32 (a high PSP value) and two samples just over the AOAC safety level (S58 and S184), whilst a sample with no toxicity was only slightly cytotoxic. Lower cytotoxic effects were observed with the NG108 cells. The most apparent effects were seen with a negative sample (S2) and AOAC level (S184) sample.

These initial results with both the STX and PSP samples suggest that the N2a cells are very sensitive to exposure to neurotoxins, whilst NG108 cells show less sensitivity but greater robustness under the same conditions. Thus, even before cell differentiation had been induced, NG108 cells showed greater stability and resilience than N2a cells, which should yield quantitatively repeatable results. A detailed comparative study to assess the overall robustness and reliability of the three clonal cell lines examined in this study, using a combination of electrophysiological and cytotoxicity methods, would be very instructive.

7.4.4. Na^+ channel modification by VER

The principle underlying the cell bioassay is a modification of the Na^+ channel after exposure to VER. This study has found that in all cell types tested, repetitive depolarisation reduces the size of the early transient conductance whilst increasing a persistent component of the Na^+ current. This is consistent with previous findings on frog nerve and muscle, lobster nerve and neuroblastoma cells and rat brain neurons (Ulbricht 1972; Yoshii and Narahashi 1984; Sutro 1986; Barnes and Hille 1988; Zong *et al.*, 1992; Dargent *et al.*, 1994).

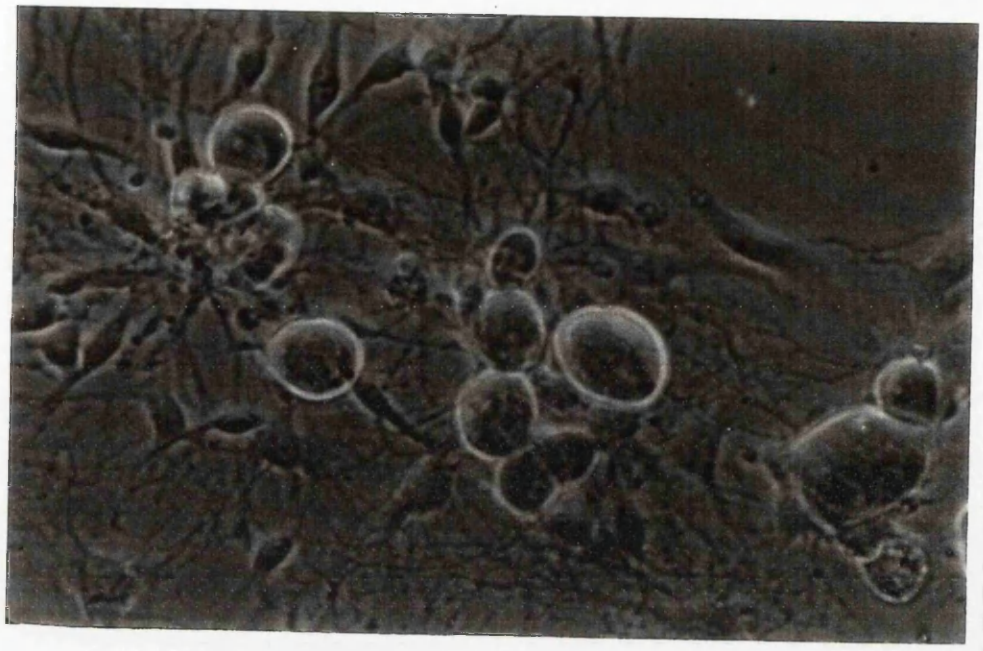
Using the single step protocol of repeated depolarisation, the modified peak transient current reduced incrementally. This suggests that there is a progressive conversion of unmodified Na^+ channels which are responsible for the transient peak current at -5 mV into modified ones that remain open at -5 mV and -80 mV The reduction in the peak current seen here provides qualitative evidence that VER binds to open Na^+ channels (Barnes and Hille 1988; Zong *et al.*, 1992) producing a sub-state that has a conductance only two thirds that of the normal value (Corbett and Krueger 1989).

Conversely, the persistent tail current was seen to grow in size with each repeated depolarisation, suggesting that it arises from the same population of VER-modified Na^+ channels that fail to close at -80 mV (Barnes and Hille 1988). This suggests that the Na^+ channel does not enter a closed (inactivated) state and thus could be immediately available for another VER modification, the occurrence of which depends on both VER concentration and diffusion time to the VER binding site (Ulbricht 1972; Honerjager *et al.*, 1992). However, at any given time, only a fraction of Na^+ channels will be activated by VER (Catterall 1977).

The repetitive depolarisation induced by the experimental protocol appeared to accelerate the pharmacological modification by VER, with the channels being activated for a few milliseconds during each stimulation, increasing the probability of VER binding. This accelerated modification provided by the protocol made it possible to observe the underlying events under voltage-controlled conditions, rather than passively over a much longer time period, as in normal cell bioassay conditions. In this situation, a steady long-term depolarisation would be expected to have little effect (because the Na^+ channels are activated only once) and the rate of depolarisation is now determined by the rate of Na^+ influx induced by VER Na^+ channel modification (which is in turn strongly dependent on external Na^+ concentration) (Catterall 1977, 1986; Jacques *et al.*, 1978). Thus the external Na^+ concentration must be kept constant once a suitable assay dose-response curve for VER+OUB has been established under a given cell culture regime.

Plate 7.1.

Micrographs of enzymically dissociated dorsal root ganglia (DRG) cells (round cells) together with spindle shaped Glial/Shwann cells. The DRG cells have not yet begun to extend any neurites and are therefore suitable for patch clamp experiments.



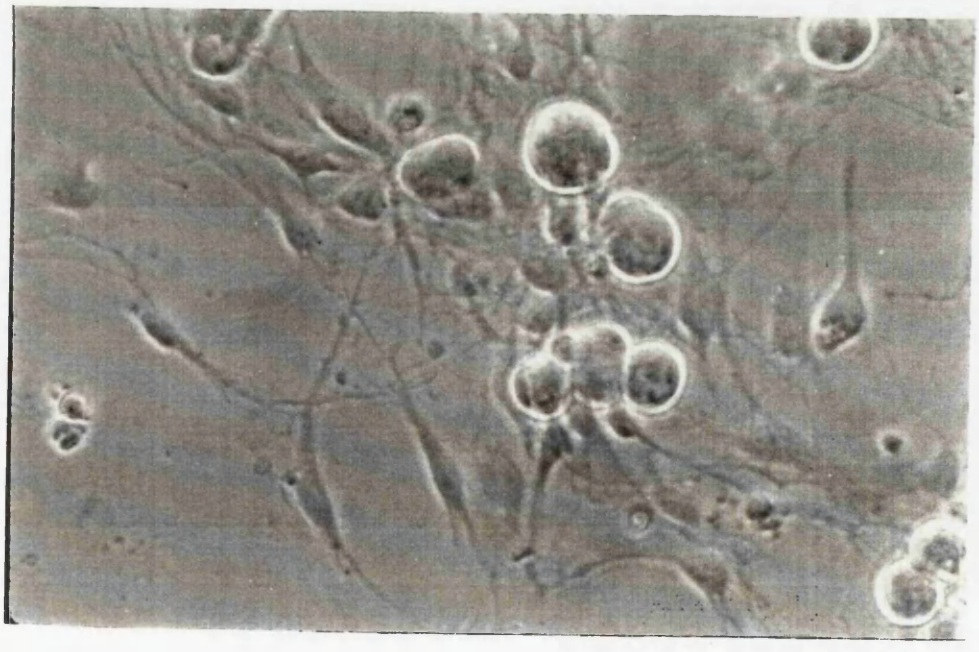
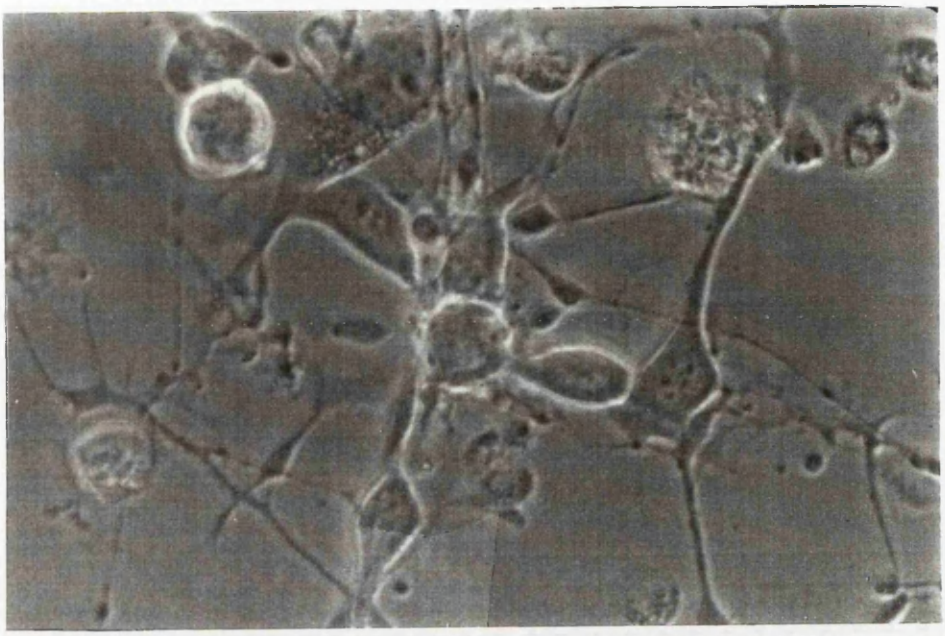
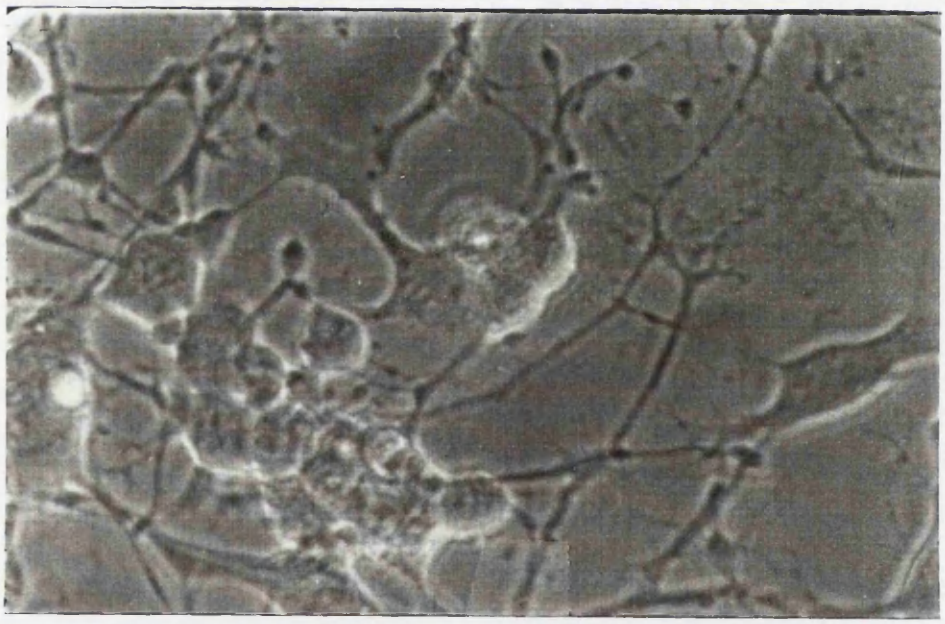


Plate 7.2.

Micrographs of neuroblastoma NG108-15 cells showing morphological changes during exposure to cAMP. Control cells (top plate), after 1.5 h incubation in cAMP (middle plate) and 48 h after exposure to cAMP (bottom plate).





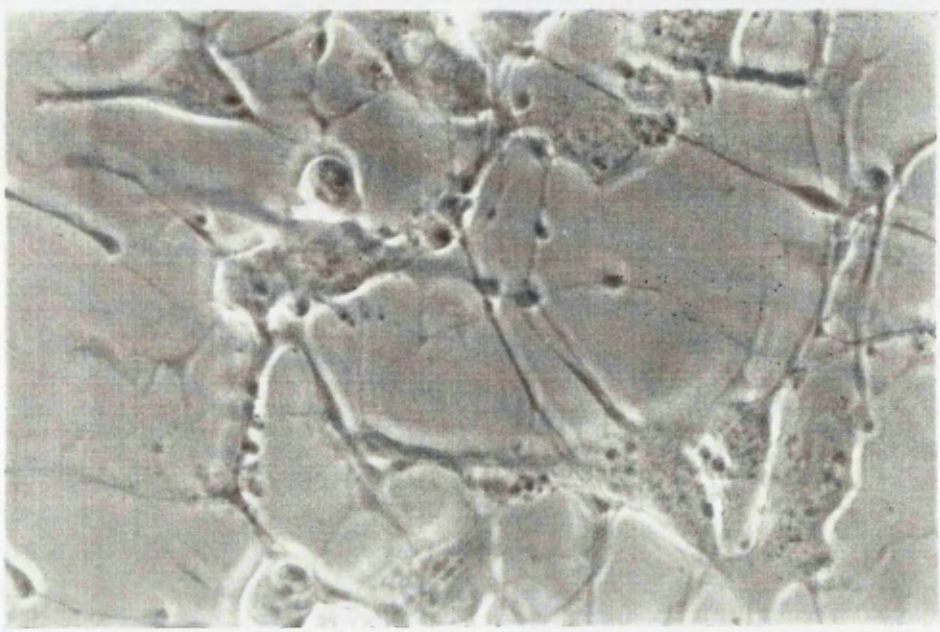


Figure 7.1.

Schematic diagram of the whole cell patch clamp circuit. **A**....preamplifier (List Medical), **A/D**....analogue/digital interface (in-house design), **E**....Electrode, **MA**....main amplifier (EPC7 List Medical), **Osc**....oscilloscope, **ST**....Stimulator (pulse generator).

