The contractility of the portal vein: role of oxygen and calcium.

A thesis presented for the degree of

Doctor of Philosophy.

BY

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DECLARATION

All experiments and results described in this thesis have been carried out by me, in the Institute of Physiology, University of Glasgow between October 1984 and October 1987. No part of this thesis has already been or is being concurrently submitted for any other degree at this or any other university.

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SUMMARY

The effects of Po_2 and $[Ca^{2+}]_o$ in the isolated rat portal vein have been investigated.

1. Po_2 tensions from a supraphysiological level (95%O_2) to normoxic levels (16%O_2) did not affect the spontaneous phasic contractile activity of the portal vein. In fact, levels down to 50mm Hg (8%O_2) did not statistically significantly affect it. On the other hand, drug induced contraction to NA, amidephrine and phenylephrine was sensitive to O_2 over this range. Responses induced by these drugs changed from being monophasic in supraphysiological O_2 to biphasic in normoxia. In general, drugs that induced an increase in the spontaneous activity without a concomitant increase in baseline tension were less sensitive to O_2 change from 95%O_2 to 16%O_2. Examples of such drugs were UK14304, xylazine and (+) m-synephrine, while clonidine was the only exception to this rule, being sensitive to O_2 changes.

2. The tonic component of the biphasic response to NA at normoxia became progressively smaller as Po_2 was lowered from 32%O_2, while the phasic component was quite resistant to Po_2 changes. Consequently the phasic component became more prominent as O_2 was lowered. However, the nature of this biphasic response at normoxia O_2 in this tissue is different from that

obtained in the aorta which is considered to involve the release of intracellular Ca^{2+}. (i). The biphasic contraction is rapidly lost in nominally "Ca^{2+}-free" saline. (ii). The Ca^{2+} threshold of the phasic component is at 1.25mM. (iii). The phasic response was also susceptible to blockade by the Ca^{2+} chelators EGTA + NTA, and the Ca^{2+} antagonist nifedipine and to facilitation by Bay K 8644. All these results suggest that both components require influx of extracelullar Ca^{2+}.

3. Assessment of the facilitating effect of Bay K 8644 (1nM - 0.3μM) at the [Ca^{2+}] (2.5mM) normally employed in pharmacological experiments and over a wide range of Po_2 (95%O_2 - 4%O_2) showed that while spontaneous activity was increased in a concentration related manner, there was little effect on responses to NA or KCl. On the other hand Bay K 8644 could facilitate responses to these activators at low [Ca^{2+}]. Under such conditions the preparation provided a highly sensitive assay for drugs which facilitate Ca^{2+} channel opening such as Bay K 8644 and CGP 28392.

4. Assay of Bay K at different O_2 tensions indicated similar potency versus responses induced by NA and KCl suggesting that both might be acting via voltage - operated channels (VOCs).

5. At hypoxia, NA- induced responses were depressed. On the other hand KCl-induced responses were resistant and in some cases were bigger than they were in normoxia. Investigation into the hypoxia- resistant KCl-induced responses showed that they were relatively resistant to Bay K 8644 and CGP 28392 when compared to those in normoxia. This could mean that these hypoxia resistant KCl -induced responses may have already been facilitated as a result of endogenous accumulation of some metabolites such as long chain acyl carnitines, which is known to occur in hypoxia/ ischaemia. Palmitoyl carnitine, an acyl carnitine, has been shown to activate Ca^{2+} channels directly (Spedding & Mir, 1987). In the present study it has been shown that the effects of palmitoyl carnitine, mimic those of Bay K and CGP in both normoxic and hypoxic conditions. The resistance to Ca^{2+} facilitators occurs because there is less scope for further facilitation by these Ca^{2+} facilitators since the channels are fully facilitated in hypoxia.

6. KCl -induced response was more susceptible to POCA, a carnitine acyl transferase in hypoxia than in normoxia suggesting that POCA can block the accumulation of acyl carnitines in hypoxia, thereby reducing their facilitating effects. The relative resistance of KCl response to POCA in normoxia suggest that acyl carnitines do not accumulate in normoxic conditions.

7. The hypoxia-resistant response was also relatively resistant to nifedipine. Such a situation could be predicted since palmitoyl carnitine acts at the dihydropryridine sites to oppose the effects of nifedipine (Spedding & Mir, 1987) and my experiments add evidence for this, showing that it may occur with endogenous PC under hypoxic conditions.

8. Despite several experimental manipulations, hypoxic vasodilatation was not reversed by Ca^{2+} facilitators Bay K and CGP, supporting the hypothesis that these compounds may be relatively ineffective in hypoxic conditions.

9. The implications of the relative ineffectiveness of the dihydropyridine compounds in patho-physiological conditions of hypoxia/ischaemia are (i) these compounds would be ineffective in alleviating ischaemic conditions, (ii) they cannot be employed in the treatment of hypoxic vasodilatation.

CHAPTER 1.

LITERATURE REVIEW.

I THE ROLE OF OXYGEN IN THE CONTRACTILITY OF VASCULAR SMOOTH MUSCLE

The importance of oxygen in the contractility of vascular smooth muscle has long been recognised. Ross et al (1962) showed that a lowered bath O_2 caused relaxation of vascular smooth muscle. This was demonstrated by perfusing isolated segments of arteries from dog skeletal muscle with blood over a range of Po_2. They found that conductance of these vessels increased 2-5 fold as Po_2 was lowered from 100 mmHg to 30 mmHg and concluded that vascular smooth muscle of these vessels was sensitive to changes within a physiologically important range. Smith and Vane (1966) superfused strips of swine carotid artery and guinea pig, rat and cat aorta with both blood and physiological salt solution with different Po_2. They found that the contractile tension of these preparations varied directly with the perfusate Po_2. This relationship was not affected by hyoscine, phenoxybenzamine, hexamethonium, bromolysergic acid diethylamide or mepyramine.

Numerous studies have supported the view that contractile strength of isolated vascular smooth muscle is dependent on bath Po_2. This is true of rabbit aorta (Furchgott, 1964; Shibata & Briggs, 1967; Detar & Bohr, 1968, Namm & Zucker, 1973; Altura & Altura, 1976), rat aorta (Freed & Meunier, 1972), ductus arteriosus (Kovalcik, 1963; Fay, 1971; Starling & Elliot, 1974; umbilical artery (Nair & Dyer, 1973; Starling & Elliot, 1974), skeletal muscle arteries (Gellai & Detar, 1974), coronary arteries (Gellai et al, 1973; Gellai & Detar, 1974), hog carotid artery (Pittman & Duling, 1973) and pulmonary, mesenteric and saphenous veins (Vanhoutte, 1976). However there exist quantitative differences between bath Po_2 and tension development in different tissues (Sparks, 1979).

A Mechanism of action of oxygen tension on vascular smooth muscle

1. Hypoxic core

One explanation for this variation is the size of the hypoxic core of isolated strips. This is determined by the diffusion constant for O_2, strip O_2 consumption and the geometry of the vessel wall. Umbilical arteries for example, which tend to be quite thick (approx. 1mm) exhibit changes in contractile tension in response to bath Po_2 far above 100 mmHg (Nair & Dyer, 1973). On the other hand rabbit aorta which is approximately 0.2

mm thick is sensitive to Po_2 changes below 100 mmHg (Detar & Bohr, 1968). Smaller vessels isolated from skeletal muscle and heart (approx 20-40 mm thick) are sensitive to Po_2 changes only below 10-20 mmHg (Gellai & Detar, 1974). Pittman & Duling (1973), showed, assuming similar diffusion coefficients and oxygen consumptions for each of these tissues, that increased sensitivity to Po_2 could be explained by a greater distance to the centre of a vessel strip and thus increased size of its hypoxic core. Smooth muscle within this hypoxic core might relax because of inadequate O_2 to support oxidative phosphorylation or because of the production of a vasodilator.

2. Limitation of energy production

Another possible cause of the variability in the effect of hypoxia on contractions of vascular smooth muscle is the relative capacity of anaerobic ATP synthesis (Sparks, 1980). In situations in which hypoxia has little effect on contractile force, removal of glucose from the bath causes almost complete relaxation. This is probably because the substrate for anaerobic metabolism has been removed. Since anaerobic capacity varies from tissue to tissue, the effect of hypoxia should also vary. Evidence has shown that it is unlikely that vascular smooth muscle contraction is limited by inadequate energy production, with the exception of situations whereby the availability of

substrates from the bath limits ATP production. Paul (1980) in his review showed that vascular smooth muscle exhibits a considerable capacity for anaerobic glycolysis. Under hypoxic conditions glycolysis is strongly stimulated in some smooth muscles (Arnqvist & Lundholm, 1976) and appears to be capable of satisfying the energy needs of the muscle at the level required before hypoxic relaxation.

3. Influence of external substrate.

In cases where glucose is the exogenous substrate, energy production in hypoxia could be limited by the bath glucose concentration, if it is below 10 mM. Namm & Zucker (1973) showed that raising the glucose concentration above 5 mM significantly elevates anoxic force development in rabbit aorta and pig carotid artery respectively. Glucose concentration in the 10 to 15 mm range appear to be optimum in terms of minimizing the contribution of substrate availability to hypoxic force depression.

4. Alteration of membrane bound protein

Altura & Altura (1976) proposed that anoxia might lead to the alteration of some membrane bound protein whose usual conformation requires a continuous supply of energy. This protein, in turn could in some way control the permeability of the sarcolemma or other membrane to

an ion (e.g. calcium) important for maintenance of contraction or could be associated with some pharmacological receptor and thus alter the binding of an activator molecule. Energy limitation (see section 3) could however prevent such energy-dependent alteration of the membrane bound protein. The possibility that the O_2 molecule per se could produce such an effect ("oxygen receptor") cannot be excluded (Paul 1980).

5. Membrane Na^+-K^+

Detar (1980) proposed that the mechanism of oxygen sensing involves the sarcolemma Na-K active transport system, although the manner in which O_2 is coupled to the system remains to be determined. Paul et al (1979), however showed that the Na-K transport in vascular smooth muscle is specifically coupled to glycolysis, while oxidative metabolism is coupled to the energetics of the contractile process. These observations taken together suggest that if O_2 sensing is accomplished through Na-K transport processes, the link is likely to be an indirect one. However, Jennett and Wallace (1981) argued against the invovlement of Na- K in hypoxic relaxation since ouabain, an inhibitor of Na- K pump, did not alter the hypoxic relaxation of NA-induced contraction of the rabbit portal vein.

6. Specialised O_2 receptor

Coburn et al (1979) suggested that O_2 might influence vascular contraction by acting on some specialized receptor. Their conclusion was based on the relationship between force development and oxygen consumption. They showed that the latter decreased with decreasing Po_2 or increasing concentration of cyanide. However, the qualitative relationship between force and O_2 uptake were quite different in the two situations. Furthermore, decreased Po_2 made an additional difference after poisoning of oxidative metabolism by cyanide. A clear interpretation of these experiments rests on the determination of the specificity of cyanide for cytochrome oxidase. The search for an O_2 receptor may continue, but more specific requirements for such an entity must be provided before definitive experimental tests can be made.

7. Formation or release of endogenous vasoactive substrates

The idea that the production rate of some vasoactive oxygen-linked substrates is altered in response to hypoxia has been suggested by many investigators (Eckenfels & Vane, 1972, Hellstrand et al, 1977, Kalsner, 1977; Roberts et al, 1979; Smith & Vane, 1977). Amongst the substrates which have been implicated as mediators of local blood flow control are adenosine and

prostaglandins:-

a. Adenosine and derivatives

Adenosine and its derivatives have been implicated in the hypoxic vasodilation. Adenosine nucleotide derivatives (adenosine, vinosine and hypoxanthine) have been shown to accumulate in hypoxic media relative to the formation rate in well oxygenated conditions in hog carotid artery (Van Harn et al, 1977). Pittman & Quinn (1970) later showed that the accumulation of the adenosine nucleotide in hypoxia leads to depression of active isometric force in the same preparation.

Adenosine deaminase which breaks down adenosine to inosine and ammonia, has been shown to reverse the hypoxic vasodilation mediated by adenosine. Since the other products of catalysis i.e. inosine and ammonia could not reverse anoxic/hypoxic vasodilation, Pittman and Quinn (1979) postulated that adenosine deaminase reversal of the effect was a result of the breakdown of adenosine. The reversal of anoxic/hypoxic vasodilation by adenosine deaminase under anoxic conditions further argues against the idea that ATP generation is limited during anoxia in smooth muscle. The hypothesis of adenosine as a mediator of hypoxic vasodilation would be more convincing if direct measurement of adenosine levels within strips during graded hypoxia and their relation to force development is determined.