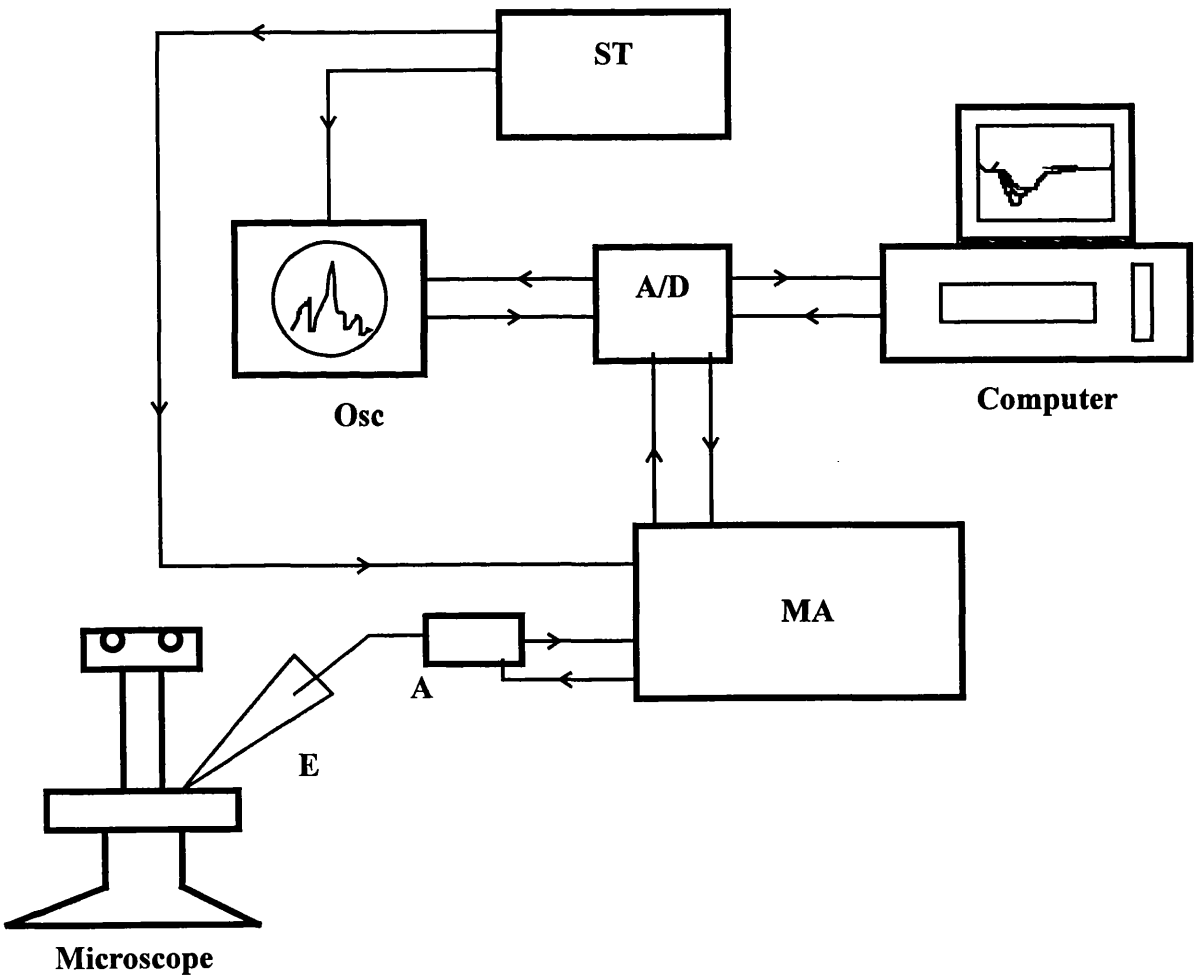
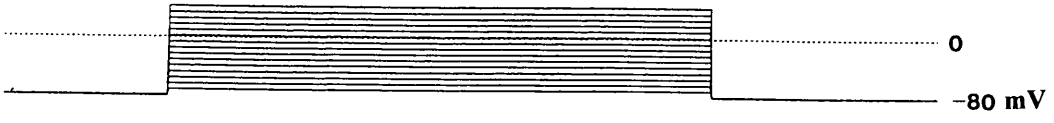
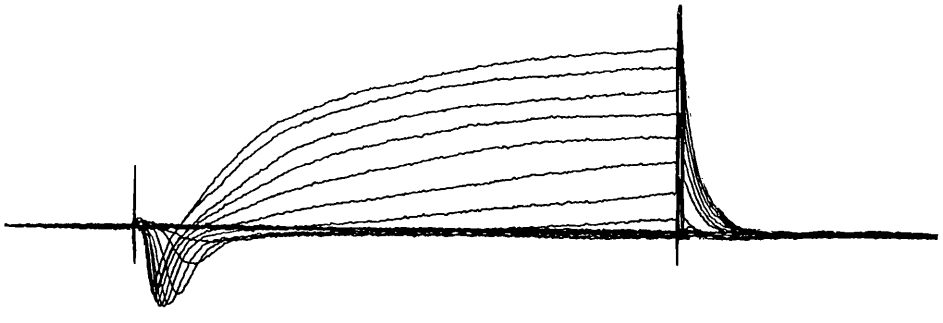

ST
A/D
Osc
Computer
MA
A
E
Microscope

Figure 7.2.

Families of ionic currents from N2a cells. The currents were elicited using a 15 step depolarising protocol (**a**), with test pulses of 30 ms, applied at 1 Hz, incrementing by 10 mV from a holding potential of -80 mV. (**b**) Currents elicited from an undifferentiated N2a cell. By convention, the upward traces represent K^+ currents, whilst the downward traces represent Na^+ currents. (**c**) A family of currents typical of those recorded from a chemically induced, differentiated N2a cell. Linear leakage currents have been subtracted.



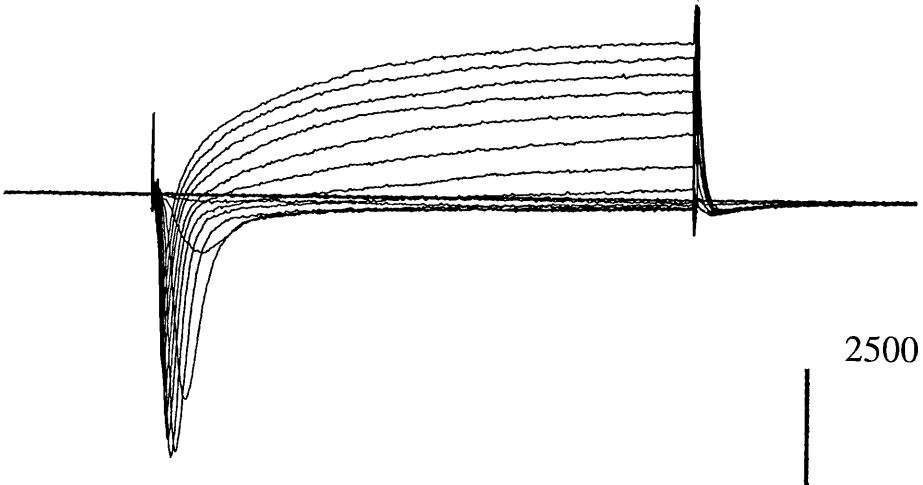
a



b

500 pA

5 ms



2500 pA

c

5 ms

Figure 7.3.

Families of currents recorded from four excitable cell types using the same voltage protocol as shown in Figure 7.2.a.

a) an undifferentiated NG108-15 cell.

b) a chemically induced differentiated NG108-15 cell.

c) a primary DRG cell which exhibited STX_S Na^+ currents.

d) a primary DRG cell which exhibited STX_R Na^+ currents.

e) a primary SCG cell.

f) a SK.N.SH clonal cell.

K^+ currents were blocked using Cs and TEA in records (**c**) (**d**) and (**f**). Leakage currents have been subtracted.

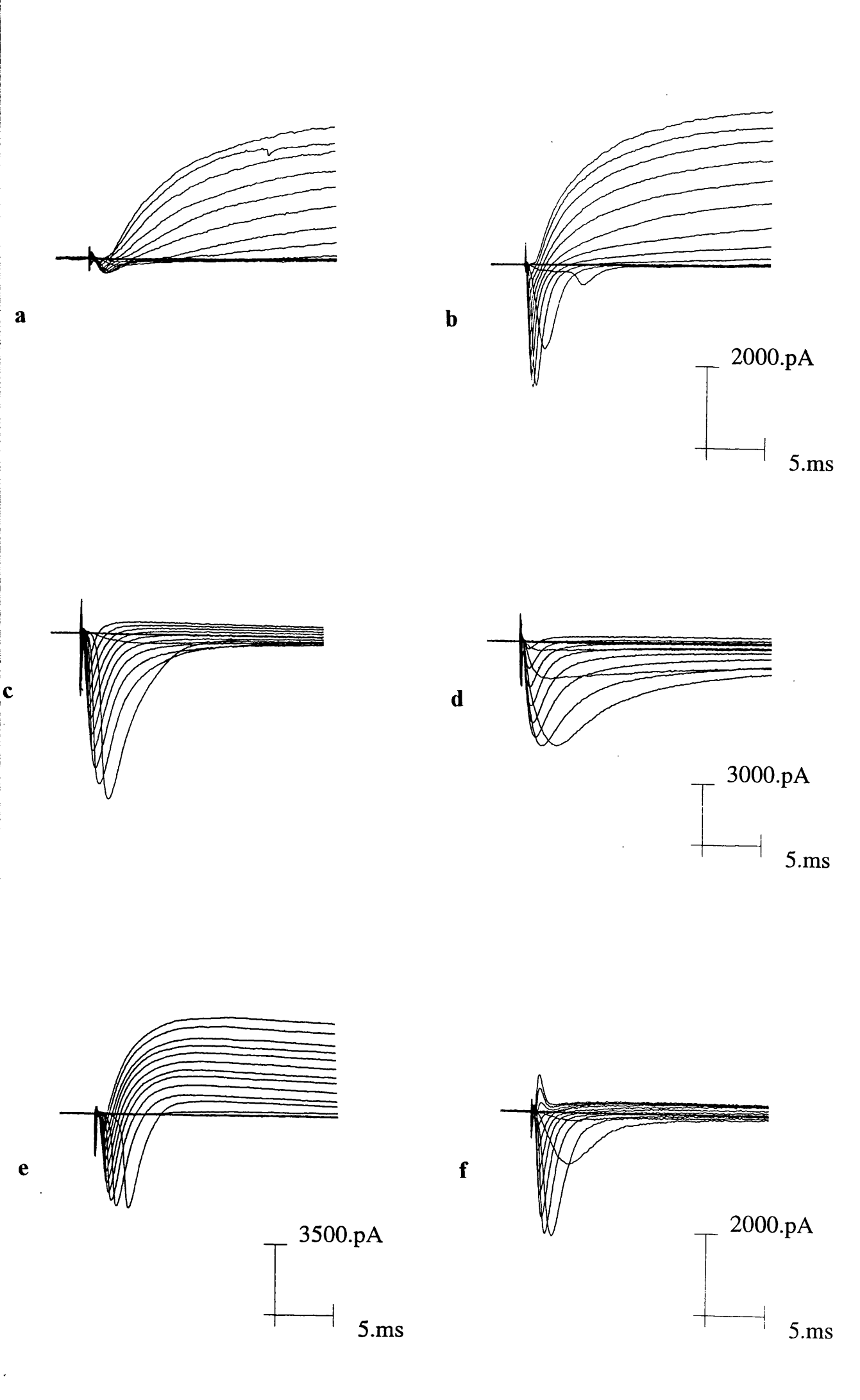
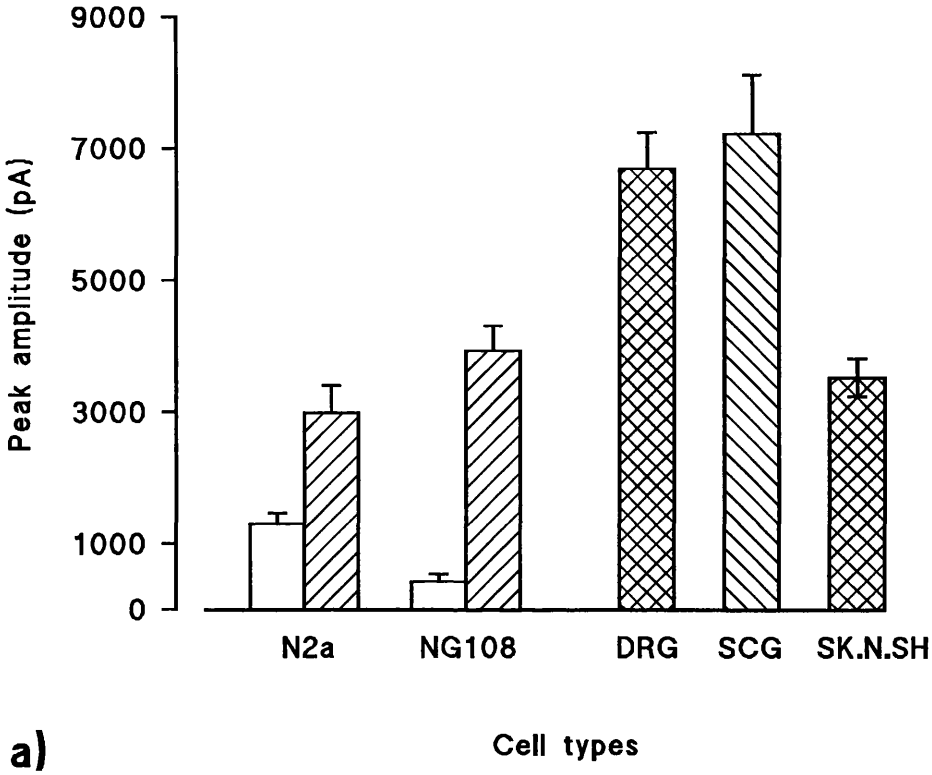

a
b
2000.pA
5.ms
c
d
3000.pA
5.ms
e
3500.pA
5.ms
f
2000.pA
5.ms

Figure 7.4.