Pittman & Duling (1973) proposed that an anoxic core is required before oxygen-linked alterations in contractile activity can take place. Wilson et al (1979) showed that substantial changes in cellular levels of adenosine nucleotides and perhaps adenosine, could occur at higher cellular Po_2 (i.e. near 12 mmHg instead of 0 mmHg). Thus if adenosine levels above the threshold for relaxation (about 10^{-8} M) could accumulate near smooth muscle cells, changes in cellular Po_2 in the 0 to 12 mmHg range could modulate contractile activity. This possibility could be tested by simultaneous measurements of intrastrip Po_2 and isometric force during changes in bathing solution Po_2.

b. Creatine phosphate (CP) involvement in adenosine-induced effect

Rubio et al (1979) proposed a biochemical mechanism for adenosine formation in skeletal and cardiac muscle which might also apply to vascular smooth muscle.

The enzyme 5' nucleotidase forms adenosine for 5' adenosine monophosphate (AMP). This enzyme is normally inhibited in vivo by ATP, ADP and creatine phosphate (CP). During graded hypoxia, Born (1956) using taenia coli & Namm & Zucker (1973) using rabbit aorta found that ATP and ADP levels remain near normoxic control

levels until severe hypoxia and anoxia are reached. Isometric force, however was observed to decline at much higher Po_2 while ATP and ADP levels remained unchanged. CP, however did fall in concert with isometric force and this parallelism has never been satisfactorily explained. The hypothesis of Rubio et al (1979), if valid for smooth muscle, could provide the key to understanding this puzzle. When a hypoxic cell cannot produce sufficient ATP by oxidative phosphorylation, the CP stores might be utilised as an immediate source of ATP during the time interval it takes for glycolysis to become fully stimulated. The decrease in CP concentration could lead to increased activity of 5' nucleotidase and thus allow more adenosine to be generated. The hypoxic cell would also probably have a higher concentration of 5' AMP, the substrate for 5' nucleotidase. Thus, adenosine could be the missing link between CP levels and isometric force development during graded hypoxia (Pittman, 1981).

c. Prostaglandins

Another group of vasodilators released during hypoxia are prostaglandins. Kalsner (1976, 1977) showed that production of a prostaglandin E-like substance by bovine coronary strips increases with lowered bath Po_2. In addition, cyclooxygenase inhibitors reduce the sensitivity of coronary strips to O_2. There is however, a lower limit on this mechanism because O_2 is needed for

the cyclooxygenase mediated transformation of arachidonate to endoperoxides. Kalsner (1977) within the same study showed that when bath Po_2 is reduced below 9 mmHg, PGE-like synthesis is reduced. Thus within a certain range of Po_2, hypoxia could induce relaxation by promoting PG release. Recently Jackson (1986) using hamster cheek, produced evidence that PG was not invloved in hypoxic vasodilatation. He showed that while cyclo-oxgenase inhibitor could prevent effects due to arachidonic acid, those by hypoxia were not prevented.

8. Calcium

Another possible explanation for the variability of tissue responses to hypoxia is the involvement of calcium. Ebeigbe et al (1980) examined the mechanism of hypoxia vasodilation of a variety of vascular strips, namely the rat portal vein, rabbit ear artery, rabbit aorta and rabbit carotid artery. They showed that the sensitivity of the tissues both to hypoxia and to external Ca^{2+} was in the order portal vein > ear artery > aorta > carotid artery. There was a correlation between tissue susceptibility to hypoxia and dependence on extracellular calcium as shown by the tissue's susceptibility to calcium-free physiological saline, which explained why tissues like the portal vein were more susceptible to hypoxia while other tissues such as

aorta, were less susceptible because they were dependent on intracellular calcium to a greater extent. This is also consistent with the observation by Marriot et al (1985), that tissues susceptible to hypoxia were more susceptible to calcium channel blockers, e.g. diltiazem, nifedipine.

Calcium flux studies with rabbit aorta by Ebeigbe et al (1980) suggested that in hypoxia there was increased calcium uptake in non- stimulated preparations whereas decreased calcium uptake was observed in tissues activated with either NA or KCl. Results obtained with the portal vein in a similar study were not consistent, therefore no meaningful interpretation could be made. Ultrastructural studies on the rat portal vein by Delvine (1972) and Ebeigbe et al (1980) showed that in hypoxia compared to normoxia the following changes occur: increase in number of surface vesicles per unit membrane length, decrease in number of cell to cell contacts per unit membrane length, enlargement of mitochondria, more frequent occurrence of sarcoplasmic reticulum - surface vesicles, mitochondria association, and loss of intramitochondrial granules. The loss of intramitochondrial granules suggests redistribution of calcium in hypoxia (microscopic analysis confirmed Ca^{2+} content of these granules).

B. Rat portal vein (RPV) in O_2 studies

The importance of local O_2 tension has long been established in the production of functional hyperaemia and autoregulation in skeletal muscle (Greyton et al, 1964; Skinner & Powell, 1967). The portal vein represents an experimentally convenient example of spontaneously active vascular smooth muscle, a kind that is thought to predominate in small resistance vessels and precapillary sphincter, but which is difficult to study in those locations (Wiedeman, 1968; Mellander & Johansson, 1968). Since local control of blood flow mainly results from variations in the myogenic component of tone in the resistance vessels (Mellander & Johansson, 1968); it appears more relevant to examine the oxygen dependence of vascular smooth muscle preparations which, in contrast to large arteries, possess myogenic spontaneous activity.

Hellstrand et al (1977) studying the effects of hypoxia and of glucose-free saline on the isolated rat portal vein showed that extra- cellular Po_2 below 50 mmHg caused inhibition of the spontaneous phasic activity and below 7 mmHg there was complete inhibition in most of the preparations. Contractile force of KCl depolarised tissue was less sensitive to O_2 than were equivalent responses to noradrenaline with the phasic contraction less affected than the tonic contraction. Responses to noradrenaline (NA) were markedly depressed at extreme

hypoxia (10 mmHg).

Sucrose gap experiments showed that hypoxia reduced the spontaneous electrical spike discharge. Mean tissue levels of phosphate creatinine (PCr), ATP and glycogen were depressed. From this study the authors concluded that physiological variations in Po_2 may influence myogenic activity of the portal vein largely through an action at the membrane and this mechanism may participate in local blood flow. This observation was later confirmed by Linke & Heinle (1981). The graded response to hypoxia, according to Hellstrand et al (1977, 1979) could not be due to diffusion limitations. Calculations based on values for the rate of O_2 diffusion through vascular tissue (Kirk et al, 1955; Pittman & Duling, 1973) and data from O_2 consumption of portal vein (Hellstrand, 1977) indicate that the difference in Po_2 from surface to the innermost layer when mounted as a sheet is only 9 mmHg. This value is outside the critical Po_2 of 40-50 mmHg. Also the argument on an increasingly larger anoxic core with proportionate fall in contractile tension as Po_2 is lowered (Pittman & Duling, 1973) is probably not applicable to the portal vein where activity is propagated electrically along the vessel wall from a small number of pacemaker areas (Johansson & Ljung, 1967; Hellstrand, 1979).

An interesting observation in the Hellstrand et al study is the apparent similarity between effects of hypoxia

and of glucose withdrawal on KCl and NA-induced contractions. A similar observation was made by Vanhoutte (1976) using the dog mesenteric vein. On the other hand Hellstrand et al (1977) showed that spontaneous phasic activity was more resistant to glucose-free medium than to hypoxia. This was explained by endogenous substrates (amino acids e.g. aspartate and glutamate) being able to support a low level of contractile activity (SA) but insufficient to support a strong excitatory stimuli such as that by NA or KCl. The same reasoning would explain why the tonic component i.e. the delayed response to KCl, was more susceptible to glucose-free solution.

The metabolic activity in the portal vein is higher than in non-spontaneously active tissue. The high rate of metabolism could be related to the 'phasic' nature of the tissue (Hellstand, 1979). The high metabolic activity in this muscle compared with the tonic smooth muscle of the conduit vessels could explain the potential of the spontaneously active vascular smooth muscle to effect a dynamic control of blood flow, e.g.in the vascular bed of skeletal muscle (Johansson & Mellander, 1975; Grande & Mellander, 1978).

The high metabolic rate of the portal vein, its spontaneous spike generation and propagated electrical activity are suggested as causes for the graded Po_2 dependence.

Hellstrand et al (1979) also showed that metabolic rates and intracellular energy stores were reduced in severe hypoxia and under glucose depletion. Anaerobic lactate production was 2.7 times the anaerobic rate. Ebeigbe et al (1980) showed that glycolysis increased in the rat portal vein (RPV) during hypoxia.

Lovengren & Hellstrand, (1985) compared the hypoxic relaxation of spontaneous activity in rat portal vein to oxygen and to respiratory inhibitors such as amobarbital and cyanide. With the inhibitors, O_2 consumption (JO_2) is uniformly decreased throughout the cell mass and thus O_2 gradients in the tissue are avoided. Hence the effects are not to be attributed to all or none inhibition in anoxic regions, a possibility that might complicate the interpretation of responses to hypoxia. With stepwise reduction in Po_2 (96%-0%) or increasing concentration of inhibitor (0-5 mmol), JO_2 decreased with a concomitant reduction in mean contractile activity (P) and increase in lactate production (J_{LA}). The calculated ATP production (J_{ATP}) was linearly related to P for P 10% of the control value in 96% O_2, with the same slope for hypoxia and both inhibitors. In this range, the reduced J_{ATP} with P can largely be attributed to decreased metabolic demand of contraction, as evident from a comparison with the responses to hypoxia of portal veins relaxed in nominally calcium free medium. With reduced Po_2 or increased amobarbital

concentration the tissue content of phosphocreatine decreased, whereas ATP remained constant for P 10% of control. Similar responses to hypoxia and respiratory inhibition demonstrate graded effects on metabolism and contractility in the vascular smooth muscle cells, correlating with reported vasodilating effects of these interventions in vivo.

II THE ROLE OF CALCIUM IN VASCULAR CONTRACTILITY

A. Introduction

The importance of calcium in vital cellular processes such as contractile, secretory and neuronal activities has long been recognised. In most of these cases calcium can be considered as a final intracellular messenger. As a free cation, or more often after formation of a complex with some macromolecule such as calmodulin, calcium is an activator of several key enzymes of the cell. In addition, calcium is an essential constituent of bone matrix.

In the mid 1960's Fleckenstein and Godfraind discovered that the function of calcium in excitation-contraction coupling could be altered by pharmacological agents that are without effect on the concentration of calcium in extracellular fluids and calcified tissues. The first drugs shown to interfere with calcium function (e.g. cinnarizine and verapamil) were already used in the

1960's for the treatment of angina pectoris, allergic reactions and vertigo. Since then several chemical families have been added to the first generation of compounds and they have been characterised as calcium antagonists. See table 1.1

A calcium antagonist compound does not necessarily compete with calcium for a binding site and may be defined as a drug that alters the cellular function of calcium (i) by inhibiting its entry and/ or its release and/ or (ii) by interfering with one of its intracellular actions (Godfraind et al, 1986). Subgroups of calcium antagonists can therefore be defined. Those that specifically inhibit calcium entry into cells due to tissue excitation by various stimuli have been called calcium entry blockers (Godfraind, 1981). This antagonistic activity is most likely due to interaction with calcium channels activated by membrane depolarisation or by receptor stimulation, and, in these circumstances, these agents may also be termed calcium channel blockers or inhibitors. When blockade occurs at the level of the "slow" channels in cardiac tissues, the term slow channel blockers has been used (verapamil, nifedipine, diltiazem and some of its derivatives). The term calcium overload inhibitors has been utilized in some classifications (Godfraind et al, 1986) but has not gained wide currency. Agents that interact specifically with calmodulin are properly labelled calmodulin antagonists. The designation "calcium agonists" has

been introduced recently to characterise dihydropyridine derivatives that increase the probability of calcium channel opening instead of blocking them (Schramm et al, 1983). The whole group of agents affecting calcium movements has received the general denomination of calcium modulators.

Since there is no suitable tool for studying the calcium channel analogous to tetrodotoxin (TTX) for the Na channel, calcium modulators are now being used to characterise the calcium channels, rather than well defined calcium channels being used to characterise the drugs.