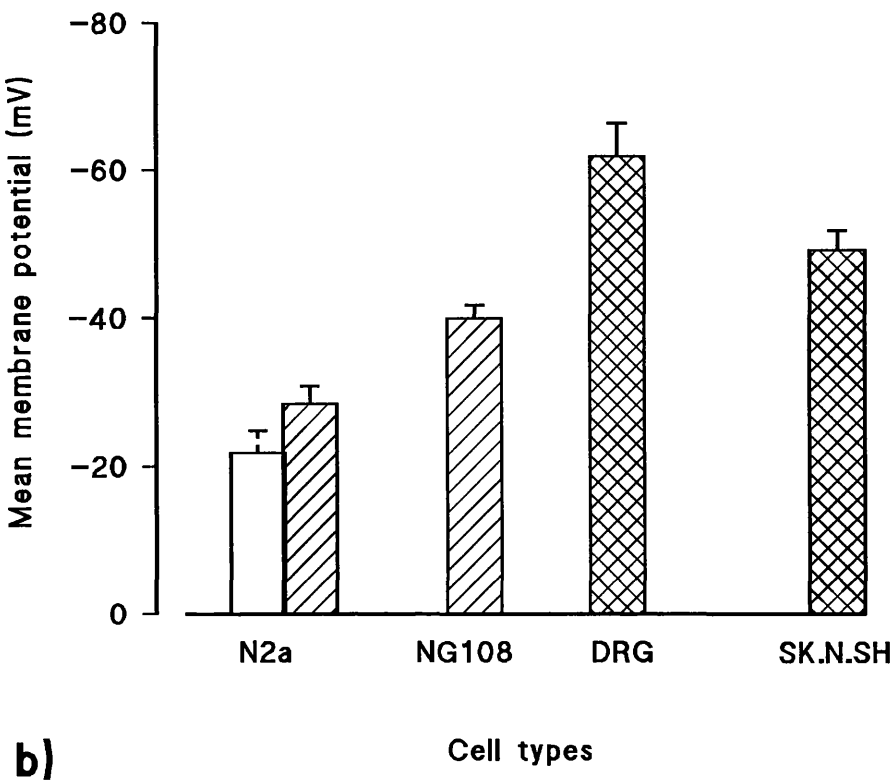
a) Peak Na^+ current amplitudes recorded from five cell types.

b) Cell resting membrane potentials from current clamp recordings.

Open bars represent undifferentiated cells. Each bar is the mean ± s.e.m. (represented by the vertical lines) for each cell data set. The *n* values for each cell type are given in the respective results sections (**sections 7.3.1 to 7.3.4**).


9000
7000
5000
3000
1000
0
Peak amplitude (pA)
N2a
NG108
DRG
SCG
SK.N.SH
Cell types

a)

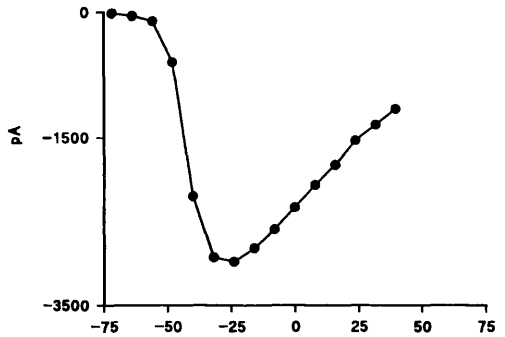

-80
-60
-40
-20
0
Mean membrane potential (mV)
N2a
NG108
DRG
SK.N.SH
Cell types

b)

Figure 7.5.

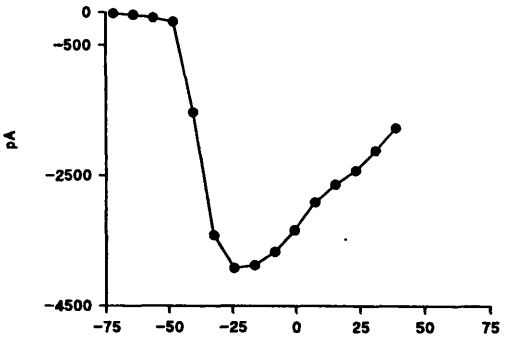
Representative current-voltage (I-V) relationships for the Na^+ current, for the five cell types used in this study: (**a**) N2a cell; (**b**) NG108-15 cell; (**c**) DRG cell; (**e**) SCG cell; (**e**) SK.N.SH cell.

N2a



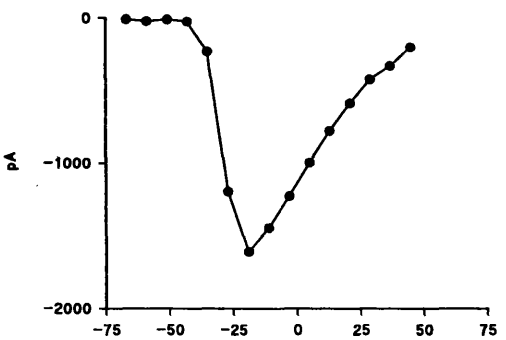
a)

NG108-15



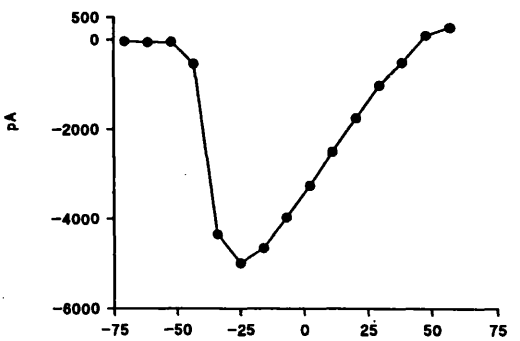
b)

DRG



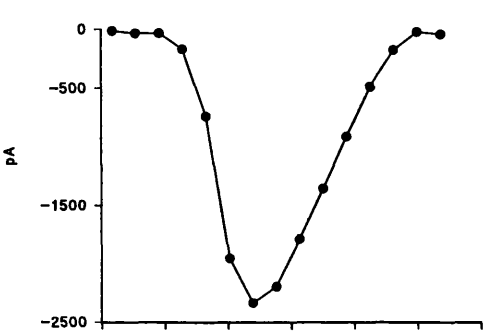
c)

SCG



d)

SK.N.SH

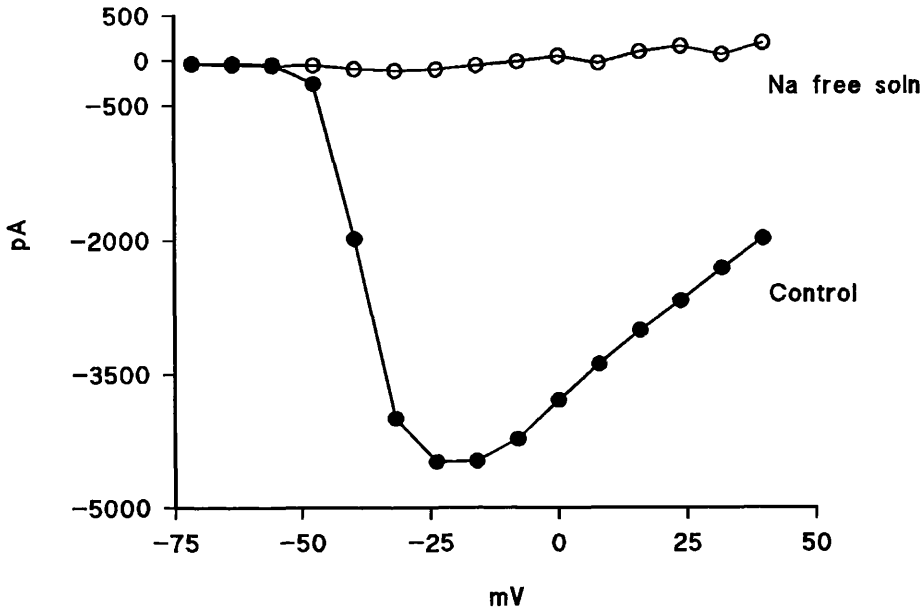


e)

Figure 7.6.

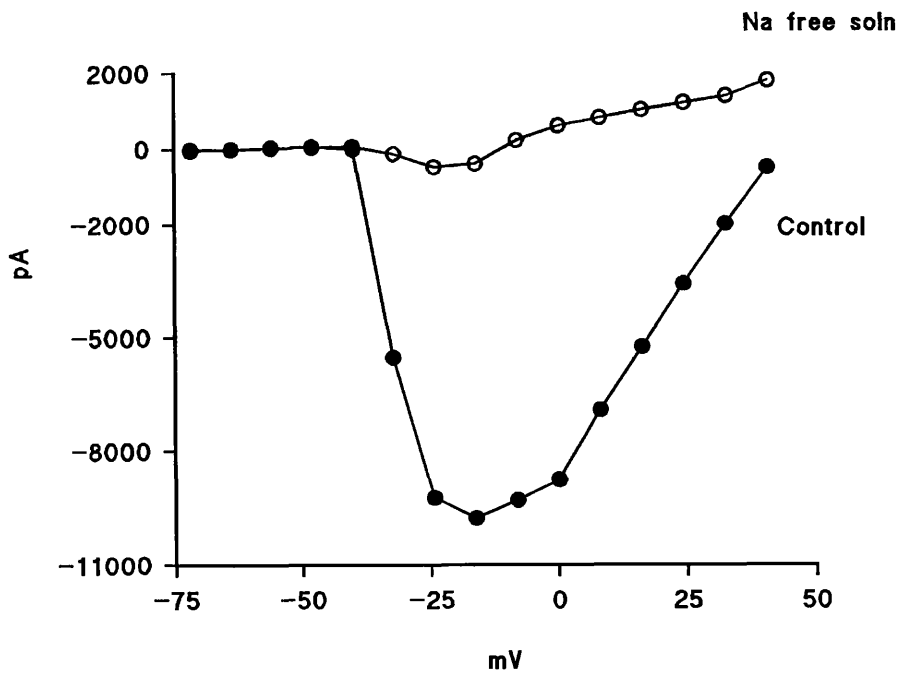
Typical Na^+ current I-V plots recorded from (**a**) an NG108-15 cell and (**b**) a DRG cell showing the normal Na^+ current curve (• — •) and the absence of the Na^+ current following removal of Na^+ ions from the external bathing solution (o — o).

NG108–15



a)

DRG

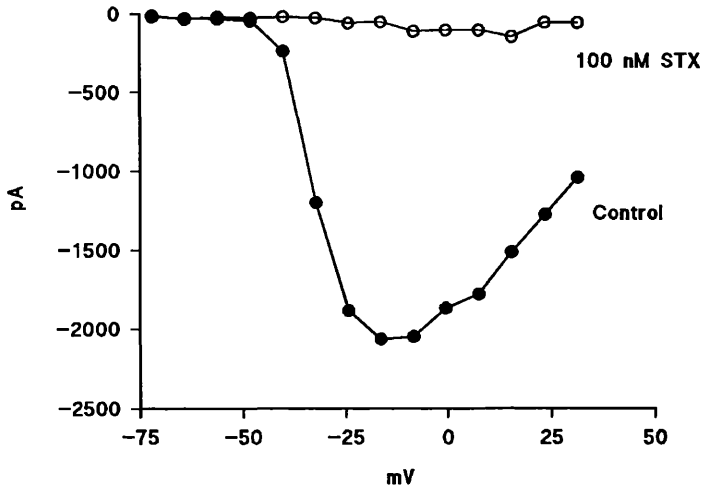


b)

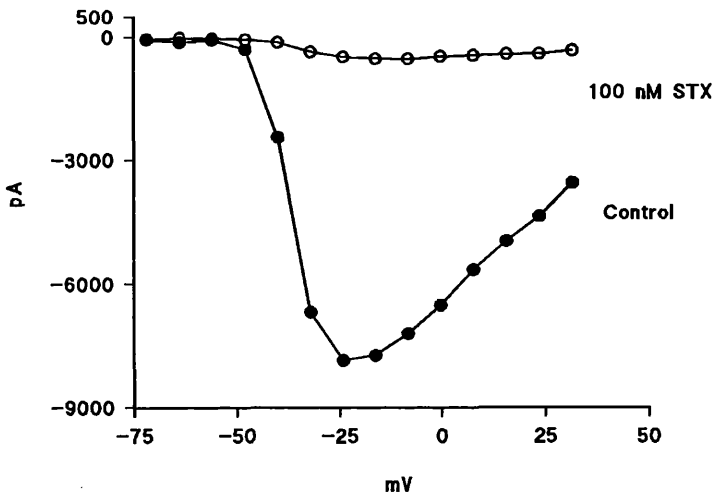
Figure 7.7.

Representative Na^+ current I-V plots for the three neuroblastoma cell types: N2a, NG108 and SK.N.SH lines, before (• — •) and after (o — o) application of either 50 nM or 100 nM STX to the external bath solution.

N2a



NG108



SK.N.SH

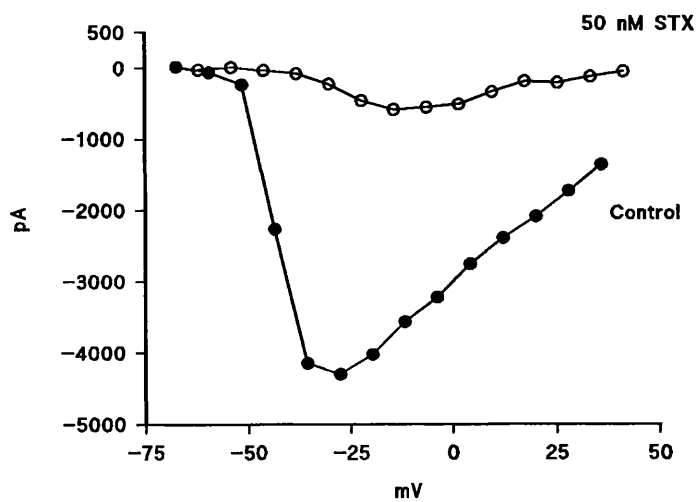
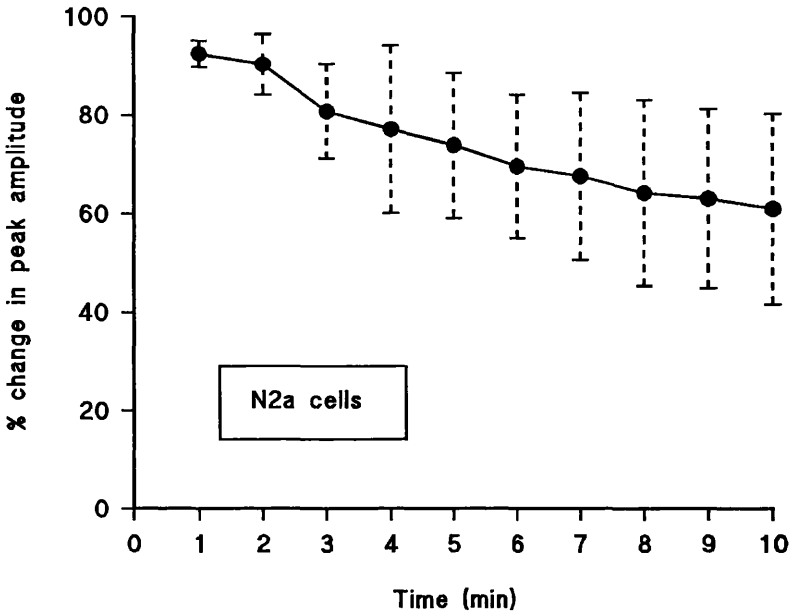
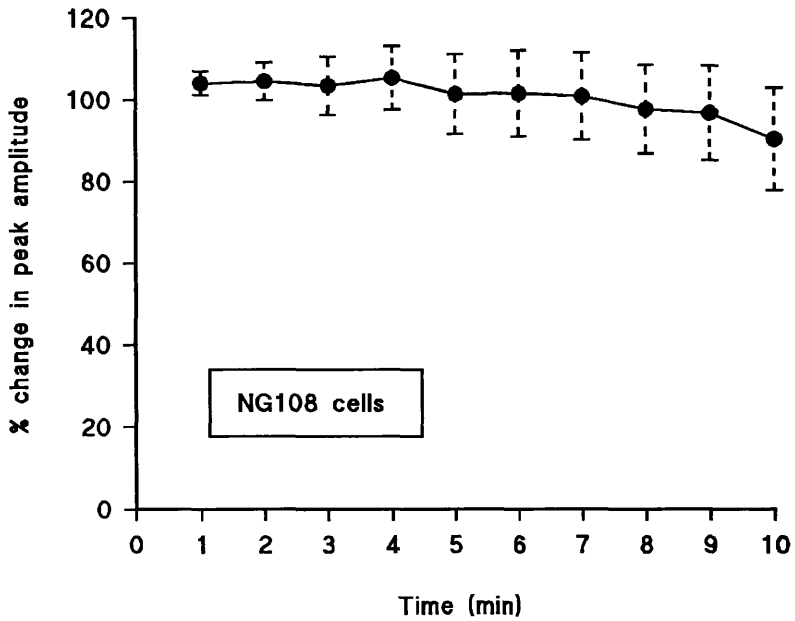


Figure 7.8.

Peak Na^+ current amplitude, time-matched control experiments, for the two neuroblastoma cell lines, N2a (**a**) and NG108 (**b**) plotted as the mean ± s.e.m. (n = 5 and 8 respectively). The peak amplitude was recorded using a single step protocol (**section 7.2.6. viii**) over a 10 min period. Change in amplitude was calculated as the percentage change from the initial control amplitude value.


100
80
60
40
20
0
% change in peak amplitude
N2a cells
0 1 2 3 4 5 6 7 8 9 10
Time (min)

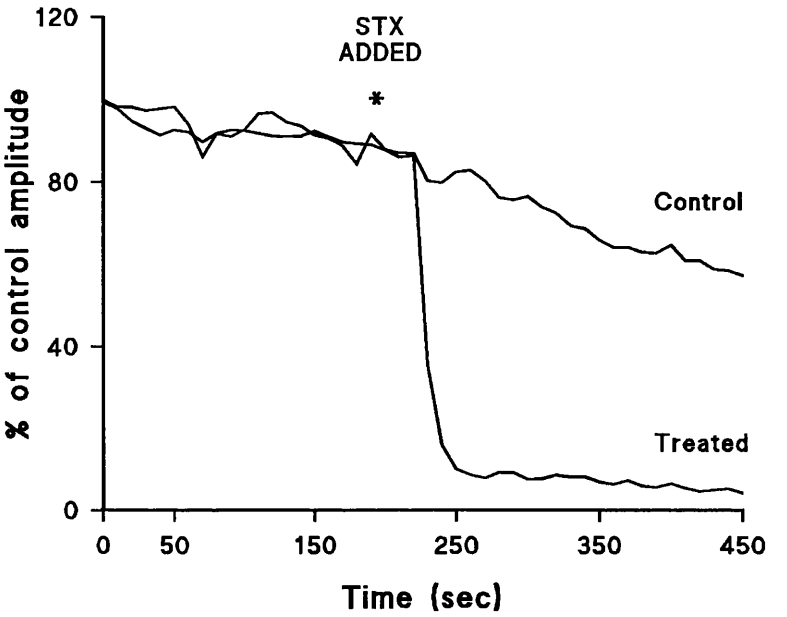
a)


120
100
80
60
40
20
0
% change in peak amplitude
NG108 cells
0 1 2 3 4 5 6 7 8 9 10
Time (min)

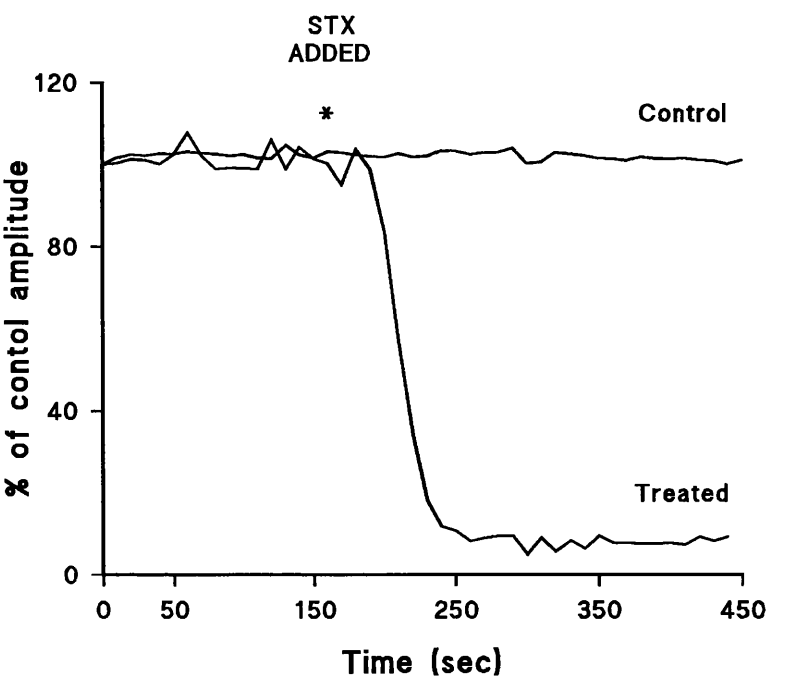
b)

Figure 7.9.

Data from a control time-matched experiment plotted against a time-matched peak Na^+ current amplitude before and following exposure to exogenously applied STX recorded from a typical N2a cell (**a**) and a NG108 cell (**b**). A sudden reduction in peak current amplitude is recorded within 30 sec of 100 nM STX being added (*) in both cases.


STX
ADDED
*
120
80
40
0
% of control amplitude
Control
Treated
0
50
150
250
350
450
Time (sec)

a)


STX
ADDED
*
120
80
40
0
% of contol amplitude
Control
Treated
0
50
150
250
350
450
Time (sec)

b)

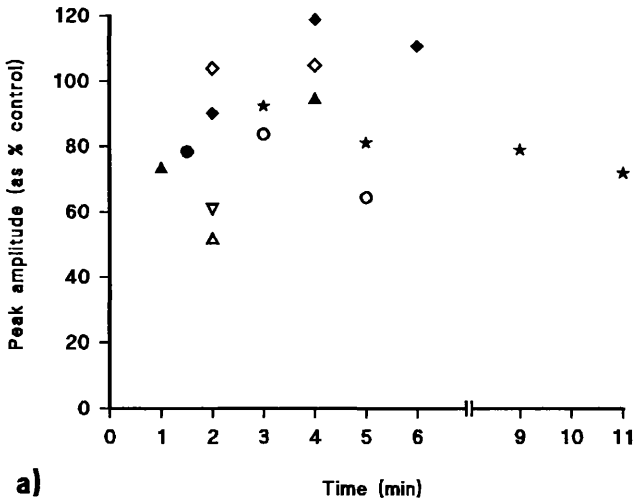
Figure 7.10.

Peak Na^+ current amplitude of N2a cells at different exposure times to a given concentration of STX. Each symbol-type on the scatter graph represents a separate data set for individual cells.

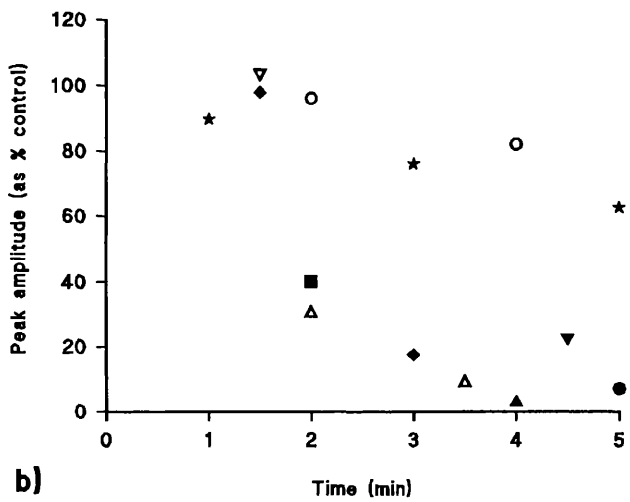
a) 1 nM STX (n = 8).

b) 10 nM STX (n = 9).

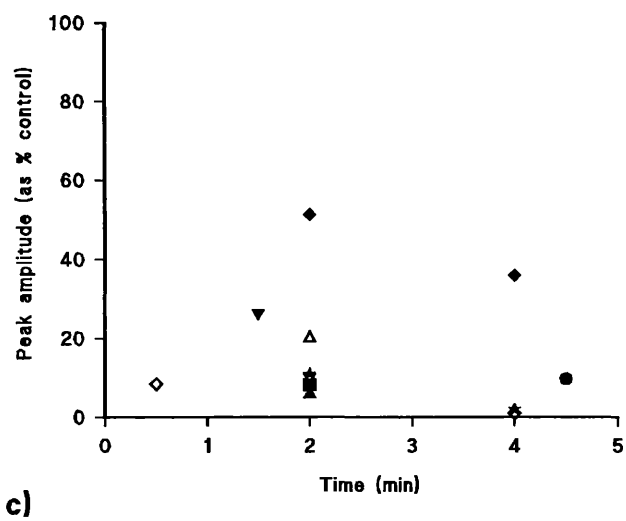
c) 100 nM STX (n = 8).


Peak amplitude (as % control)
120
100
80
60
40
20
0
0 1 2 3 4 5 6 9 10 11
Time (min)

a)


Peak amplitude (as % control)
120
100
80
60
40
20
0
0 1 2 3 4 5
Time (min)

b)


Peak amplitude (as % control)
100
80
60
40
20
0
0 1 2 3 4 5
Time (min)

c)

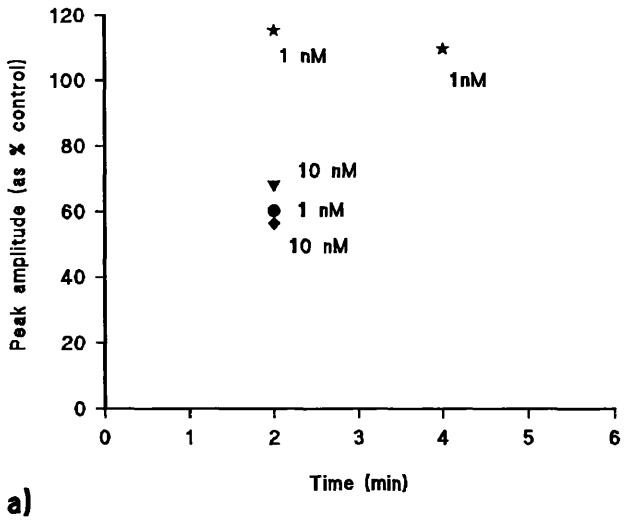
Figure 7.11.

Peak Na^+ current amplitude of NG108-15 cells at different exposure times to a given concentration of STX. Each symbol-type on the scatter graphs represents a separate data set for individual cells.

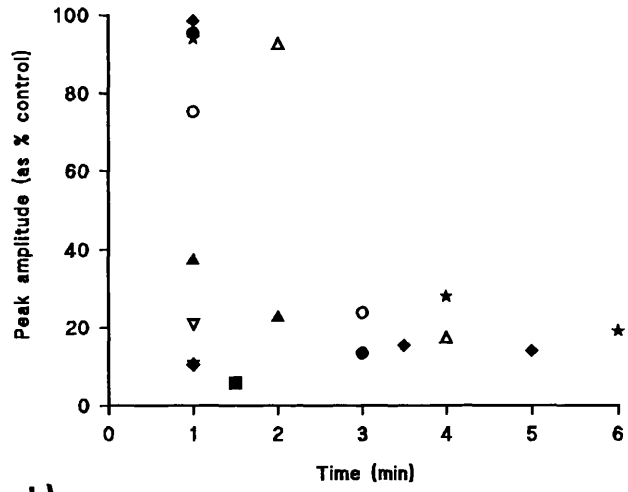
a) 1 nM and 10 nM STX (n = 4).

b) 100 nM STX (n = 8).