B. Calcium homeostasis in smooth muscle

Calcium has been known to be important for contractile activity of smooth muscle since the time when it was realised that it was an essential constituent of artificial physiological salt solution in which smooth muscle and other excitable tissues would continue to function for many hours after removal from the body (Ringer, 1896).

The contractile activity of smooth muscle is regulated by the free calcium concentration in the cytosol. In the resting state, the calcium concentration is held very low at around 100 nM, and the ratio of extracellular to intracellular calcium exceeds 10,000. The total calcium

inside the cell is much higher than 100 nM, and the bulk of this calcium is either bound to proteins, membranes or other cellular constituents or most importantly sequestered inside intracellular organelles such as mitochondria, endoplasmic reticulum (er), golgi apparatus and nuclei (see review Irvine, 1986).

Upon stimulation, the cytosolic calcium concentration rises partly as a result of an influx of extracellular calcium, and partly following the mobilisation of intracellular calcium stores, to reach 10μM. It has been proposed that this mobilisation in vascular smooth muscle is mediated by a calcium-induced calcium release (van Breemen, 1969; 1977; Saida & van Breemen, 1984; Cauvin & Malik, 1984; Leijten et al 1984, 1985).

Alternatively, agonist-stimulated calcium release from the sarcoplasmic reticulum may be triggered by inositol 4,5-biphosphate (see section C.2 for more detail) (Berridge, 1984; Somlyo et al, 1985). To restore the low resting cytosolic calcium and to maintain calcium homeostasis, the cell uses calcium transport systems that are able to operate against large electrochemical gradients. Such calcium transport systems have been demonstrated in the plasma membrane and in intracellular organelles, mainly the endoplasmic (sarcoplasmic) reticulum. There are two main mechanisms allowing "uphill" calcium transport. The first relies on the direct utilisation of energy from ATP (calcium transport

ATPase), and the second uses the sodium electrochemical potential to drive the extrusion of calcium in exchange for sodium (sodium-calcium exchange). ATP-dependent calcium pumps with different properties are present in the plasma membrane and the endoplasmic reticulum. Their identification in smooth muscle has relied mainly on cell fractionation techniques, including the digitonin shift method (Godfraind et al, 1976; Wuytack et al, 1978; Wibo et al, 1980, 1981; Morel et al, 1981; Daniel et al, 1982).

The plasma membrane calcium pump is activated by calmodulin, which in rat aorta acts mainly by enhancing the affinity of the pump for calcium (Morel et al, 1981). The nucleotides cyclic AMP and cyclic GMP may also regulate calcium availability in smooth muscle (Lincoln, 1983; Pfitzer, et al 1984) perhaps by regulation of calcium pumps. Cyclic AMP-dependent phosphorylation seems to enhance calcium accumulation into the endoplasmic reticulum, as indicated by studies on skinned arterial smooth muscle (Saida & van Breemen, 1983). The role of cyclic GMP in relaxation of vascular smooth muscle appears to be well established in the action of nitro-compounds and in endothelium-dependent relaxation by acetylcholine (Holzmann, 1982; Rapoport & Murad, 1983).

Stimulation of the plasma membrane calcium pump following cyclic GMP-dependent phosphorylation may contribute to vascular relaxation (Suematsu et al,

1984).

Participation of plasmalemmmal sodium-calcium exchange in the physiological control of calcium in smooth muscle is controversial (van Breemen et al, 1979; Brading, 1979; Hirata et al, 1981). Nevertheless, such a system has been clearly demonstrated in various smooth muscle tissues (Grover et al 1981; Morel & Godfraind, 1982, 1984). However, in smooth muscle, the calcium transport capacity of the sodium-calcium exchange system and its calcium affinity seem to be distinctly lower than those of the plasmalemma calcium transport ATPase (Morel & Godfraind, 1982, 1984), in contrast to the heart sarcolemma where the transport capacity of the exchanger is much higher than that of the calcium pump (see review by Godfraind et al 1986). The role of mitochondria in the regulation of calcium in smooth muscle is currently believed to be limited to slow processes associated with pathological anoxia or ischemia (Albertini & Ferrari, 1982; Godfraind-de Becker & Godfraind, 1980)

The foregoing section dealt with general mechanisms by which calcium homeostasis is maintained. However, the relative importance of such processes varies between vessels, owing to the considerable heterogeneity in vascular muscle (Ratz and Flaim, 1982).

Sources of activator calcium are extracellular Ca^{2+} and intracellular Ca^{2+}. The extracelluar Ca^{2+} arrives via transmembrane influx of Ca^{2+} through voltage-operated

channel, receptor-operated channels and passive 'leak' channels (Flaim, 1982; van Bremeen et al, 1982d). The extracellular activator Ca^{2+} will be discussed in greater detail in the section below.

C. Transmembrane calcium influx in vascular smooth muscle.

Evans et al (1958) found that acetylcholine could contract completely depolarised smooth muscle which was incapable of action potential generation. The contraction of depolarised muscle produced by acetylcholine was shown to involve inter alia an increase in the permeability to calcium. This experiment was the first indication of a "receptor-operated channel"- (ROC) through which calcium can enter the cell upon receptor activation. Subsequently, Somlyo and Somylo (1968) proposed that excitation-contraction coupling mechanisms in vascular muscle could be divided into 2 main types (1) electro-mechanical coupling process which operated in response to membrane potential changes and (2) pharmaco-mechanical processes, which were independent of membrane polarity.

The concept of pharmaco- and electro-mechanical coupling was extended by Bolton (1979) and Van Breemen et al (1979) who independently proposed the existence of distinct calcium channels in vascular smooth muscle which were activated by agonist-receptor binding

(receptor-operated channels, i.e. ROC) or membrane depolarisation (voltage operated channels i.e. VOC). Subsequently various workers demonstrated, principally in the rabbit aorta, that distinct receptor and voltage operated channels might be present in this tissue. Evidence for this is as follows:

(i) NA (10 nM-10 μM) does not produce a change in the membrane potential of the rabbit aorta (Cauvin et al, 1984b) yet the resultant tonic contraction appears to be dependent upon extracellular calcium.

(ii) Meisheri et al (1981) demonstrated that the undirectional ^{45}Ca influxes in response to potassium (80 mM) and NA (1 μM) are additive.

(iii) In the same study, D600, diltiazem and nisoldipine abolished the ^{45}Ca influx produced by potassium yet the same concentration of each agent only produced a small (10-20%) reduction of the corresponding influx produced by NA.

(iv) More recent evidence for a separate existence of ROC and VOC comes from a study on the isolated rat mesenteric artery by (Mulvany et al, 1982). NA (1-10 μM) contracts this vessel. At lower concentration of the agonist (1-2.2 μM) this contraction (of up to 50% of the maximal response) was associated with a

depolarisation of the smooth muscle cell resting potential from about -59 mV to about -34 mV. Higher concentration of NA did not produce further depolarisation. On the other hand an increase in potassium concentrations from 20-125mM in the bathing solution produced a linear increase in the degree of membrane depolarisation corresponding to 50 mV/decade. Tension increased over the range 30 to 85 mM potassium (Mulvany et al, 1982). In the potential range over which NA produced depolarisation, potassium produced only up to 38% of the maximal NA response, and the authors concluded that the mechanical response to NA may not generally be dependent upon a depolarising effect.

1. Voltage operated channels (VOC)

Many workers have demonstrated that KCl-induced contractions in a number of vascular preparations are totally dependent upon extracellular calcium (see Weiss, 1977). As the extracellular concentration of KCl is raised, depolarisation of vascular smooth muscle occurs and a contracture develops (Kreye 1981). Bolton (1979) and van Breemen and co-workers (1979) independently proposed that depolarisation of vascular membrane produces activation of voltage operated channels (Casteels, 1980). The evidence for the existence of such channels has been outlined in the previous section. VOC's are thought to resemble slow calcium channels in

cardiac muscle (Harder and Sperelakis, 1979; Fleckenstein-Grun and Fleckenstein, 1981) although the exact nature of the slow calcium channel in cardiac muscle has not been elucidated. Reuter (1979) has suggested an arrangement of membrane proteins with at least two regulatory gates. Triggle (1982) has outlined a schematic represention of model of a VOC. This model has been devised from patch clamp experiments in which current flow through individual channels can be recorded. Owing to the high calcium concentration of the extracellular fluid compared to that of the intracellular fluid, calcium will move passively across membranes and can be measured as a membrane current (ICa). The movement of calcium might occur through a proteinaceous channels in the lipid membrane. In the case of VOC's these protein structures are envisaged to open and close in response to membrane potential changes (Reuter, 1983). Voltage sensors in the channels (possibly a protein with a dipole) are modified by the change in electrical field, resulting in a minute "gating current" which may be measured (Reuter, 1983, Nayer & Dillon, 1986).

Cauvin et al (1983) showed that there was a difference of 1-2 log cycles in IC_{50} values for individual calcium entry blockers in different tissues. Kadza and co-workers (1983) compared the effects of a range of calcium entry blockers against contractions of the rabbit aorta to 42.7 mM KCl. They also found that the

IC_{50} for individual agents varied, usually over 3 log cycles. This indicates that the sensitivity of VOC's to individual calcium entry blockers might vary considerably in vascular smooth muscle.

Hurwitz and co-workers (1980b) have proposed that two distinct subgroups of VOC are present in guinea-pig ileum. Ratz and Flaim (1982) extended this hypothesis in order to explain heterogenity of vascular responses from different anatomical sites. These workers proposed 3 separate VOC's including a 'gate' for calcium (or sodium) generating spike potentials which opens and closes rapidly and two other VOC's which are activated by graded membrane depolarisation; one remaining active during membrane depolarisation and the other closing rapidly despite continued membrane depolarisation.

Hogestatt and Andersson (1984) have suggested that a heterogenous population of fast and slow voltage operated channels are present in rat cerebral arteries. The responses of the rat middle cerebral and basilar arteries to KCl 124 mM were biphasic and a fast and tonic component could be separated by cooling or by actions of calcium entry blockers. However both components were abolished by calcium withdrawal. The kinetics of each component led Hogestatt and Andersson (1984) to suggest that two populations of VOC's were present. A rapidly activated (and de-activated) calcium channel was suggested to gate calcium entry during the

fast component of the response to KCl. A corresponding slowly activated channel was proposed to admit calcium during the tonic component. These workers did not find any evidence that the fast calcium channel was controlled by spike depolarisation as the response to calcium in a previously depolarized medium was identical to that elicited by the addition of potassium. However, electrophysiological measurements were not made in this study.

In conclusion calcium entering into vascular smooth muscle in response to membrane depolarisation moves through channels which are operated by change in membrane potential.

2. Receptor operated channels (ROC)

Bolton (1979) and van Breemen and co-workers (1979) proposed that a calcium channel is activated in response to receptor occupation in vascular smooth muscle. The exact nature of ROC's or the coupling mechanisms between receptor and channel are however unknown. Putney (1978) has proposed a system in which membrane receptors and calcium channels co-exist in close association or possibly as a single functional unit. Other coupling processes between receptor and calcium channel have been proposed. One of these is the

phosphatidylinositol system (PI system). This concept of PI is widely gaining acceptance and shall be discussed in more detail below.

a. PI system

Stimulated incorporation of ^{32}P into phospholipids was first observed by Hokin and Hokin over 30 years ago, but it was only recently that the function(s) of this phenomenon has (ve) been clarified. The link between calcium and phosphoinositides was first suggested in a seminal review by Michell (1975) in which he pointed out the remarkable coincidence between those agonists which raised intracellular calcium and those which stimulated inositide turnover. This coincidence has continued to hold good, with the proviso that it is calcium mobilisation which is coincident.

The principal receptor-stimulated initial event in inositide turnover is generally agreed to be the phosphodiesteratic hydrolysis of phosphatidylinositol (4,5) biphosphate (PIP2) into inositol, 1,4,5-triphosphate (IP3) and diacylgylcerol. These latter two compounds are second messengers.