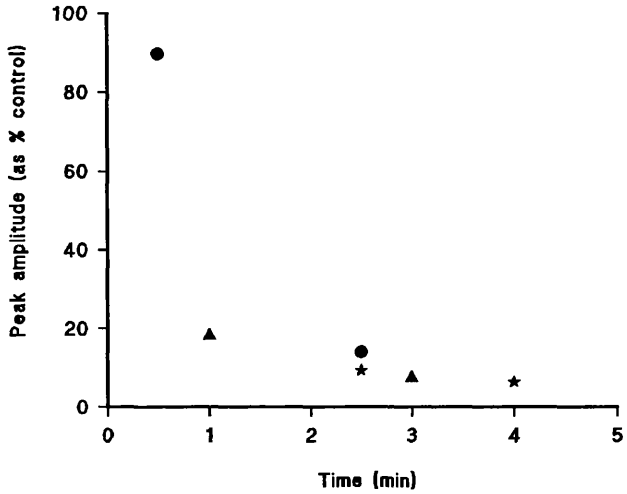
c) 300 nM STX (n = 3).


Peak amplitude (as % control)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (min)
1 nM
1nM
10 nM
1 nM
10 nM

a)


Peak amplitude (as % control)
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (min)

b)

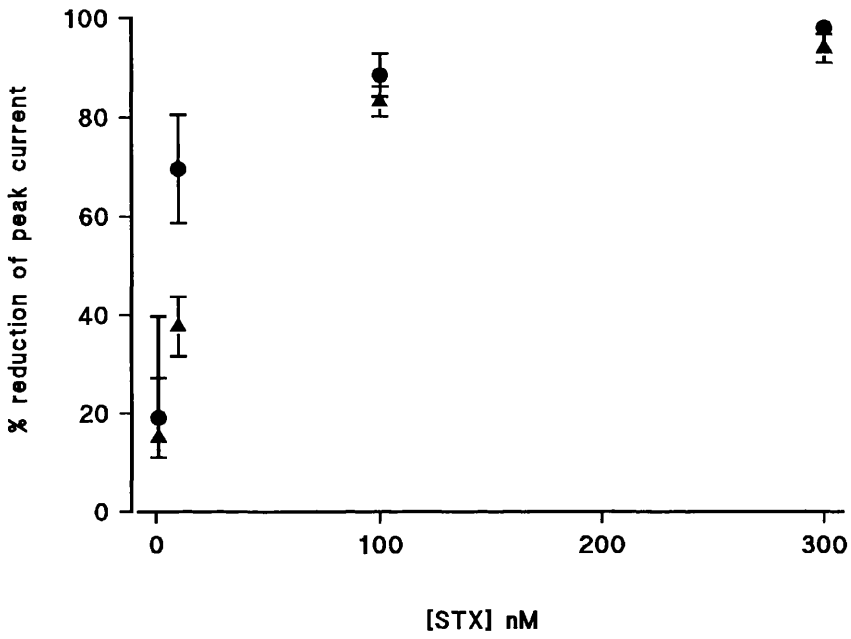

Peak amplitude (as % control)
100
80
60
40
20
0
0
1
2
3
4
5
Time (min)

c)

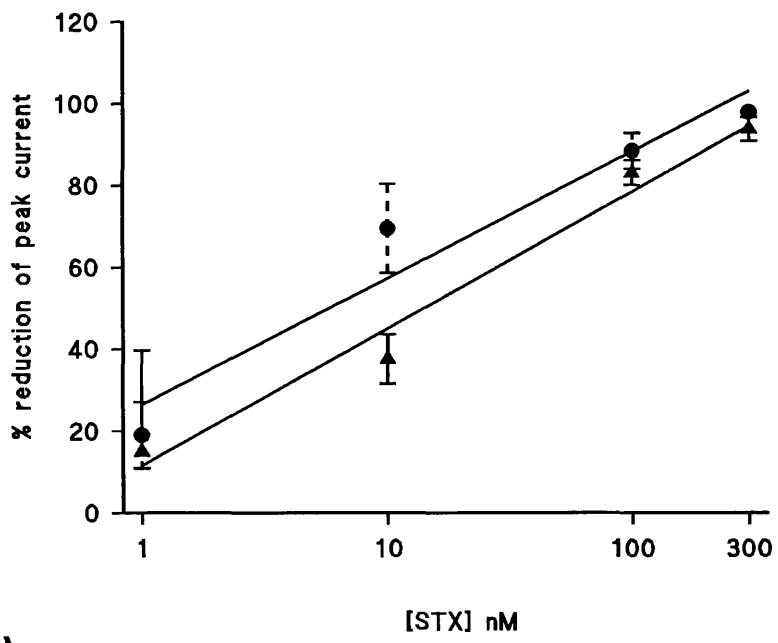
Figure 7.12.

a) Standard curve for reduction in mean (± s.e.m.) peak Na^+ current amplitude recorded from N2A (•) and NG108 (⋄) cells with increasing concentrations of STX. A plateau effect was observed after 100 nM STX.

b). Dose-response curve for N2a and NG108 cells derived from the data in (a). Data have been transformed to a log scale and a linear regression fitted to the mean data ($r^2 = 0.938$, N2a cells; $r^2 = 0.979$, NG108 cells).



a)



b)

KEY:

● N2a cells

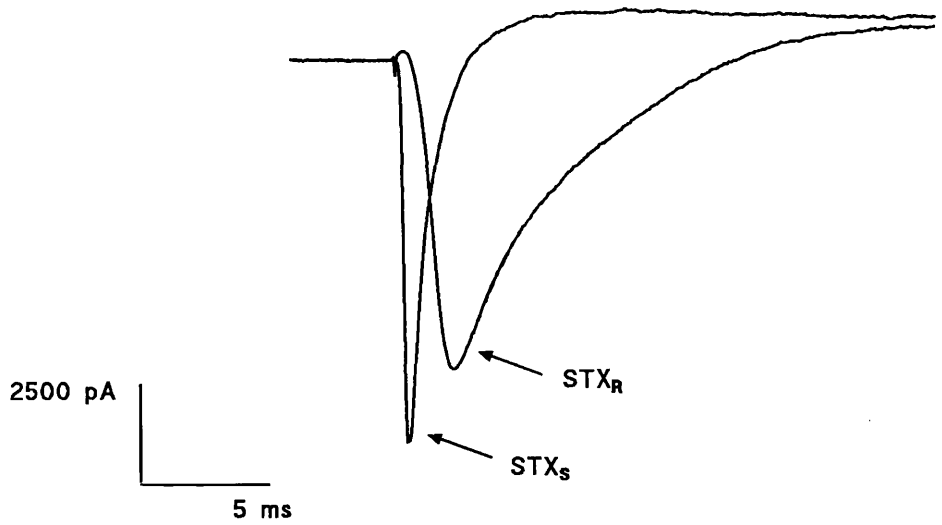
▲ NG108 cells

Figure 7.13.

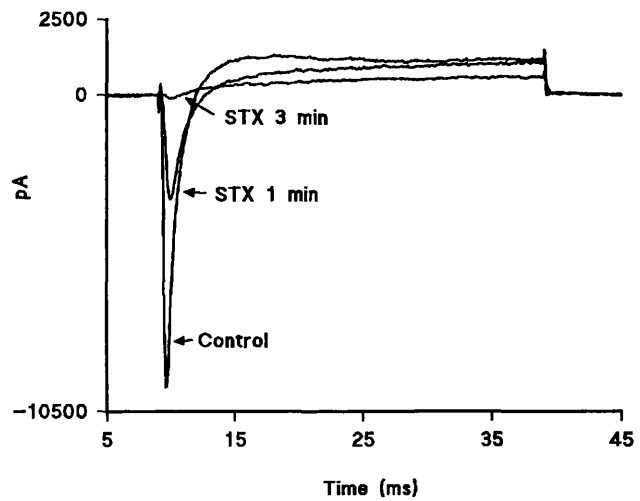
a) The peak Na^+ current from a STX_S DRG cell superimposed on the peak Na^+ current from a STX_R DRG cell.

b) The effect of 100 nM STX on the peak STX_S Na^+ current. The current is reduced by 63% by 1 min and is completely inhibited by 3 min.

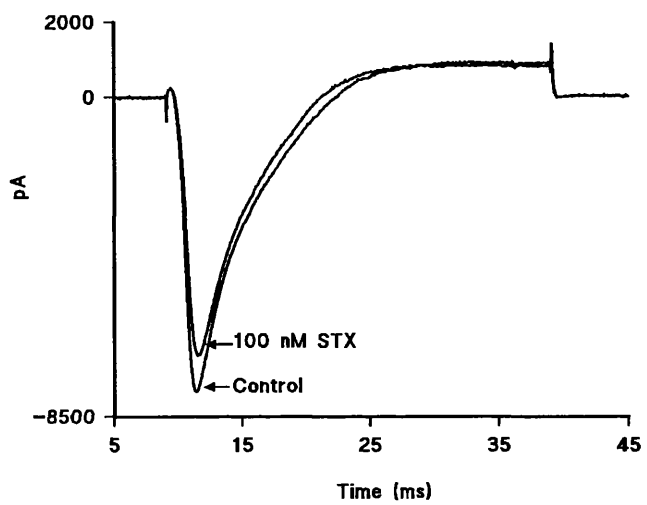
c) The effect of 100 nM STX (after 3 min application) on the peak STX_R Na^+ current. The reduction relative to the control is 12.3%.


2500 pA
5 ms
STX$_R$
STX$_S$

a)


2500
0
−10500
pA
STX 3 min
STX 1 min
Control
5
15
25
35
45
Time (ms)

b)


2000
0
−8500
pA
100 nM STX
Control
5
15
25
35
45
Time (ms)

c)

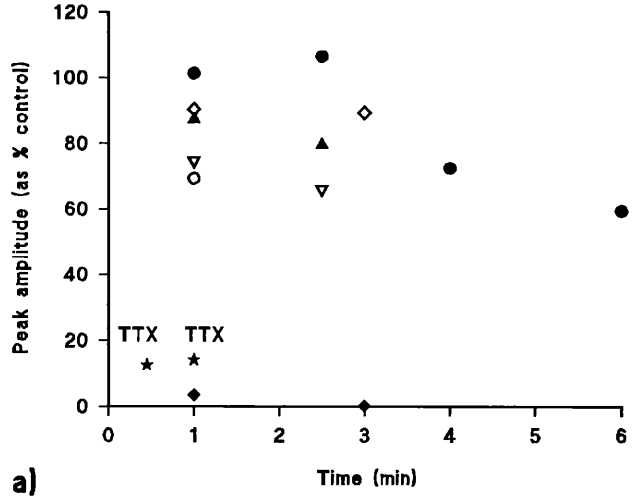
Figure 7.14.

Peak Na^+ current amplitude of DRG cells at different exposure times to a given concentration of STX or TTX. Each symbol-type on the scatter graphs represents a separate data set for individual cells. Unlabelled symbols represent results from STX experiments.

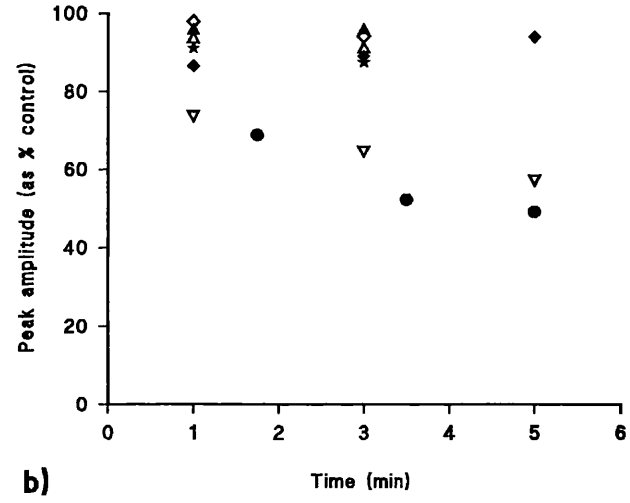
a) Peak Na^+ currents from cells exposed to 100 nM STX/TTX, whose currents did not saturate the patch clamp amplifier and the maximum peak value could therefore be measured.

b) Na^+ currents elicited from a given voltage step, based on the size of the maximum measurable current elicited from cells exposed to 100 nM STX whose Na^+ currents saturated the amplifier.

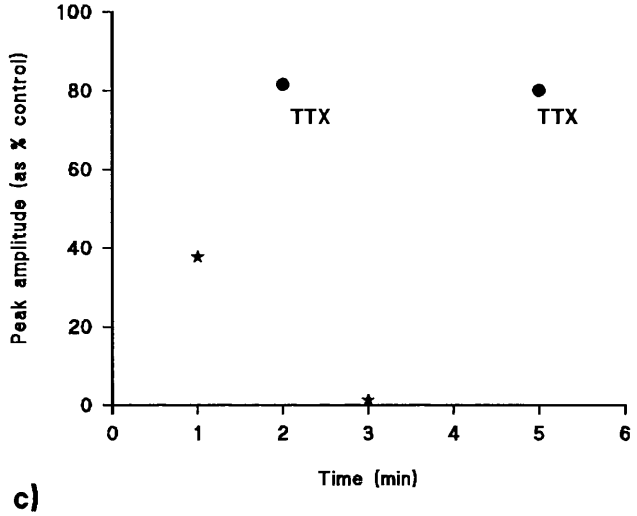
c) Reduction in amplitude of STX_S or STX_R currents in the presence 300 nM STX or TTX.