(i). Inositol, 1-4,5-triphosphate (IP3)

The idea that IP3 would be the other primary product of inositide turnover, because hydrolysis of PIP2 is the

A. Inhibitors: calcium antagonists

1. Agents acting at the plasma membrane
1.A. Calcium entry blockers
Group 1: selective calcium entry blockers
Subgroup I A: agents selective for slow calcium channels in myocardium (slow channel blockers)
Phenylalkylamines: verapamil, gallopamil (D 600†); under investigation: anipamil, desmethoxyverapamil (D 888), emopamil, falipamil (AQ-A-39), ronipamil
Dihydropyridines: nifedipine, nicardipine, niludipine, nimodipine, nisoldipine, nitrendipine, ryosidine; under investigation: amlodipine, azodipine, dazodipine (PY 108-068), felodipine, flordipine, FR 7534, FR 34235, iodipine, isrodipine, mesudipine, ni(*l*)vadipine, oxodipine, PN 200-110, riodipine
Benzothiazepines: diltiazem
Subgroup I B: agents with no perceived actions on the slow calcium inward current in myocardium (voltage clamp)
Diphenylpiperazines: cinnarizine and flunarizine
Group II: nonselective calcium entry blockers
Subgroup II A: agents acting at similar concentrations on calcium channels and fast sodium channels
Bencyclane, bepridil, caroverine, etafenone, fendiline, lidoflazine, perhexiline, prenylamine, proadifen (SKF 525A), terodiline, tiapamil
Subgroup II B: agents interacting with calcium channels while having another primary site of action
They include, among others: agents acting on sodium channels (local anesthetics, phenytoin); on catecholamine receptors (benextramine, nicergoline, phenoxybenzamine, phenothiazines, pimozide, propranolol, WB 4101, yohimbine derivatives); on benzodiazepine receptors (diazepam, flurazepam); on opiate receptors (loperamide, fluperamide); on cyclic nucleotide phosphodiesterases (amrinone, cromoglycate, papaverine); barbiturates; cyproheptadine; indomethacin; reserpine
1.B. Sodium-calcium exchange inhibitors
Amiloride and derivatives

2. Agents acting within the cell
2.A. Acting on sarcoplasmic reticulum
Dantrolene, TMB-8
2.B. Acting on mitochondria
Ruthenium red
2.C. Calmodulin antagonists
Phenothiazines: trifluoperazine, chlorpromazine
Naphtalene derivatives: W-7
Local anesthetics: dibucaine
Dopamine antagonists: pimozide, haloperidol
Calmidazolium (R-24571)

B. Facilitators
1. Agents acting at the plasma membrane
Calcium agonists
Dihydropyridines: Bay K 8644; CGP 28392; YC-170
2. Agents acting on sarcoplasmic reticulum
Inositol 1,4,5-trisphosphate
Caffeine
3. Ionophores
A 23187, ionomycin

Table 1.1. Agents affecting Ca^{2+} movements (calcium antagonists and calcium agonists). [Reproduced from Godfraind, T., Miller, R. & Wibo, M. (1986)].

primary event (as opposed to a secondary, calcium stimulated event (Abdel-Latif et al, 1977) was suggested by Michell and co-workers (1981), and the first clear evidence for this was supplied by Berridge (1983) working on blowfly salivary gland. The rapid formation of IP3, which was not secondary to the calcium mobilisation and which was indeed rapid enough to account for that mobilisation (Berridge et al, 1984), led Berridge to suggest that IP3 is the missing link between inositides and calcium, and between the receptor and the intracellular stores. Similar observations were made by other workers using GH3 cells (Rebbechi and Gershengorn, 1983; Martin, 1983, Drummond et al, 1984). It has been recognised that the alpha 1 and alpha 2-adrenoceptors systems are linked with IP3 and adenylcyclase respectively (Neylon and Summers, 1987). Experimental evidence has been produced to back up the link between alpha 1 adrenoceptor activation and the PI system in many tissues: brain (Brown et al, 1984; Minneman & Johnson 1984) many peripheral tissues including salivary glands (Michell, 1975; Berridge et al, 1982), thyroid (Uzumaki et al 1982), smooth muscle (Villalobos - Molina et al, 1982), adipocytes (Garcia-Sainz et al, 1980) liver (Harrington and Eichberg, 1983) and rat kidney (Neylon and Summers, 1987). Thus IP3 is considered to be a "second messenger" in the receptor activated events.

(ii) Diacylglycerol

1,2-diacylglycerol, which is the other product of hydrolysis of PIP2 is also a second messenger in the PI system and probably contributes to control of cell function through the activation of protein kinase C. Recently Spedding (1987) showed that protein kinase C may not be playing a direct modulating role on calcium channel function, but may exert effects indirectly (e.g. by modifying intracellular sensitivity to calcium, calcium extrusion or cellular depolarisation).

The PI system may be involved in the activation of other agonists (histamine, acetylcholine and 5HT, see Ratz and Flaim, 1982).

Cauvin and co-workers (1983) in a similar study to that on IC50 for calcium antagonists on KCl induced effects (see section II.C.1 -VOC) showed that IC50 values versus NA for calcium antagonists also varied between different tissues and calcium channel antagonists. This might indicate that ROCs vary throughout the vasculature. Van Breemen's group suggested that ROCs in vascular smooth muscle vary widely depending upon the concentration of NA used to stimulate preparations and upon the vessel studied (Van Breemen et al, 1981; Van Breemen et al, 1982a). Responses to NA of vessels which utilized intracellular calcium (e.g. rabbit) become more

resistant to calcium entry blockade as increasing concentrations of NA are used to stimulate the tissue. Such resistance is not conferred by increasing utilization of intracellular calcium, as $^{45}Ca^{2+}$ influx in response to increasing concentrations of NA also becomes more resistant to calcium entry blockade in the rabbit (see Cauvin et al, 1984b). This relationship is not observed in vessels which do not utilize intracellular calcium (e.g. rabbit mesenteric resistance vessels, see Cauvin et al, 1984b). Thus it would appear that the release of intracellular calcium might control the sensitivity of calcium influx through ROCs in vascular smooth muscle. This release of intracellular calcium might also affect the VOCs in a similar manner, though to a lesser extent (Cauvin et al, 1984b).

The preceding review details the events which might occur in vascular smooth muscle which exhibit electro- or pharmaco-mechanical coupling. Responses in such tissues may be mediated by an influx of extracellular calcium through voltage or receptor operated channels in response to KCl or NA respectively. However, owing to the heterogeneity in vascular muscle, these ideal cases might not be expected. NA might depolarize vascular muscle producing a secondary activation of voltage operated channels or KCl might release endogenous NA (Casteels, 1980) resulting in secondary activation of ROCs.

D. Mode of action of Ca^{2+} antagonists

1. Specific binding sites (or receptor sites)

Since [3H]nitrendipine was synthesized a few years ago (Bellemann et al, 1981) a new research area, dealing with specific binding sites, has developed in the field of calcium antagonism. Using classical saturation and displacement binding techniques the binding sites for the various classes of calcium antagonists, namely dihydropyridines, phenylalkylamines, diltiazem and flunarizine have been identified (Nayler and Dillon, 1986; Godfraind et al, 1986). These binding sites are chemically distinct and stereospecific.

Some of these binding sites are highly selective for the dihydropyridine calcium antagonists (Triggle and Janis, 1984), whilst others preferentially bind the diphenylalkylamines including verapamil and D600 (Hulthen et al, 1982, Reynolds et al, 1983). Within each group, however, low and high affinity binding sites have been identified and characterised. In cardiac myocytes these binding sites are predominantly concentrated in the cell membranes, a factor which may explain why the primary action of these drugs is at the cell surface. A similar situation exists in vascular smooth muscle cells (Janis et al, 1982). However, receptor binding sites for calcium antagonists are not limited to the cardiovascular system. For example, they

occur in the brain (Janis et al, 1984) and are present in large numbers in skeletal muscle cells (Borsotto et al, 1984). It follows, therefore that the mere presence of binding sites or 'receptors' for these compounds only, does not dictate a tissue's sensitivity because skeletal muscle, for example , is relatively insensitive to the calcium antagonists despite the presence of more low than high affinity sites (Nayler & Dillon, 1986). This lack of sensitivity of skeletal muscle can be most easily explained by the fact that unlike the cardiac and smooth muscles, the skeletal muscle derives calcium for excitation contraction coupling from its own intracellular stores.

However correlations between binding and pharmacological activity have been observed in many tissues; porcine coronary artery (De Pover et al, 1982); rat aorta (Godfraind et al, 1985); rat myometrium (Batra, 1985; Grover & Oakes, 1985); cat or dog mesenteric arteries (Triggle et al, 1982) and ileal smooth muscle (Bolger et al, 1983; Godfraind & Wibo, 1985).

For more details on calcium antagonists and binding studies see reviews by Godfraind et al (1986) and Glossmann (1987).

2 Interactions with voltage dependent calcium channels

a. Structure-activity relationships and stereoselectivity.

Several reviews have dealt with the structure activity relationship of the calcium antagonists (Rosenberger & Triggle, 1978, Janis & Triggle, 1983; Mannhold et al, 1982; Rodenkirchen et al, 1982).

The essential structural feature for nifedipine is the bulky substituent in the 4' position of the heterocycle, while the benzene rings in the verapamil structure are essential for activity. Small structural modifications of the nifedipine molecule give rise to compounds that can be described as calcium agonists (Bay K 8644, CGP 28392). The Bay K 8644 molecule is formed by substitution of a CF_3 group for a NO_2 group in the aromatic ring of nifedipine (see Fig 1.1) and of a NO_2 group at position 3 of the dihydropyridine ring instead of the ester group of nifedipine. Only the latter modification seems to be essential for the calcium agonistic property. See section II.E for more on Ca^{2+} agonists.

b. Use-dependent and voltage-dependent inhibition.

Electrophysiological studies show that calcium channel blockade by organic agents obeys laws similar to these of fast sodium channel blockade (Bayer et al, 1975; Bayer & Ehira 1978; Ehara & Kaufmann, 1978; Kohlhardt & Mnich, 1978) . These workers showed that the inhibitory effect of verapamil on the slow inward current depends on the rate at which cardiac preparations are stimulated (i.e. use-dependent block). Thus the degree of blockade by verapamil, D600 and diltiazem in voltage clamp experiments depends on the frequency of stimulation and also on the resting membrane potential, whereas these factors have been considered as less important in the action of nifedipine and other dihydropyridines (Trautwein et al, 1981; Bayer et al, 1982; Pelzer et al, 1983; Lee & Tsien, 1983; Tung & Morad, 1983). However more recent studies, using voltage clamp techniques, have shown that blockade of calcium channel current by dihydropyridine derivatives is modulated by membrane potential (Bean, 1984; Sanguinetti & Kass, 1984). Sanguinetti and Kass, (1984), proposed that the modulated receptor hypothesis that explains block of sodium channels by local anaesthetics in nerve and skeletal muscle (Hille, 1977) could be applied to calcium channel blockade by calcium antagonists. This hypothesis proposes that binding of a drug to a site located within the channel is influenced by the state of the channel and that this is determined by membrane

potential. This model predicts that ionized drugs can only gain access to the channel-associated binding site via a hydrophilic pathway that is available only when channels are in an open configuration. Neutral drugs can reach this site through this pathway and by a hydrophobic route through the lipid membrane surrounding the channel. Verapamil is almost entirely in a charged form at pH 7.4, whereas nifedipine, nisoldipine, and nitrendipine are neutral. Sanguinetti and Kass (1984), then proposed that, in a depolarised membrane, calcium channels may be in a resting (R), open (O) or inactivated (I) state. The sequence of channel states in the presence of verapamil (V) would be the following:

$$R \rightarrow O \rightarrow OV \rightarrow IV$$

For verapamil and related drugs, block is frequency dependent because repetitive depolarizing stimuli increase the probability for the channel to be in an open state. Neutral dihydropyridine ((D) may reach their site of interaction via two pathways:

$$R \rightarrow O \rightarrow OD \rightarrow ID$$

or

$$R \rightarrow I \rightarrow ID$$

Thus for these drugs, the frequency of stimulation is less important, but block is voltage dependent, because the second pathway of interaction is possible at

depolarised holding potentials, which increase the proportion of inactivated channels in the absence of repetitive stimulation. Sanguinetti and Kass (1984) found some use-dependent block with nicardipine, a partly charged dihydropyridine. In contrast to the Sanguinetti and Kass hypothesis Kanaya et al (1983) proposed that diltiazem and verapamil preferentially bind to inactivated cardiac calcium channels.

The voltage dependency of dihydropyridine action might explain why these drugs are more potent on vascular smooth muscle than on cardiac muscle. Indeed, in vascular smooth muscle preparations, resting membrane potential may be more positive than in cardiac cells. Moreover, the inhibitory potency of these drugs on potassium depolarised smooth muscle may be expected to be very high (Godfraind et al, 1986).

c. Subtypes of voltage dependent Ca^{2+}- channels

Recently, it has become apparent that there are distinct subtypes of potential-dependent calcium channels in neuronal cells (Carbone & Lux, 1984a, b; Bossu et al, 1985). Tsien and co-workers have reported distinct types of calcium channel in cultured sensory neurones of the chick dorsal root ganglion (Nowycky et al, 1985) and in mammalian cardiac cells (Nilius et al, 1985). In cardiac cells, they have found two types of unitary conductance with different kinetic features, which can

be attributed to the opening of 2 distinct calcium channels. The most commonly observed in many tissues, has been called the L type. Repeated opening of these procedures produces long-lasting inward calcium current through the membrane. A second type of channel, termed the T type opens at more negative membrane potentials than the L type and produces a transient inward membrane current. In chick dorsal root ganglion cells, a third type of conductance (N type) has been detected by Nowycky et al, (1985). N type channels require strongly negative potentials for complete removal of inactivation (unlike L) and strong depolarisation for activation (unlike T) and they inactivate rapidly.

In addition to the different potential ranges at which they are gated, the channel subtypes can be distinguished by different sensitivities to pharmacological agents. In cardiac and neuronal cells only L type channels are influenced by dihydropyridines, including Bay K 8644 (Nicola Siri et al, 1980; Nowycky et al, 1985a, b). In cardiac cells, nimodipine (5 uM) halved the average current through the L type channels but the same concentration failed to reduce T channel activity. It is likely that the sensitivity of a given tissue to dihydropyridines will ultimately depend upon the respective proportion of various calcium channel subtypes.

(Hidaka et al 1978, 1979 & 1980) were the first compounds demonstrated to antagonise the activity of calmodulin (see review by Weiss et al, 1980). These compounds inhibit contractions in isolated rabbit aorta elicited by agonists and by depolarisation (Kanamori et al, 1981). Recently, however, these compounds have been shown to possess calcium antagonistic properties (Shibata et al, 1968; Hennessey & Kung, 1984 & 1985; Flaim et al, 1985).

These observations all tend to suggest that inhibition of calcium influx is associated with antagonism of calmodulin.

In addition, in acellular test systems an interaction of some calcium entry blockers with calmodulin, that resembles that of calmodulin antagonists has been demonstrated. Diphenylalkylamines and dihydropyridines bind to calmodulin and inhibit enzyme activation by calmodulin (Bostrom et al, 1981; Itoh et al 1984; Johnson & Fugman, 1983; Lugnier et al, 1984; Silver et al, 1985). The binding of dihydropyridine, felodipine, to calmodulin was allosterically modulated by D600 and diltiazem (Johnson et al 1983; Johnson, 1984) in a manner analogous to dihydropyridine binding to calcium channels (De Pover et al, 1982; Ferry & Glossmann, 1982). Furthermore, Luchowski et al, (1984) showed that the affinity of calmodulin antagonists for nitrendipine binding sites correlates well with their binding to

calmodulin.

Considering these coincidences of effects between calmodulin antagonists and calcium entry blockers, the hypothesis has been put forward that a regulatory protein in or associated with calcium channels might have similar binding properties to calmodulin (Johnson et al, 1982; Johnson, 1984; Luchowski et al, 1984). However evidence against involvement of calmodulin in calcium antagonist actions is that verapamil and diltiazem as well as most dihydropyridine derivatives, have a very low affinity for calmodulin and do not inhibit contraction in skinned smooth muscle fibres (Itoh et al, 1984; Kreye et al, 1983; Metzger et al, 1982; Spedding, 1983) and effectiveness of many dihydropyridines as inhibitors of purified phosphodiesterases is generally about 3 orders of magnitude less than their effectiveness at displacing [^{3}H]nitrendipine binding (van Inwegen et al, 1984). Generally higher concentrations of calcium antagonists are needed to antagonise calmodulin than to inhibit calcium channels in many vascular smooth muscle preparations. In rat and rabbit aorta, most calcium entry blockers do not affect markedly the efflux of ^{45}Ca, a process known to be dependent on calmodulin at concentrations of calcium entry blockers up to 3 uM and which abolish ^{45} Ca influx (Church & Zsoter, 1980; Godfraind & Dieu, 1981; Godfraind & Miller, 1984; van Breemen et al, 1980). Furthermore, calcium entry

blockers do not inhibit contractions mediated by the release of intracellular calcium stores, even though some of them such as bepridil and cinnarizine (Cramb & Dow, 1983; Godfraind & Morel, 1977; Mrais & Sperelakis, 1982; Pang & Sperelakis, 1983) appear to be concentrated in cells to an extent several fold (>10) that of the nominal extracellular concentration. It therefore seems that calcium entry blockers do not normally exert effects associated with calcium antagonism of calmodulin in whole cells, and it is questionable if calmodulin itself constitutes part of the calcium channel and a receptor for some calcium entry blockers (Godfraind et al, 1986).

E. Ca^{2+} agonists

1.a Dihydropyridines: synthetic Ca^{2+} agonists.

Recently, new nifedipine analogues have been reported that have vasoconstricting and positive ionotropic properties. Bay K 8644 (Schramm et al, 1983) and CGP 28392 (Erne et al, 1984). The chemical structures of these compounds are shown in Fig.1.1. While only relatively few reports have been published on CGP 28392, Bay K 8644 has been extensively characterised in a number of experimental models (Schramm et al, 1986). Bay K contracts partly depolarised smooth muscle in a concentration (3 nM - 0.3 nM) dependent manner. This contraction can be explained by an increase in calcium

influx (Schramm et al, 1983, 1985).

A dihydropyridine 'receptor' site has been proposed. Evidence for the existence of such sites has been obtained with the use of high specific activity tritiated calcium antagonist dihydropyridines such as nitrendipine and nimodipine in the 1980s (Bellemann et al, 1981; Glossmann et al, 1982; Bolger et al, 1982; Murphy, et al, 1983). This 'receptor' is now widely accepted as an integral subunit of the calcium channels (Lazdunski et al, 1986).

The chemical similarity between calcium agonist and antagonist dihydropyridines and their pharmacological profile suggest that these compounds also bind to the dihydropyridine receptor. This has been proven by several workers (Lazdunski et al, 1986).

Electrophysiological experiments show that Bay K 8644 enhances the calcium current through the voltage-dependent calcium channel (Thomas et al, 1985; Bechem et al, 1985). Whole-cell patch clamp experiments on isolated guinea pig atrial cell show that Bay K 8644 enhances calcium influx at negative membrane potentials, but has only minor effects on the slope of the calcium current voltage curve at positive membrane potentials near the reversal potential (Bechem & Pott, 1985; Bechem et al, 1985). This suggests that the number of functional, available calcium channels is not increased

by the drug. The shift of the calcium current voltage curve to the left at negative membrane potentials can be explained by a change in the open probability of the single calcium channel. It seems therefore that Bay K 8644 mainly increases the mean open time of the calcium channel (Hess et al, 1984; Kokubun & Reuter, 1984; Ochei et al, 1984), resulting in an increased open probability in the voltage ranges normally covered in such patch clamp experiments. Hess (1986) proposed a model to explain the opposite actions of different 1,4-dihydropyridines. He proposed three so called gating modes to explain these effects. The modes are characterised by their different single channel calcium current behaviour. Mode 0 is characterised by no calcium currents, mode 1 short openings and mode 2 by long lasting openings. Generally, dihydropyridines are proposed to bind preferentially to one mode, stabilising the channel in this mode. Hess interpreted, a calcium antagonist as a compound that stabilises the calcium channel in mode 0, whereas a calcium agonist does so with mode 2.

Although this is an interesting model that can explain many of the effects of dihydropyridines, it cannot easily explain (i) the concentration dependence of action: in heart preparations at high concentrations, Bay K 8644 shows negative inotropic effects (Thomas et al, 1984; Vaghy et al, 1984), which can also be demonstrated in calcium current measurements (Bechem &

Schramm, 1986). (ii) the effects of Bay K 8644 are voltage dependent. Normally it behaves like a calcium agonist, but it has calcium antagonist effects under partly depolarised conditions (Sanguinetti & Kass, 1984). Another possible explanation of the effects of calcium modulating dihydropyridineS was proposed by Bechem & Schramm (1986); all dihydropyridines shift the open probability curve of the single calcium channel in a concentration-dependent manner to more negative potentials, which is probably due to a prolongation of the mean open time. Increasing the probability of the channel being open at a given membrane potential enhances the probability that the calcium channel will inactivate. The main difference between calcium agonists and calcium antagonists lies in the efficacy of shifting the open probability curve to the left. The bigger this shift, the more channels inactivate and the greater is the calcium antagonist, and the greater is the calcium antagonist action of a compound. This would explain why calcium modulating dihydropyridines generally have a concentration range in which they show calcium agonist behaviour, and another one in which they act as calcium antagonists (for calcium antagonist dihydropyridines, positive ionotropic actions at very low concentrations have been reported (Himori et al, 1976). In addition, the voltage-dependence of the Bay K 8644 action can easily be explained by this hypothesis. If the cells are partly depolarised, then the Bay K 8644-induced shifts of the open probability curve to negative

membrane potentials causes an increase in single channel probability at the holding potential. Therefore inactivation of calcium channels at the holding potential is accelerated, resulting in a decrease of current during the action potential (Schramm et al, 1986).

1. b. Are there any structural reasons for the opposite actions of different dihydropyridines?

Do simple constitutional or conformational parameters exist that decide whether a given dihydropyridine molecule has calcium antagonist or calcium agonist activities? The fact that the action of dihydropyridines are voltage dependent makes it unlikely that such parameters exist (Schramm et al, 1986). Another argument against this, is that optical isomers of Bay K 8644 have opposite effects on the calcium channel (Franckowiak et al, 1985). The authors showed that (-)-Bay K contracts rabbit aortic rings in a concentration dependent manner, as has been shown for the racemic compound , whereas its antipode has no effect on partly depolarised rings. In contrast, this antipode relaxes (in a concentration dependent manner) vascular smooth muscle previously contracted by depolarisation caused by addition of potassium to the bathing solution. Similar effects of the enantiomers of another 1,4-dihydropyridine have recently been reported (Hof et al, 1985).

Therefore it seems that the opposite effects of calcium antagonist and calcium agonist dihydropyridines result from only quantitative differences of qualitatively similar effects on the calcium channel. This hypothesis is supported by the finding that two enantiomers of Bay K 8644 show opposite effects.

1.c. Any possible uses for Bay K ?

Since Bay K 8644 has been shown to possess positive inotropic effects in guinea pig atria (Schramm et al, 1983a,b; Thomas et al, 1985; Mir & Spedding, 1986); calf myocardial cells (Thomas et al, 1985); Thomas et al proposed that it could be useful as a cardiotonic agent. The most often used cardiotonic agents such as the cardioglycosides and sympathomimetics, like Bay K 8644 increase cardiac sarcolemmal Ca^{2+} transport. Several questions have been raised about the usefulness of these drugs in chronic therapy to increase cardiac output. The increase in intracellular Ca^{2+} produced by these drugs can aggravate the effects of ischaemia and cause arrythmias (Smith, 1975; Branwald et al, 1976; Hamer, 1979; Fleckstein, 1983). Most of the currently available cardiotonic agents are plagued by the difficulties associated with Ca^{2+} overload and, consequently have a low therapeutic index. Katz has suggested that this

problem is inherent to the positive inotropic agents and questions the usefulness of drugs of this type (Katz, 1979). Bay K 8644 is a particularly interesting drug because it increases Ica in a unique way that should allow the extra Ca^{2+} influx to be used very efficiently. Consequently, less Ca^{2+} entry should be needed to achieve a given inotropic effect. Bay K increases Ca^{2+} primarily during the first 50ms of systole, with smaller increases taking place during the action potential, and probably no increment in influx during diastole. Recent studies suggest that Ca^{2+} entry at the beginning of an action potential has a greater effect on tension development than entry at other times (Thomas et al, 1985).