Peak amplitude (as % control)
120
100
80
60
40
20
0
TTX
TTX
0
1
2
3
4
5
6
Time (min)

a)


Peak amplitude (as % control)
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (min)

b)


Peak amplitude (as % control)
100
80
60
40
20
0
TTX
TTX
0
1
2
3
4
5
6
Time (min)

c)

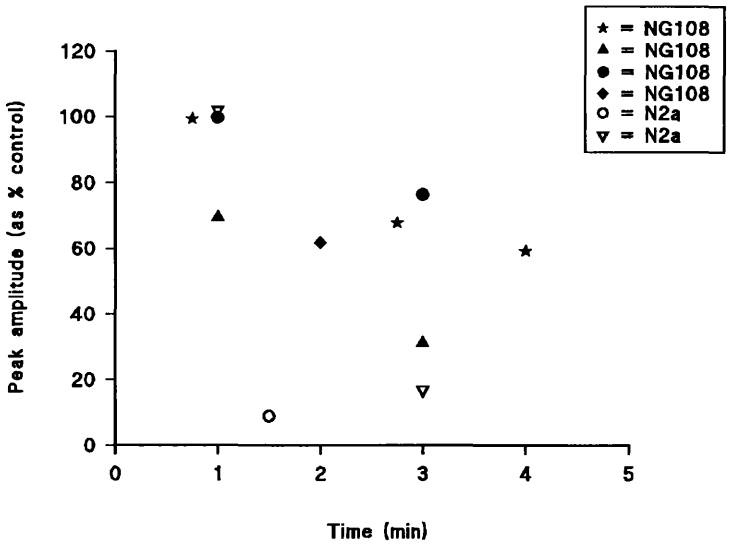
Figure 7.15.

Peak Na^+ current amplitude of N2a and NG108 cells after exposure to PSP sample S70 containing 565 μg STX eq. Each symbol-type represents a separate experimental data set of individual cells.

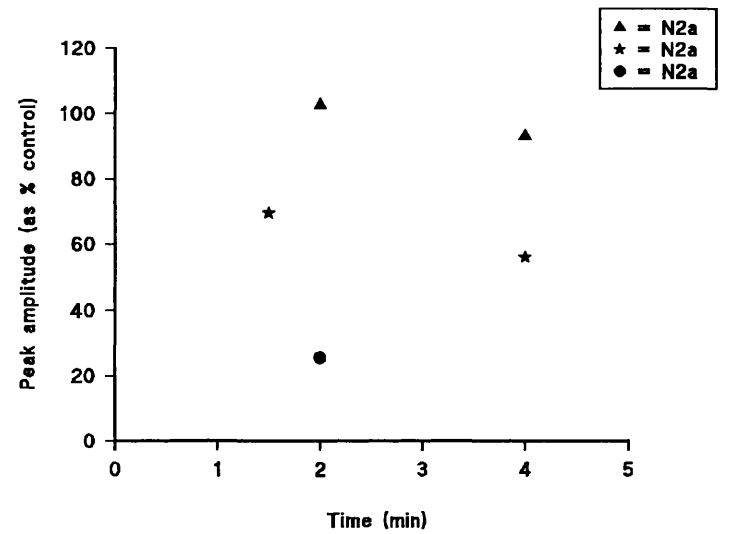
a) 1:10,000 parts dilution of toxin to solution.

b) 1:1000 parts dilution of toxin to solution.

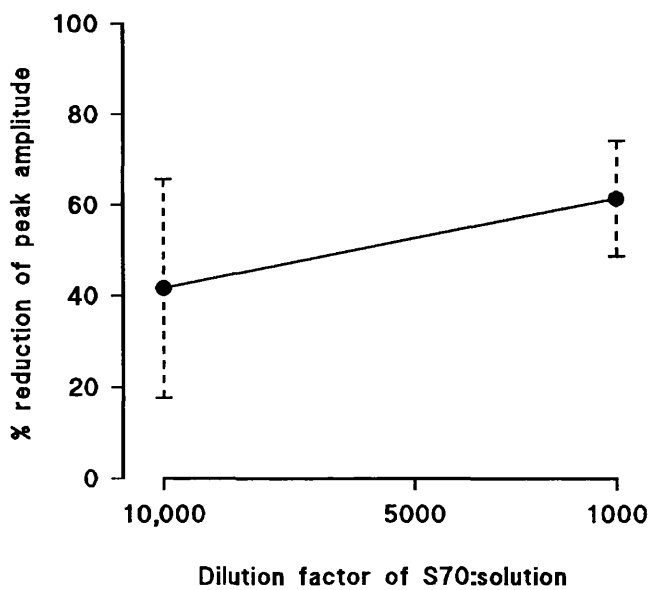
c) The mean data ± s.e.m. for both dilutions.


★ = NG108
▲ = NG108
● = NG108
◆ = NG108
○ = N2a
▽ = N2a
Peak amplitude (as % control)
120
100
80
60
40
20
0
0
1
2
3
4
5
Time (min)

a)


▲ = N2a
★ = N2a
● = N2a
Peak amplitude (as % control)
120
100
80
60
40
20
0
0
1
2
3
4
5
Time (min)

b)

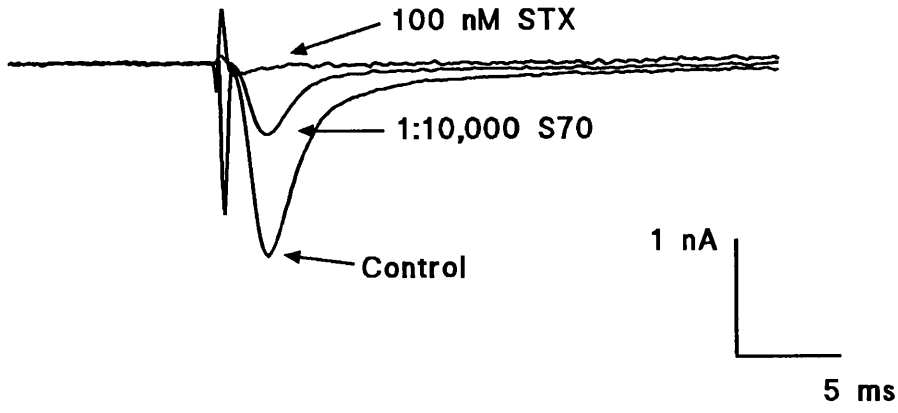

% reduction of peak amplitude
100
80
60
40
20
0
10,000
5000
1000
Dilution factor of S70:solution

c)

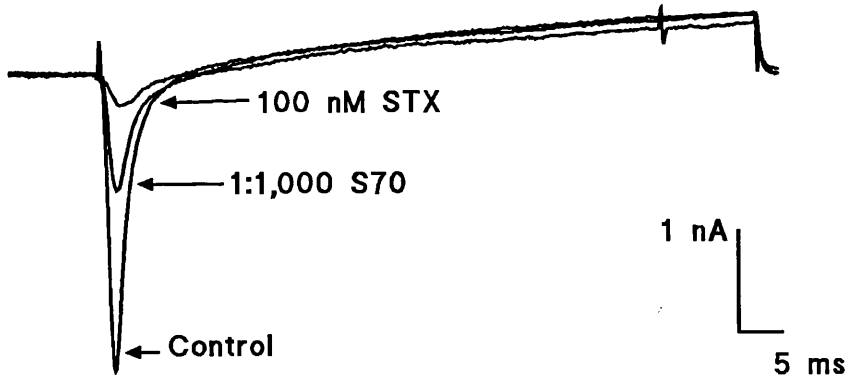
Figure 7.16.

Representative traces of reduction in the peak Na^+ current in response to exposure to 100 nM STX and sample S70 for an N2a cell (top traces), an NG108-15 cell (middle traces) and a DRG cell (bottom traces). Cells were exposed to sample S70 following the application of STX and an appropriate wash period. Sample dilutions are indicated on each set of traces.

Neuro 2A



NG108-15



DRG

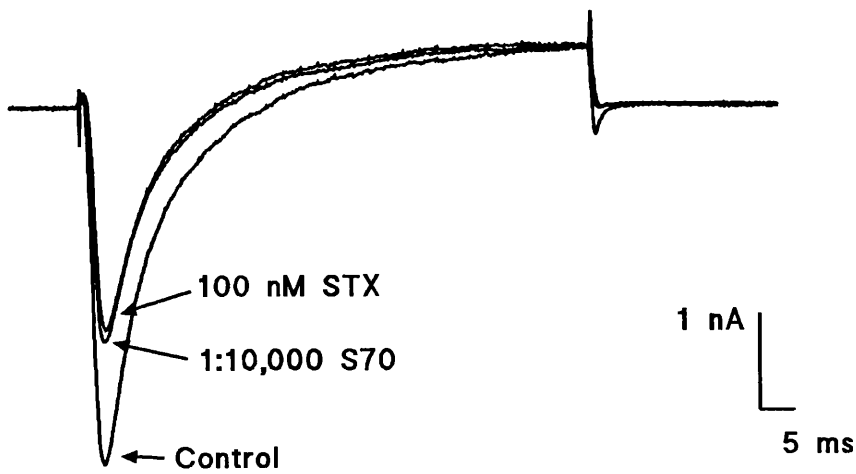


Figure 7.17.

Effects of 100 nM STX and a negative (0μg STX eq) PSP shellfish sample, S1, on the peak Na^+ current of an NG108-15 cell. Sample S1 was applied to the cell following the application of STX and an appropriate wash period. The negative sample caused a reduction in peak current amplitude that corresponded to an equivalent STX concentration of 6 nM.

NG108

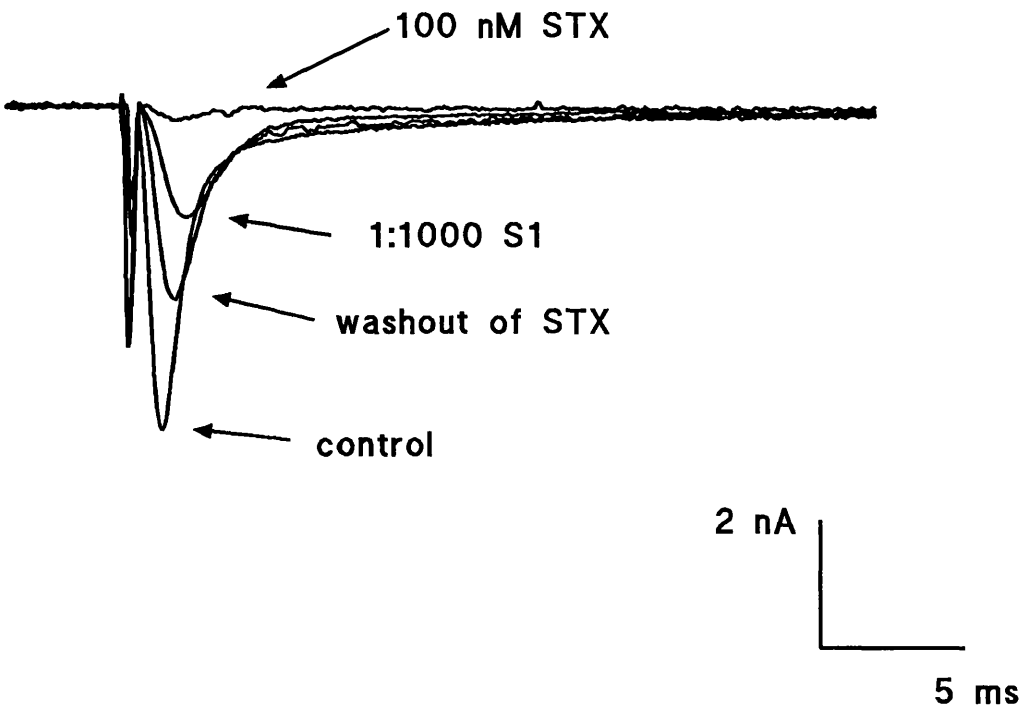


Figure 7.18.

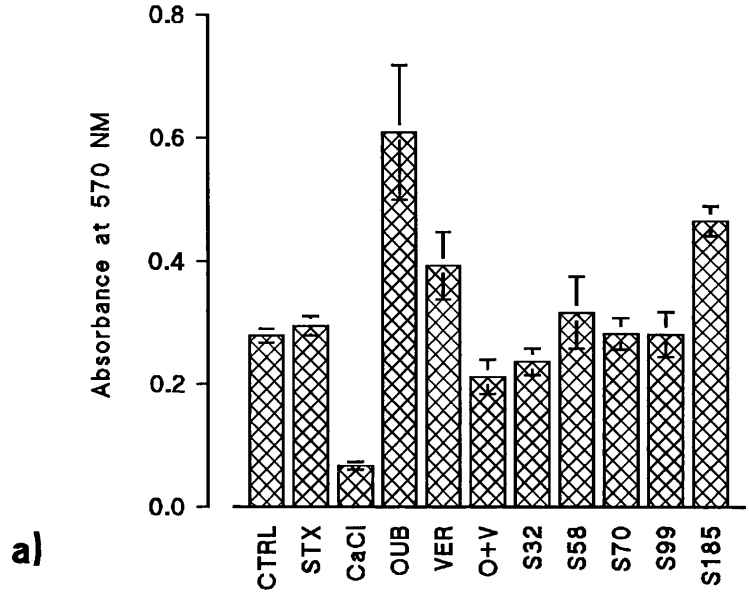
Results from the MTT cytotoxicity study of the effects of PSP samples on the neuroblastoma N2a and NG108 cell lines. STX, $CaCl_2$, OUB and VER were used as internal controls

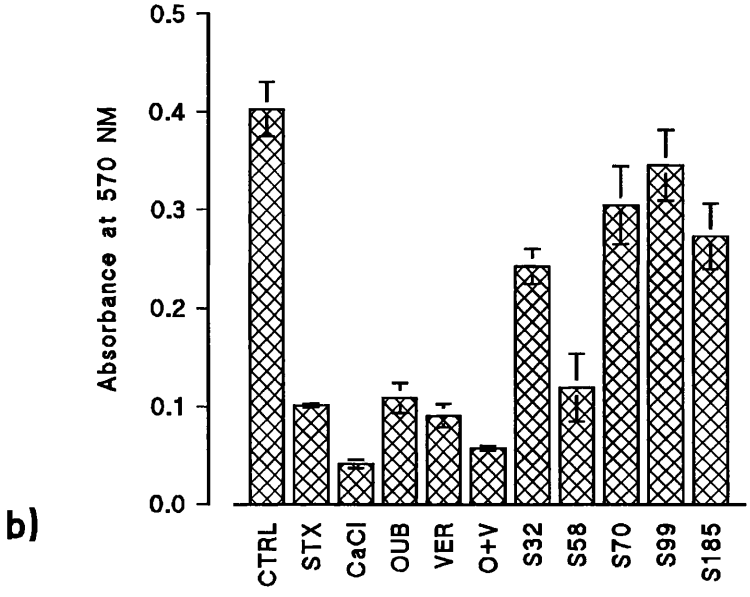
a) N2a cells incubated with a test solution for 2 h.