Bay K does not increase Ica via cylic AMP (Thomas et al 1985). Agents that alter Ica cyclic AMP are prone to produce Ca^{2+} overload for three reasons (i) Ca^{2+} entry is not preferentially increased at the beginning of systole, so that Ca^{2+} release from the sarcoplasmic reticulum is not optimized. (ii). The sensitivity of myofibrils to Ca^{2+} is reduced, so that a greater increment in intracelullar Ca^{2+} must occur for a given inotropic effect (Ray & England, 1976; McClellan & Winegrad, 1978; Allen & Kurihara, 1980: Marban et al, 1980; though Fabiato & Fabiato 1975b did not observe this effect; (iii) the positive inotropic effect of increased cyclic AMP is always accompanied by an increased heart rate. Bay K 8644 has none of these

effects and thus offers a promising new way to increase cardiac output. Unfortunately, Bay K 8644 is not clinically useful because it increases coronary resistance, apparently by a direct effect on vascular smooth muscle (Schramm et al, 1983a, 1983b). Although it is uncertain whether the vasoconstrictor properties of this dihydropyridine can be eliminated, it is clear that the discovery of superior positive ionotropic agent is in principle, possible.

Bay K 8644 is the most specific positive inotropic agent for increasing Ca^{+} currents. As such, it is a useful pharmacological tool for defining the role of Ca^{2+} in physiological processes, complementing the use of the Ca^{2+} channel blockers.

2. Endogenous calcium activators

Are there any endogenous calcium activators?

Two endogenous calcium activators have been identified, namely palmitoyl carnitine and substance P (Mir & Spedding, 1986; Bigaud & Spedding, 1986; Spedding, 1987). Accumulation of endogenous cardiac amphiphiles, such as lysophosphoglycerides and long chain acylcarnitines (LCA), in ischemic myocardium was reported in the late 1970s (Idell-Wenger et al, 1978; Liedtke et al, 1978; Shug et al, 1978, Sober et al, 1978; Opie, 1979). These amphiphiles also induce

electrophysiological alterations in vitro resembling those seen in ischaemic myocardium in vivo (Corr et al, 1979, 1981, 1984; Arnsdorf and Sawicki, 1981; Clarkson & Eick, 1983). Their amphiphilic properties may facilitate their incorporation into sarcolemma with consequent perturbation of ion transport (Katz & Messino, 1981; Corr et al, 1984; Knabb et al, 1986) as well as channel gating (Inoue & Pappano, 1983).

a. Palmitoyl carnitine

Palmitoyl carnitine has been reported to increase calcium in the heart by a surface charge effect (Inoue & Pappano, 1983). Recently Spedding & Mir 1986, 1987) using K^+-depolarised taenia coli of the guinea-pig, showed that palmitoyl carnitine behaved like Bay K 8644 in several respects. Palmitoyl carnitine could facilitate calcium channels similarly to Bay K 8644, though to a lesser extent. Like Bay K , such facilitation does not impair the ability of the tissues to relax to EDTA. In addition the interaction of palmitoyl carnitine with different classes of Ca^{2+} antagonists i.e classes 1 (nifedipine), 11 (diltiazem and verapamil) and 111 (flunarizine and cinnarizine) according to Spedding's (1985a, b) classification of Ca^{2+} antagonists, is similar to that of Bay K 8644 (Spedding & Mir, 1987). These findings correlate well with ligand binding studies. Palmitoyl carnitine can reduce [^{3}H]nitrendipine binding to rat cortical membrane

preparations. It can also displace [^{3}H]diltiazem binding and this was temperature independent (Spedding & Mir, 1986, 1987).

These authors concluded that the endogenously occurring lipid metabolite, palmitoyl carnitine, can directly affect VOCs calcium channel. In addition, its detergent properties could confer a surface charge effect on the VOCs resulting in selective interactions with such VOCs, different from the dihydropyridine sites (Spedding & Mir, 1987). They suggested that palmitoyl carnitine could modulate calcium channel function in certain conditions such as ischaemia.

b. Substance P

Substance P has been found to increase binding of [^{3}H]nitrendipine and of the verapamil analogue [^{3}H]D888 in the rat hippocampus and also to increase potassium-stimulated calcium uptake in this tissue (Govoni, 1987). Perney (1987) demonstrated that substance P release from cultured dorsal root ganglion neurones was particularly sensitive to calcium antagonists and augmented by the calcium-channel activator Bay K 8644. These findings indicate a close coupling of substance P with calcium channels.

c. Physiological role for endogenous Ca^{2+} facilitators

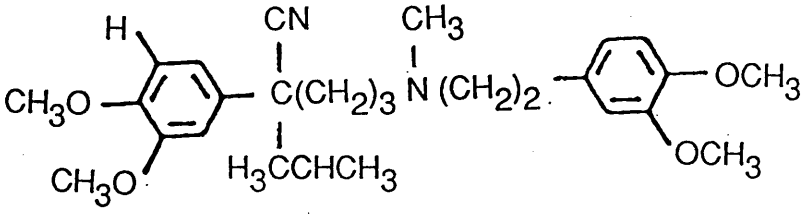
The physiological implication of endogenous calcium facilitators is that since, for example, palmitoyl carnitine accumulates in the sarcolemma and the cytosol during ischaemia, it could exacerbate calcium overload. Acyl carnitines could therefore be responsible for some of the massive Ca^{2+} overload which follows reperfusion of ischaemic tissues (Spedding & Mir, 1987). Already it has been shown that it has affinity for the [^{3}H] verapamil binding site (pIC_{50} 42 μM), which is within the range found during myocardial ischaemia (Spedding, 1987).

d. Pharmacological implication of endogenous Ca^{2+} facilitators

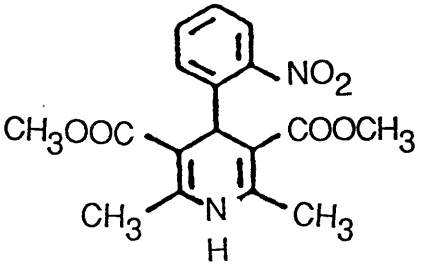
Since palmitoyl carnitine could antagonise classes 1 (nifedipine) and 11 (diltiazem and verapamil) antagonists but could not antagonise class 111 antagonists (flunarizine and cinnarizine) as shown by Spedding & Mir (1987), it then means that in ischaemia VOCs activated by acyl carnitines would be relatively resistant to compounds such as nifedipine, verapamil and diltiazem, but not flunarizine or cinnarizine (Spedding & Mir, 1987).

Ca^{2+} antagonists.

Verapamil

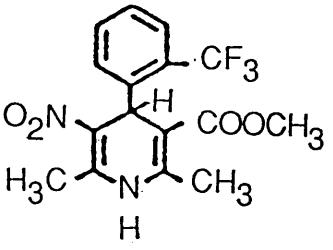


Nifedipine



Ca^{2+} agonists.

BAY K 8644



CGP 28392

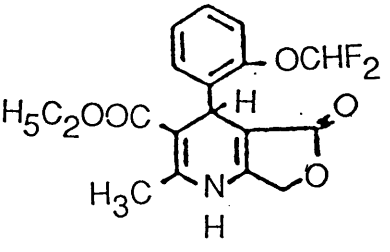


Fig 1.1. The chemical structures of the Ca^{2+} antagonists and Ca^{2+} agonists used in this study.

AIMS OF THIS STUDY

The aim of this study was to investigate the influence of oxygen and Ca^{2+} on the rat portal vein. This tissue has been shown to have properties similar to resistance vessels, which regulate local blood flow in response to local factors, i.e. O_2 & other metabolites (Hellstrand, 1979). In addition the spontaneous activity of the tissue is associated with the spontaneous action potentials, which open the voltage- operated channels (VOCs) (Funaki & Bohr, 1964; Godfraind et al, 1986).

Most pharmacological experiments are usually carried out at supraphysiological levels of Po_2 ($95\%O_2$) whereas the normoxic level is equivalent to only $16\%O_2$. Most *in vitro* experiments are carried out to simulate *in vivo* conditions, i.e. maintained at body temperature and extracellular ionic composition. Therefore should the Po_2 be kept at *in vivo* extracellular level i.e. would normoxia alter the pharmacological / physiological interpretation of drug effects?

It has been shown previously that there is a causal relationship between the influences of O_2 and Ca^{2+} (Ebeigbe et al, 1980). Therefore the first aim of the study was to find out the extent to which O_2 influences the excitation -contraction coupling mechanisms. Ca^{2+} modulators which alter Ca^{2+} sensitivity of the tissue

were employed to this end.

Following this a study was made of Ca^{2+} sensitivity and Ca^{2+} modulators under patho-physiologically simulated conditions associated with hypoxia/ ischaemia with the aim of establishing whether Ca^{2+} channel function is radically different under such conditions.

The ultimate aim was to understand any changes which occur between normal and hypoxic conditions in the excitation- contraction coupling process in vascular smooth muscle in order to be able to devise strategies for controlling blood flow in ischaemic conditions.

CHAPTER 2

EFFECT OF OXYGEN TENSION ON THE NON-STIMULATED AND DRUG-INDUCED RESPONSES OF THE RAT PORTAL VEIN.

SUMMARY

1. Full agonists NA, amidephrine and phenylephrine at low concentrations up to 30nM increased the contractile phasic activity of the RPV. Higher concentrations induced increased the baseline tension (tonic response).
2. At 95%O_2, full agonists produced monophasic responses particularly at high concentrations. At 16%O_2, these monophasic responses became biphasic. The biphasic response was characterised by a transient phasic contraction usually lasting 15s, followed by a slower tonic contraction. The tonic component at 16%O_2 was usually smaller than that at 95%O_2. A progressive lowering of O_2 tension from 32%O_2, made the tonic component progressively smaller, while the transient component was relatively resistant to reduction in O_2.
3. Partial agonists, UK14304, xylazine, and (+) m-synephrine increased the contractile phasic activity of the RPV without increasing the baseline tension. In general, the effects of most of these compounds were quite resistant to O_2 change from 95%O_2 to 16%O_2.
4. Clonidine produced a qualitatively similar response to those of UK14304, xylazine and (+) m-synephrine, but unlike these drugs clonidine's responses were sensitive

to O_2 changes.

5. pA_2 for prazosin against NA (phasic response) was 11.0, while that for prazosin against NA (tonic response) was 9.1 at 16%O_2. The latter pA_2 is comparable with published pA_2 data for prazosin against NA at 95%O_2. pA_2 for prazosin against UK14304 at 16%O_2 was 7.9.

INTRODUCTION

Most in vitro studies of smooth muscle have been carried out using hyperoxic and supraphysiological level of oxygen caused by bubbling with, for example i.e 95%O_2 in 5%CO_2. The reasons advanced for this are that better responses are obtained at hyperoxic O_2 (Smith & Vane, 1966) and the development of an anoxic core is prevented by the use of such O_2 tension (Paul & Duling, 1973-see Introduction). Given that tissues in vivo are exposed to a range of O_2 tensions equivalent to those generated by bubbling with 16%O_2 (arterial) to about 2%O_2 (low end of venous range), clearly, the use of 95%O_2 could lead to oxygen toxicity which might be deleterious to the tissues. Use of supraphysiological Po_2 could cause some extra activator effects or mask normal responses which would clearly not be a true reflection of an in vivo situation. A typical example is the recent study done by McGrath & McLennan (1986), using the human umbilical artery. They showed that O_2 tension could modulate 5-HT

induced responses. At the physiological P_{O_2} level for that tissue, two components to 5-HT-induced responses were identified, but only one was observed at the supra physiological O_2 level.

A series of studies were carried out to find what differences in response were made in isolated RPV by gassing with different O_2 tensions.

METHODS

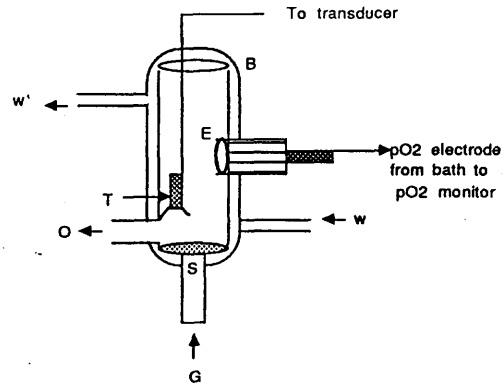
Rat portal vein

Male rats of weights between 245 and 255g were stunned and killed by cervical dislocation. Portal veins were dissected out and cleared of connective tissue under a binocular microscope. The vessel was cut open longitudinally and the blood in the lumen was removed.

Recording of the mechanical activity.

Isometric contractile activity of the longitudinal muscle layer was monitored from 10mm long strips which were mounted in 50ml organ baths. Contraction was recorded via Grass FT 03 force transducers on a Linseis recorder, model Typ LS. The veins were allowed to equilibrate in standard Krebs solution under a passive tension of 1g for 1.5hr. The composition of the saline was as follows (mM): NaCl, 119; KCl, 4.7;

a



B = 30ml tissue bath

W,W'= Water (37oC), Inlet,Outlet.

E = pO2 electrode

T = Tissue

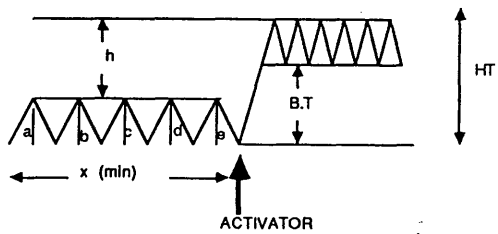
O = Outlet for physiological saline

S = Sinthered glass

G = Gas, O2 in 5%CO2.

Schematic representation of the tissue bath used in the experiments.

b



Mean spontaneous activity = a + b+ c+ d.....+n / n (g)

Mean frequency of spontaneous activity = No of phasic contractions/ x (spikes/min)

B.T = Increase in baseline tension (g)

h = change in tension (g)

HT = absolute height of contraction (g).

Schematic representation of the parameters measured.

Fig 2.1

$NaHCO_3$, 24.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; Glucose, 11.5.

Different additives were included in the Krebs in a series of initial experiments designed to determine the best combination for NA studies. These additives were propranolol 1μM, cocaine 1μM and EDTA 23μM.

All experiments were carried out at 37°C.

In the early experiments the tissues were equilibrated with Krebs solution gassed with a mixture of either 95%O_2 & 5%CO_2, but in later experiments they were equilibrated with 16%O_2, 79%N_2 & 5%CO_2; each giving a pH of 7.37- 7.40. The gas was bubbled through sintered glass to achieve a rapid equilibration (Ebeigbe et al 1979).

The oxygen tension in the bath was recorded by an oxygen electrode attached to an O_2 monitor (Strathkelvin Instruments). The bath Po_2 tensions at 95%, 64%, 32%, 16% 8% and 4% were 590 ± 12, 380± 10, 228± 9, 110 ± 10, 76±8, and 30 ± 2mm Hg respectively. See appendix for measurement of bath Po_2.

Non- cumulative concentration response studies were made instead of cumulative concentration response in order to study the nature of the biphasic response to activators which occurred at 32%O_2 and below. The initial "phasic" component of such biphasic responses would not normally be observed in cumulative concentration response studies.

After equilibration the tissues were contracted with NA

10μM for 5min, with 10min intervals until reproducible responses were obtained.

Non-cumulative concentration response curves to agonists /activators were constructed by tripling drug concentrations with each addition. 5min was allowed for the drug response after which the drug was washed out and 10min was allowed for tissue recovery before adding the next concentration of the drug. In each preparation, two concentration response curves were produced; one at each of 95%O_2 or 16%O_2.

In the initial experiments, tissues were first gassed with 95%O_2 and later with 16%O_2. This order was reversed in the later experiments. A time dependent decrease in response was observed at low agonist concentration during the second concentration/response curve. Time control experiments (i.e. repeating at the initial gas tension) were carried out simultaneously with each series of experiment. No more than two concentration response curves (CRCs) were produced with each tissue. 30min was allowed between the first and the second CRCs.

Initially the drugs were washed out by the overflow method, in order not to expose the tissues to air, but this was discontinued as it became increasingly difficult to wash out the drugs at high concentrations, even after prolonged washing, which led to a large amount of Krebs being consumed. In order to overcome

this problem, the physiological saline was washed out by draining a little at a time, and replacing it with fresh Krebs, ensuring that the tissue was not exposed at any time.

Spontaneous activity (S.A) increased as the experiment progressed. Therefore the net change became smaller and the drug did not give a geometric increase in response as the concentration increased. On the other hand if a second CRC was to be constructed in the same tissue, the S.A significantly decreased during a rest period of 15-30min before constructing the 2nd CRC. Therefore the net change in this latter situation may be greater than the 1st CRC. In order to take care of the fluctuations in spontaneous activity during the course of the experiments, the steady spontaneous activity during each equilibration prior to the construction of each CRC was used to compute the change in tension in the presence of the drug.

The time course of the response, shows the changes induced by the drugs such as increased height of response, increased baseline tension as well as increased spontaneous activity.

The time course of responses to agonists were determined at the usual speed of the chart, usually, 20mm/min. Sometimes a faster speed of 50mm/min was used in order to pick up the fast event. This was particularly useful for discerning the biphasic contraction to NA as well as phenylephrine and amidephrine at low O_2 tension. Time

course of the response to the agonist was examined at a few concentrations, usually 2 or 3. Such responses were reproducible throughout the course of the experiment.

In experiments examining the effects of antagonists, after the construction of the concentration response curve to the agonist, a concentration of the antagonist was added to the baths for 30min before a further concentration response curve was constructed. Only one concentration of antagonist was examined in each preparation. pA_2 values for the antagonists were calculated according to Arunlakshana & Schild (1959). Plots were constructed of log (concentration ratio - 1) against log (antagonist concentration). Concentration-ratios were calculated at the EC_{50} level.

Perfusion experiments.

These experiments were carried out with the portal vein and the rat anococcygeus muscle. The protocol for setting up the latter preparation is as follows.

Rat anococcygeus muscle preparation.

Rats of similar weight as for portal vein were used. The tissue was dissected out according to the method described by Gillespie (1972).

The tissue was set up under 1g tension and after

equilibration isometric contractions were recorded using the same recording device as for the portal vein.

Protocol for the perfusion experiments

Infusion pump (medical SE 300) and a Watson Marlow perfusion pump were used. Both pumps were calibrated by varying the dial positions and determing the flow rate for the volume of solution collected per unit time.
The desired concentration of NA infused into the tissue bath was calculated from the following equation:

$$\text{Syringe concentration} = \text{Mean flow rate of perfusion pump} \times \frac{\text{Desired concentration}}{\text{Syringe flow rate}}$$

NA was made up in 0.01M HCl and placed in the 20ml syringe connected to an infusion pump. The needle of the syringe was connnected to the rubber tubing which in turn had been connected to the Watson Marlow pump which perfused the tissue with Krebs.
When the effect of infused NA was to be compared with the bolus adminstration of NA, both pumps were stopped and NA was injected directly into the baths. Bolus adminstration of NA was also examined in the presence of additives.
Sometimes a faster response to infused NA was achieved by increasing the rates of both pumps in the same proportion.

In the results section, perfusion refers to the rate at which Krebs was perfused while infusion refers to the rate at which NA was infused.

Results are mean±s.e.m

Results, were expressed either as a percentage of a maximum response at 16%O_2 or as absolute tension in gram.

Results have been expressed in two ways:

(1) the increase in the height of response.

(2) increase in baseline tension.

Fig 2.1 b. shows the parameters measured in these experiments.

Some agonists could increase these two parameters e.g NA, phenylephrine and amidephrine, while others such as clonidine, UK14304, xylazine, (+)m- synephrine only increase (1).

The 15s and 5min responses of (1) represented the phasic and tonic components of the agonist-induced responses, respectively.

The 5min measurements were used when comparing the CRCs of the various agonists.

EC_{50} values were determined relative to the maximum produced by each agonist at 16%O_2.

Drugs:

Noradrenaline bitartrate (Sigma), phenylephrine hydrochloride (Sigma), amidephrine hydrochloride

(Sigma), caffeine hydrochloride (Sigma), choline chloride (Sigma), (+)m-synephrine (donation from Prof. John Midgley, of University of Strathcylde), prazosin hydrochloride (Pfizer), xylazine hydrochloride (Bayer), UK14304 (Pfizer), ouabain octahydrate: strophantin-G (Sigma), Wyeth 26703 (Wyeth).
All drug solutions were made in distilled water and were added to the bath in volumes of 0.1 or 0.3ml.

RESULTS

1. Effect of O_2 tension on the non-stimulated spontaneous phasic activity of the portal vein.

Spontaneous activity (S.A) was not affected by the O_2 tension from 95% down to 8%O_2 which was the lowest O_2 tension used in this series of experiments. The mean levels of spontaneous activity recorded in different O_2 tensions 1.5hr after setting up were 0.34 ± 0.05, 0.34 ± 0.04, 0.33 ± 0.04, 0.30 ± 0.07 and 0.35 ± 0.04 (n=6) at 95%, 64%, 32%, 16% and 8%O_2 respectively.

However there was a time-dependent decrease in the non-stimulated spontaneous activity. Starting off at 95%O_2, the spontaneous activity of the tissue was monitored over 1hr. The spontaneous activity by 5min was 0.34 ± 0.05. This decreased to $0.30\pm.07$ and 0.27 ± 0.07 by 30min and 1hr respectively. When the gas was then switched to 16%, the S.A increased from 0.27 ± 0.07 to

0.30±0.09 (n=6) by 5min and subsequently decreased to 0.18±0.06g and 0.17±0.08 by 30min and 1hr respectively.

This therefore shows that the S.A of the non-stimulated RPV decreases with time in either 95% or 16% O_2.
The frequency of the spikes was 3 spikes per min and remained unaffected by O_2 tension in the range examined.

2. Non-cumulative concentration/ response curves for NA

The pattern of the response to NA (particularly with EDTA present) is described below.
At low NA concentrations (3nM - 30nM), there was an increase in the height of the contractile spikes without a concomitant increase in baseline tension. At 0.1μM, these concentration-related contractile spikes were accompanied by increased baseline tension within the first 2min of response. Above this the baseline tension increased further and lasted throughout the 5min observation. At 1uM, there was a tonic contracture with or without contractile spikes superimposed on it. Above this concentration the response was a smooth tonic contracture. Even though the maximum of the height of contraction is usually reached by 1uM NA, the maximum of the baseline tension is not reached until 10μM.

There was a qualititative as well as a quantitative

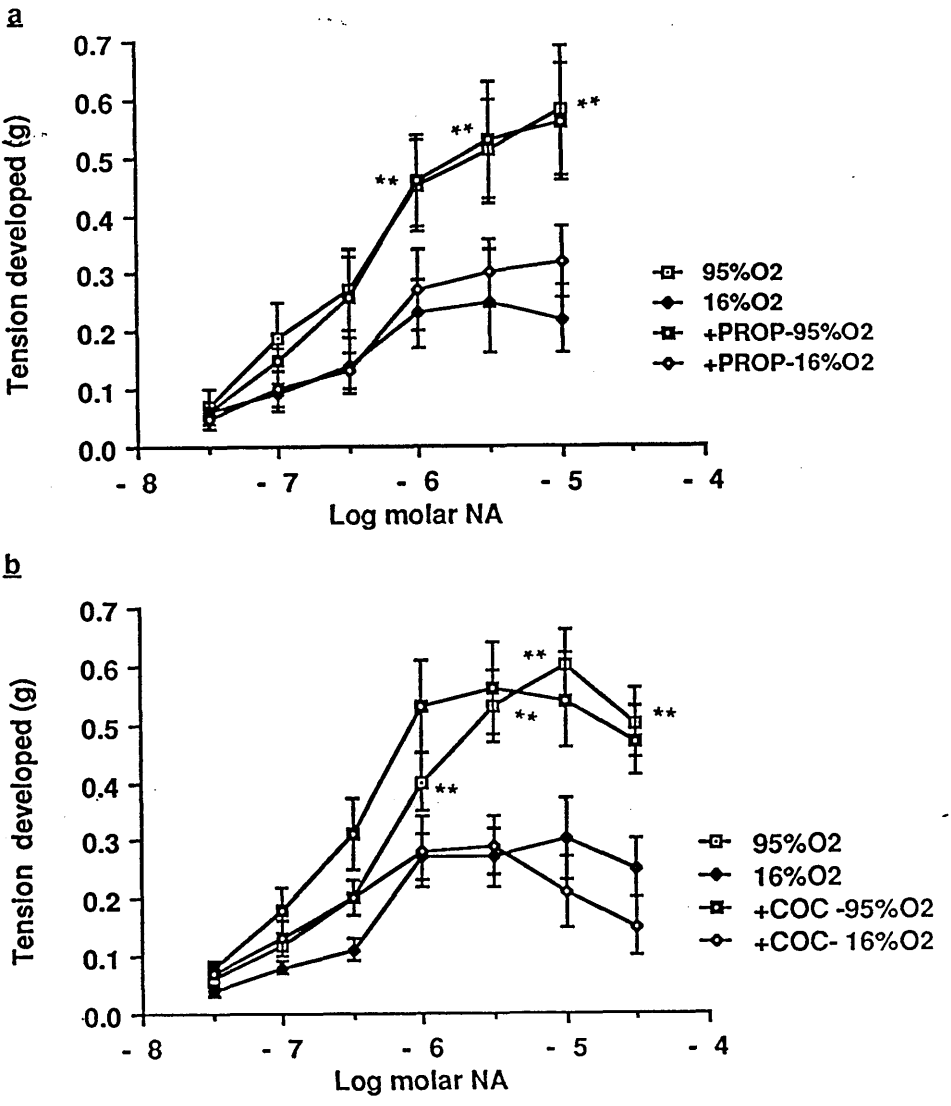


Fig 2.2

Concentration response curves for NA at 95%O_2 and at 16%O_2 in the rat portal vein. Effect of (a) propranolol (PROP) 1μM after 30min pretreament was determined on NA -induced response.