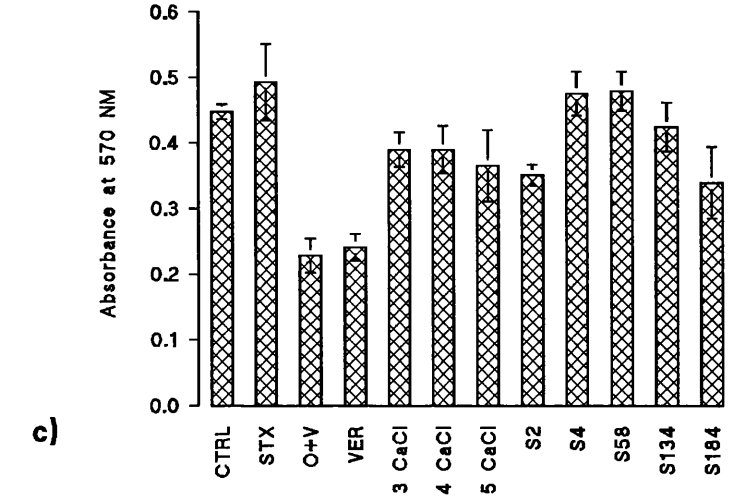
b) N2a cells incubated with a test solution for 24 h.

c) NG108-15 cells incubated with a test solution for 18 h.

Values represent the mean of 4-5 replicates. The error bar represents ± s.e.m.







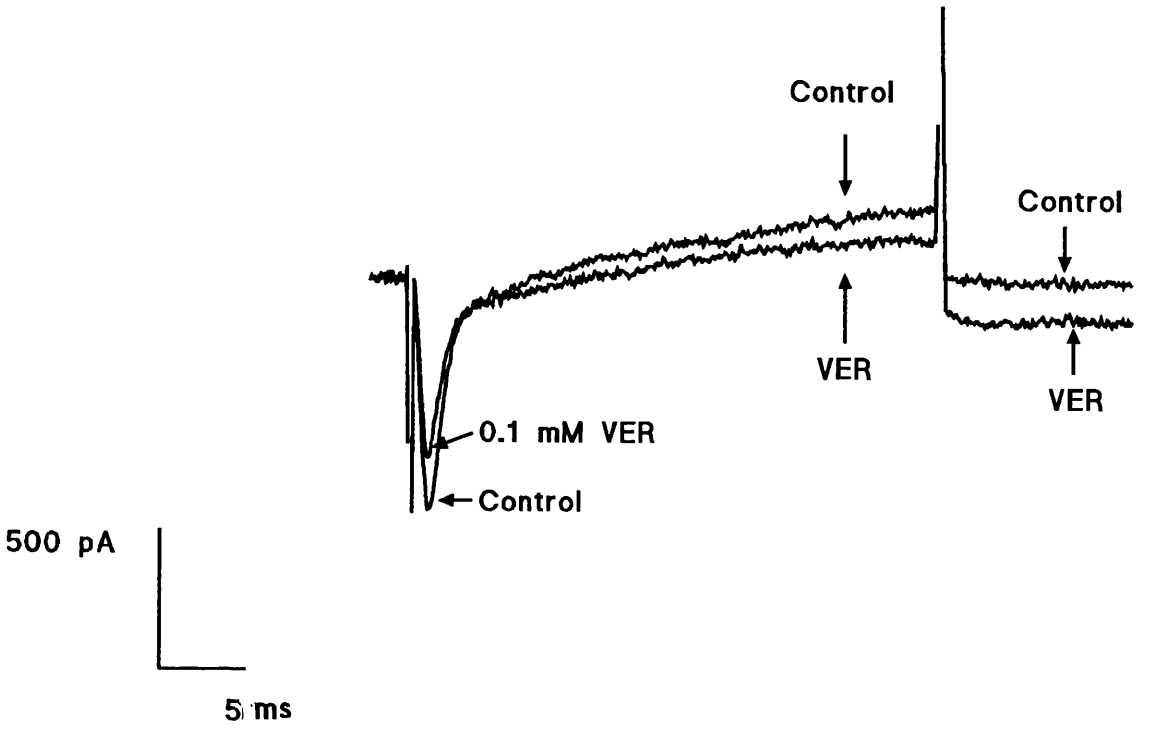
KEY:

STX = 100 nM
OUB = 0.4 mM
VER = 0.05 mM
S's = 1:60 diln

Figure 7.19.

The effect of VER on the peak transient Na^+ current and standing tail current in N2a (**a**) and NG108-15 (**b**) cells under voltage clamp conditions. In both traces a reduction in the peak transient current can be seen, together with the appearance of a non-inactivating current, indicated by an incomplete return to baseline, and an increase in the standing tail current evident after the voltage pulse.

N2a


Control
Control
VER
VER
0.1 mM VER
Control
500 pA
5 ms

NG108-15

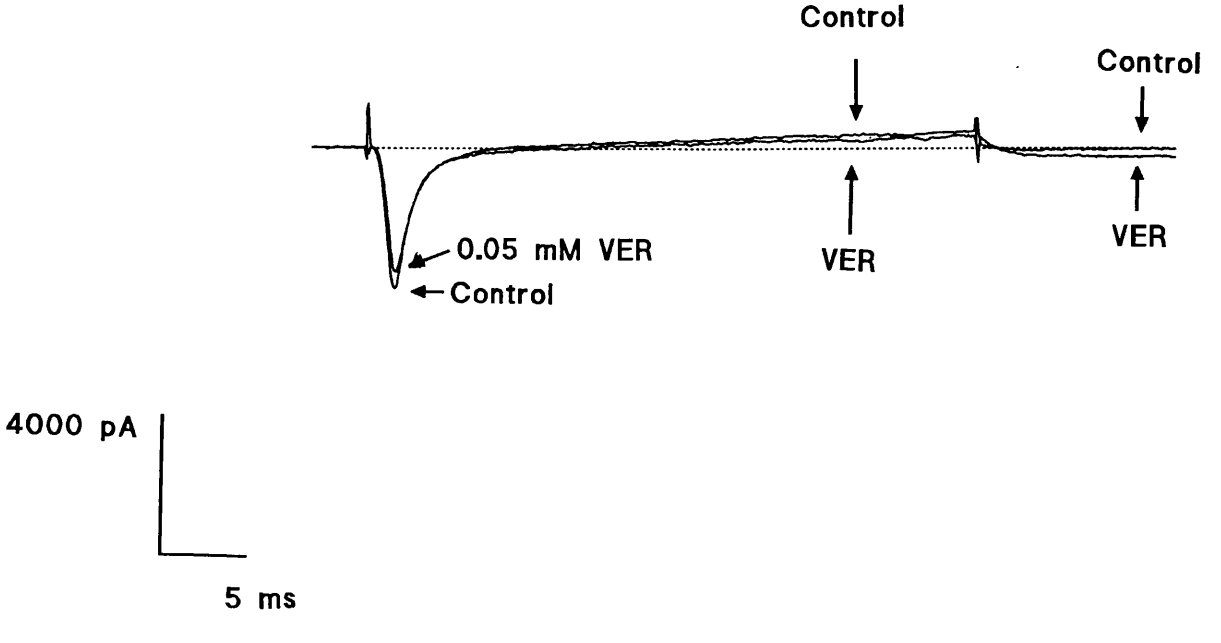
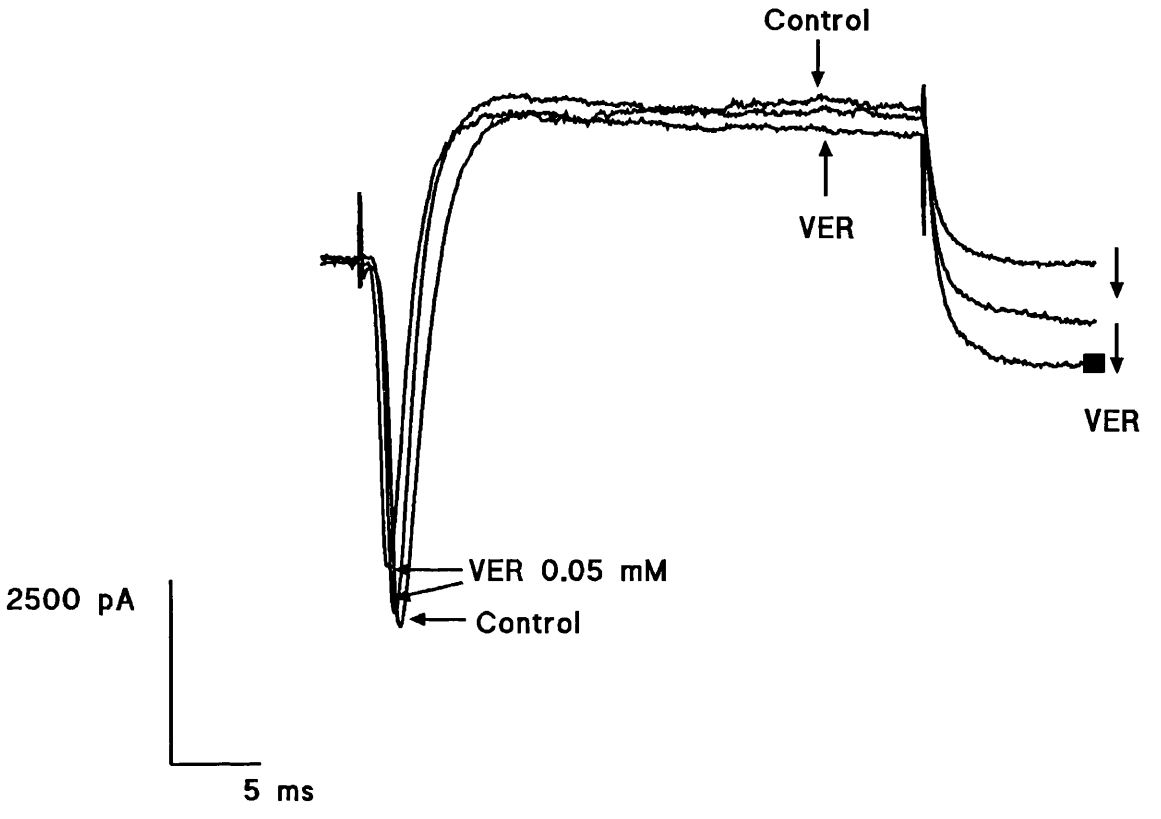

Control
Control
0.05 mM VER
VER
VER
Control
4000 pA
5 ms

Figure 7.20.

The effect of VER on the Na^+ current in the primary neuronal cell types: (**a**) SCG and (**b**) DRG. With repeated pulsing a gradual reduction in the peak transient Na^+ current and an increase in the standing tail current are clearly seen with recordings from an SCG cell (**a**). VER caused a marked reduction in the transient peak Na^+ current of the DRG cell (**b**) but had little effect on the tail current.

a) SCG


Control
VER
VER
VER 0.05 mM
Control
2500 pA
5 ms

b) DRG

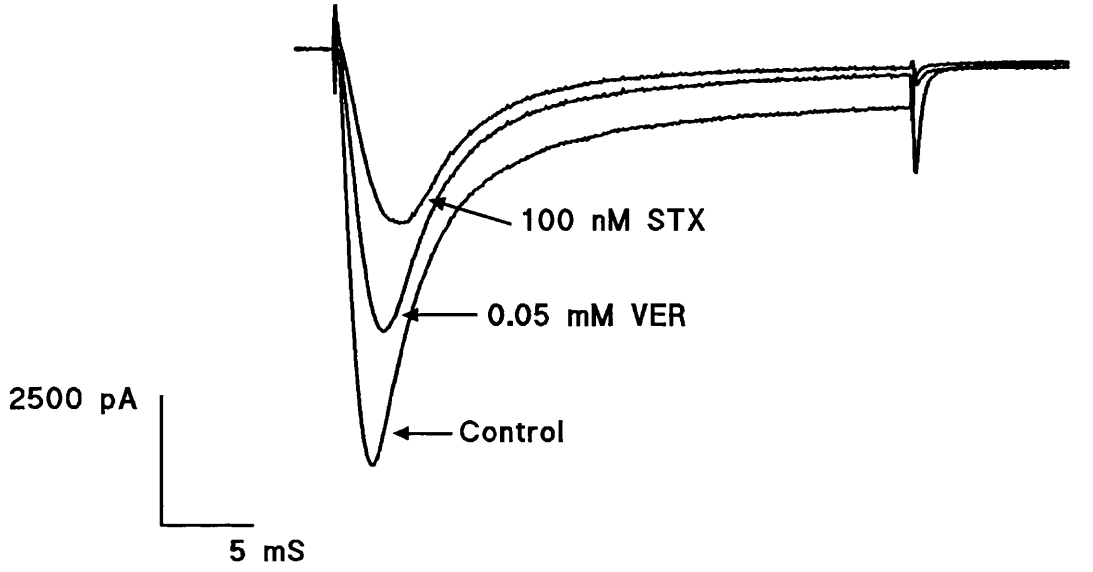

100 nM STX
0.05 mM VER
Control
2500 pA
5 mS

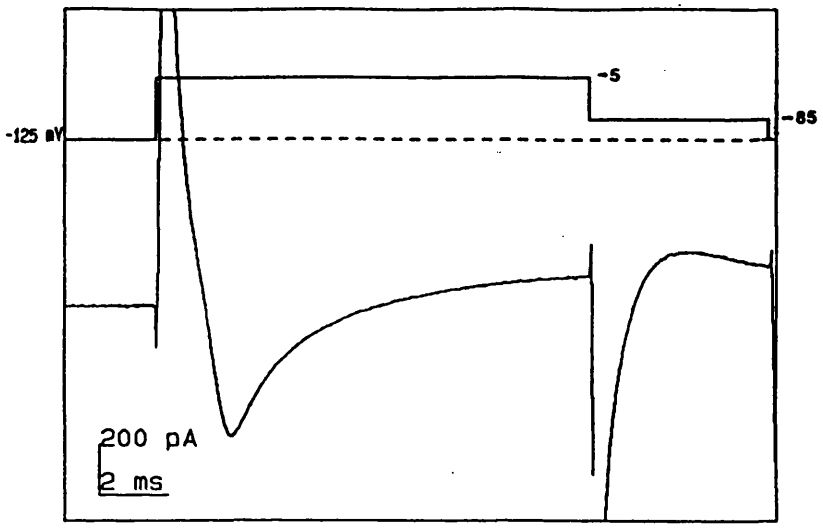
Figure 7.21.

a) A single depolarising step protocol (see **section 7.2.6.viii**) elicits an inward Na^+ current in a N2a neuroblastoma cell

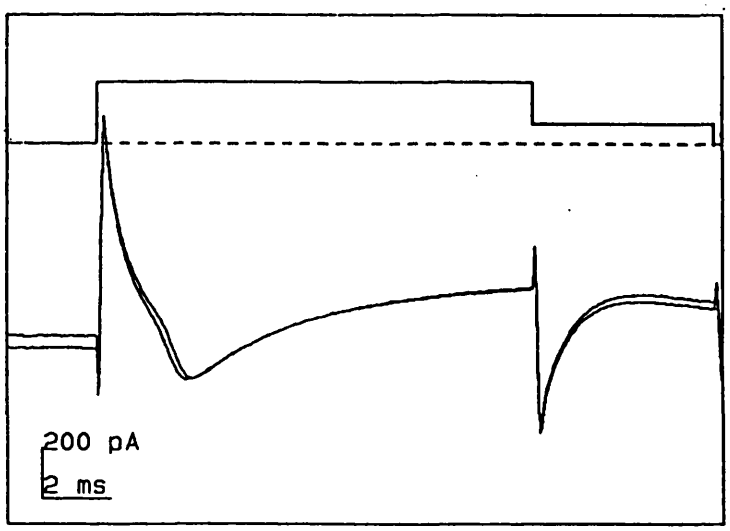
b) Application of 0.05 mM VER modifies the inward transient current and there is evidence of an increase in the standing tail current after the voltage pulse.

c) Exposure to 50 nM STX suppresses the modification induced by VER by inhibiting the inward Na current. This also has the effect of normalising the tail current.

a) NORMAL RINGER



b) VERATRIDINE



c) SAXITOXIN

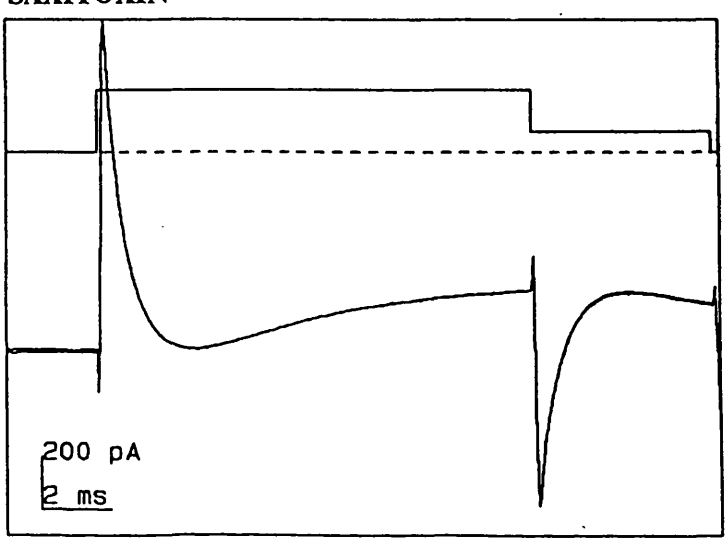
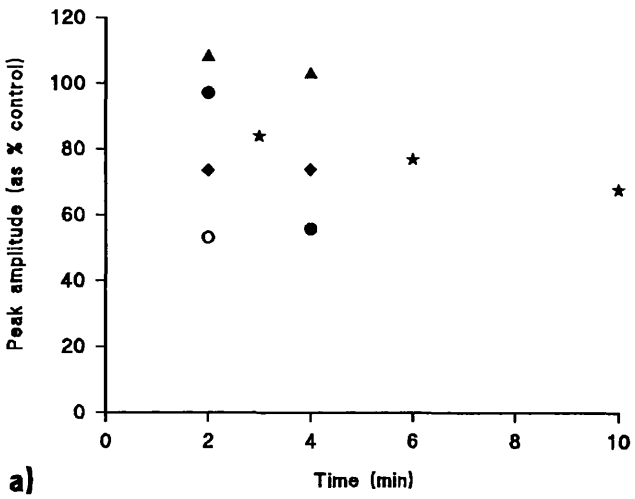
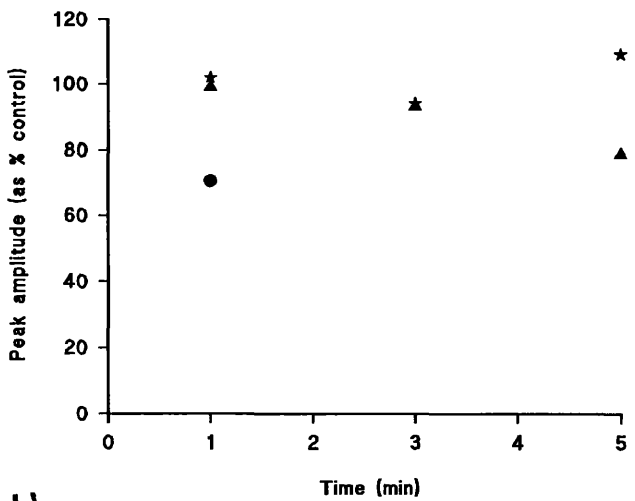


Figure 7.22.

Reduction in peak Na^+ current amplitude following exposure to either (**a**) 0.05 mM VER or (**b**) 0.1 mM VER. Each symbol-type on the scatter graphs represents a separate data set for individual cells.


120
100
80
60
40
20
0
Peak amplitude (as % control)
0
2
4
6
8
10
Time (min)

a)


120
100
80
60
40
20
0
Peak amplitude (as % control)
0
1
2
3
4
5
Time (min)

b)

Chapter 8

Concluding Remarks on the Comparative Studies

To explain all things is too difficult a task for any one man or even for any one age. 'Tis better to do a little with certainty, and leave the rest for others that come after you, than to explain all things.

Sir Isaac Newton (*ca.* 1680)

8.1. Concluding remarks

This study has effectively, been a two part approach to the examination of the effects of PSP extracts on a variety of nerve based tissues. In the first half of the study where the nerve and nerve-muscle preparations were investigated, the electrophysiological methods employed were used to evoke a specific response from the preparation that could be used as a measuring device. In the second part of the study, the electrophysiological techniques were not used as a part of the assay *per se*, but rather, as a tool to examine the neuronal membrane channel properties that underlie the currently used *in vitro* cell bioassays (Gallacher and Birkbeck 1992; Jellet et al., 1992; Manger *et al.*, 1995; Hawasaki *et al.*, 1995). Examination of such properties required the high time resolution that intracellular and patch clamp electrophysiological techniques can provide.

The aim of the study as a whole was to look at the key attributes offered by the various neuronal assays with a view to the introduction of new approaches and to offer suggestions for improvement in the robustness, reliability, and sensitivity of the *in vitro* cell bioassays.

The comparative studies on the nerve and nerve-muscle preparations showed that each system has merits. All displayed at least one of the key properties required for a good screening assay. All were in fact very sensitive, the CBC and frog being at least one order of magnitude more sensitive than the AOAC mouse bioassay (40 μg/100 g tissue), based on the further dilutions (1:100 to 1:1000) of the value of the MAFF samples (**section 4.2.3**). The lobster also showed good sensitivity with $1x10^{-12}$ M as its lower detection limit. At the same time these assays, unlike others based on animal tissue (**section 2.4.4**). were robust enough to remain viable when exposed to high toxin concentrations, e.g. 10^{-6} M STX and the high PSP samples of >200 μg

STX eq/100 g tissue. Application of the toxin directly to the site of action, i.e. the nervous tissue, is likewise an improvement on the intraperitonial (i.p.) injection procedure the mouse bioassay.

Another advantage over the mouse bioassay is that these assays could be used several times per preparation, and therefore despite being of animal origin, they could in fact reduce the number of animals used. Two muscles and associated nerve supplies were obtainable from each CBC assay (Chapter 4), plus at least two samples per nerve (if the twitch height recovered to control amplitudes). Two nerves were also usable from the frog, which proved to be very robust (Chapter 5) and similarly the crustacean assays (Chapter 3) could yield several preparations each of which could be used for multiple toxin application. According to Sullivan (1993) the mouse bioassay is only quantitative between death times of 5 and 7 min, with substantial variations either side of these times. Because of this very limited range, multiple dilutions may be required to obtain concentrations within that range and then testing must be repeated on the diluted extract. If toxicity is high then ten or more mice may be necessary to test one sample.

The principal disadvantage of the nerve-muscle assays was that the nerve-synapse-muscle configuration produced too many variables. Thus systems like the lobster and CBC, whilst initially appearing simple, were complicated by the post-synaptic events incorporated into the assay. However a single length of nerve composed of many axons which exhibits a graded effect, as seen with the frog, is more site directed, with no "contamination" from additional variables. A large section of nerve from crustacea would be just as suitable. Such a system could provide a rapid screening tool before multiple repeats of samples were required. Then automated assays, such as the cell bioassays, based on 96 wells containing samples, could be used.

The electrophysiological studies on the cell types, especially the N2a cells, provide extremely valuable information with regard to the stability of cell lines and their electrical properties at the time of use in the bioassay. Whilst the primary DRG and SCG cell types are closer representations of *in vivo* mammalian cells, compared

to the neuroblastoma lines (derived from tumour origin), they were clearly not a suitable substitute. The expression of at least two Na^+ channel types, STX_S and STX_R, makes them unreliable as a measuring tool. However, primary cells expressing STX_S channels were affected by either STX or the PSP samples in a similar manner to the neuroblastoma cells.

This in itself reinforces the presumption that the tissue culture cell lines have not lost the ability to behave as normal nerve cells to Na^+ channel blocking toxins. Furthermore, the STX_R channels in these primary cells may provide an explanation to to the fact that organisms that sequester the PSP toxins remain relatively unaffected by the sodium channel blocking action of these compounds.

The patch clamp experiments which investigated the Na^+ channel properties of the N2a cells at the stage of differentiation at which they are used in the cell bioassay, revealed some important properties that require further consideration. The key factors, discussed in Chapter 7, may relate to inconsistencies in Na^+ channel expression in these cells and two other neuroblastoma cell lines, NG108-15 and SK.N.SH, under the present assay-culture regime investigations, and further highlight that the choice of cell line is of critical importance.

These latter two neuroblastoma lines, one a hybrid (NG108-15) and the other from human origin (SK.N.SH), (which is perhaps worthy of further investigation) exhibited greater culture robustness than the N2a cells. Thus, although Jellet *et al.* (1992) specifically advocate the use of actively growing cells to produce a greater assay reliability (but combined with a less sensitive detection limit), this study found that there was no significant difference in sensitivity to STX between undifferentiated (i.e. actively growing) and differentiated cells (**section 7.3.5.ii**). Despite the increase in Na^+ current size there was in fact, a greater reliability in the Na^+ current amplitude and expression. Manger *et al.* (1995b) appear to be adopting a similar approach to this study, having found that reducing the concentration of serum in the culture medium promotes differentiation, and increases the reliability of their assay system.

A combination of electrophysiological techniques with binding experiments would be an instructive method to identify and evaluate tissue culture cell lines that

might be suitable for use in competitive binding assays (**section 2.4.6**). These have already been shown to have high reliabilty as well as sensitivity. These could then be developed as a replacement for rat brain membranes synaptosomes currently used. Such a binding system would eliminate the need for the chemical compounds presently used (i.e. VER and OUB) to depolarise the cell membranes and for an incubation period required, making a faster assay system available for screening purposes.

The way forward for the detection of PSP toxins would appear to lie with the use of tissue culture cell lines, whether through binding assays or the cytotoxicity method of the Gallacher and Birkbeck (1992) assay. The need to replace the mouse bioassay is unquestionable. That stated, there is still a reasonable argument for the use of simple nerve preparations, with a proven robustness and toxin inhibition responses, for the periodic screening of extracts as a quality control for the cell assays. A similar quality control for the condition of the cell lines themselves, employing methods such as patch clamping, would ensure a regulated and well-maintained cell line.

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Anderson, D.M. (1994) Red tides. *Sci. Amer.*, **271**(2), 52-58.

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The roots of education are bitter, but the fruit is sweet

Aristoteles, Diogenes Laertius