Similarly, in a seperate set of experiments effect of (b) cocaine (COC) 1μM was determined.

The fig is a plot of change in tension (spontaneous phasic activity or tonic) against log concentration of NA.

Points represent means ± s.e.m, n = 6.

**, represents $P < 0.01$.

difference between the response at 95% and that at 16%O_2. NA-induced responses were monophasic at the high O_2 tension, while they were biphasic from 0.3μM - 10μM at 16%. An initial rapid but short- lived "phasic" contraction reached a peak by 15s and then declined subsequently the slower and better maintained "tonic" contracture developed.

The concentration response curve to NA at 16%O_2 usually lay to the right of that at 95%O_2, with its maximum significantly depressed ($P < 0.01$), see fig 2.2. These responses correspond to the tonic contracture which in these experiments was more sensitive to changes in O_2 than was the phasic contracture.

a. The choice of additives for NA experiments.

The effects of different additives alone and in combination were observed on NA-induced responses.

(i). Propranolol 1μM

The threshold concentration for NA in the control was 30nM.

The mean control $-\log EC_{50}$ values in six experiments were 6.91 ± 0.14 and 6.46 ± 0.08 ($P < 0.05$) at 95% and at 16%O_2 respectively. In the presence of propranolol, these values were 6.87 ± 0.14 and 6.34 ± 0.12. Therefore the $-\log EC_{50}$ values of the control and the treated were not statistically significant ($P > 0.05$) from each other.

Comparing the responses at each concentration of NA, the responses in the presence of propranolol were statistically ($P < 0.05$) significant different only at NA 10uM, 16%O_2 where the mean was larger than in controls. See fig 2.2a.

(ii). Cocaine 1μM

The mean control $-\log EC_{50}$ values were less than those obtained in the propranolol experiments. The values were 6.67 ± 0.14 and 6.29 ± 0.03 ($P < 0.05$) at 95% and 16%O_2 respectively. In the presence of cocaine, the log concentration response curves were shifted to the left in each O_2 tension, consequently the $-\log EC_{50}$ values were increased to 6.94 ± 0.1 ($P > 0.05$) and 6.49 ± 0.3 ($P > 0.05$) at 95% and 16%O_2 respectively (Fig 2.2b). The P values in bracket show significance levels compared with the controls.

In both O_2 tensions the maxima were not enhanced by cocaine. See fig 2.2b.

(iii). EDTA 23uM

EDTA significantly increased responses to lower concentrations (30nM-0.3μM) of NA. The log concentration/response was shifted to the left of the control. The concentration response curve was less sigmoid. About 50% of the maximum response was attained at 30nM, in other word, the threshold response was

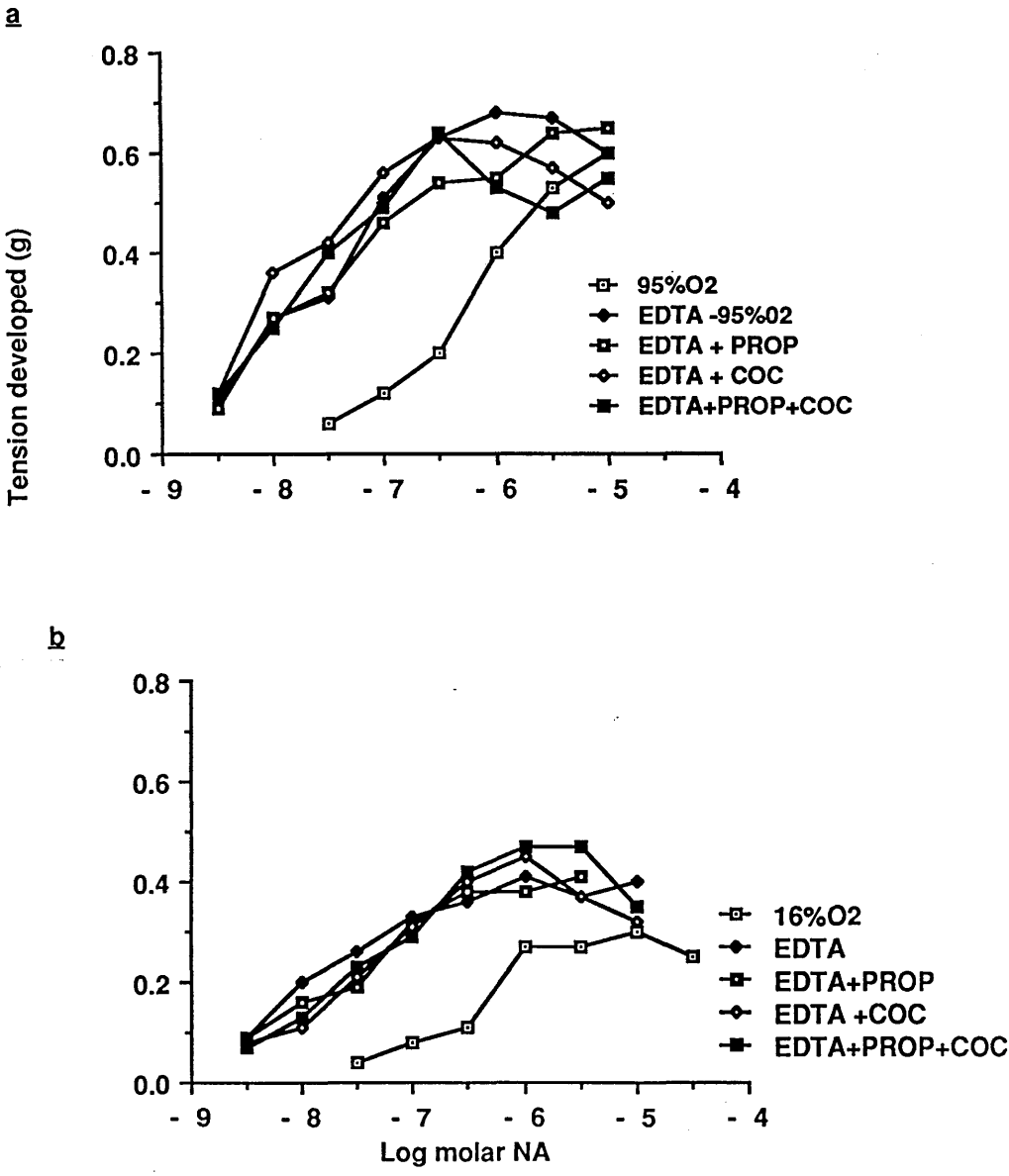


Fig 2.3

Concentration response curves for NA in the presence of various additives i.e. EDTA 23μM, cocaine (COC) 1μM and propranolol (PROP) 1μM at (a) 16%O_2 and (b) 95%O_2 .

In the presence of EDTA the CRCs to NA at 16%O_2 and at 95%O_2 were statistically significantly ($P < 0.01$) shifted to the left. A combination of EDTA with cocaine or propranolol or the three together was not statistically different from EDTA alone.

The fig is a plot of change in tension (spontaneous phasic activity or tonic) against log concentration of NA.

Points represent mean, n = 6. The vertical error bars have been omitted for clarity.

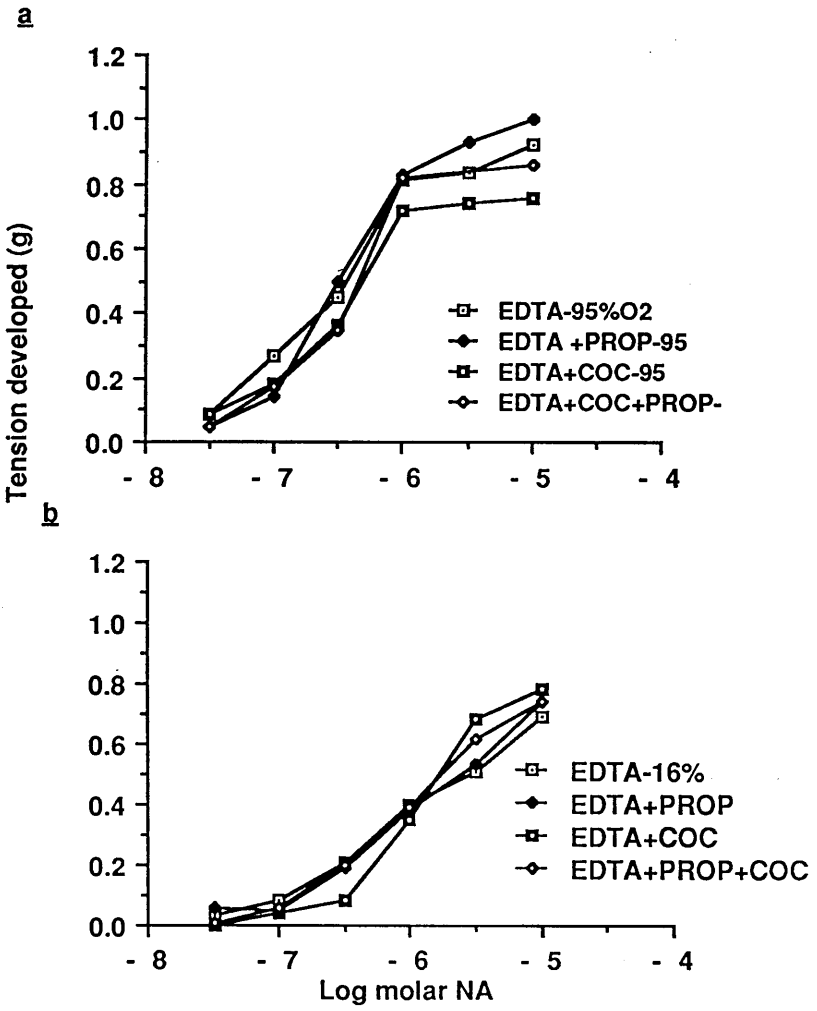


Fig 2.4

Concentration response curves for NA in the presence of various additives i.e. EDTA 23μM, cocaine (COC) 1μM and propranolol (PROP) 1μM at (a) 16%O_2 and (b) 95%O_2 .

Fig is a plot of change in baseline tension (note that this is different from response measured in figs 2 & 3) against log concentration of NA.

Points represent means, n = 6. The vertical error bars have been omitted for clarity.

Since the increase in sensitivity in the presence of EDTA was much greater than for either of the two other additives, an experiment was designed to see whether marked potentiation of NA-induced response by EDTA was due to its ability to prevent oxidation of NA by chelation of heavy metals that catalyse such oxidation in the bath or to some other effects by EDTA per se.

b. Perfusion experiments with either the RPV or rat anococcygeus.

(i). RPV

The object was to compare the effects of constant infusion of NA with those to bolus administration of NA either with EDTA alone or in combination with cocaine or propranolol.

NA 30nM or 0.1μM (in 0.01M HCl) was infused into the bath along with the perfusate for 5min and the tissue reponse was taken. The infusion pump was then switched off, leaving the perfusion on to wash off NA. 10min after infusion was stopped, a bolus of NA was added to the bath. The resulting responses to infused or bolus NA were compared in both 95% and 16% O_2. The protocol was repeated in the presence of various additives alone and in combination.

Responses to NA by infusion were higher than by bolus

Fig 2.5

NA -induced response without (i.e. bolus NA) or with infusion at (a) 95%O_2 and (b) 16%O_2 in the rat portal vein. The bolus administration of NA was carried out in the absence or presence of additives, EDTA, cocaine (COC) and propranolol (PROP) [same concentrations as above].

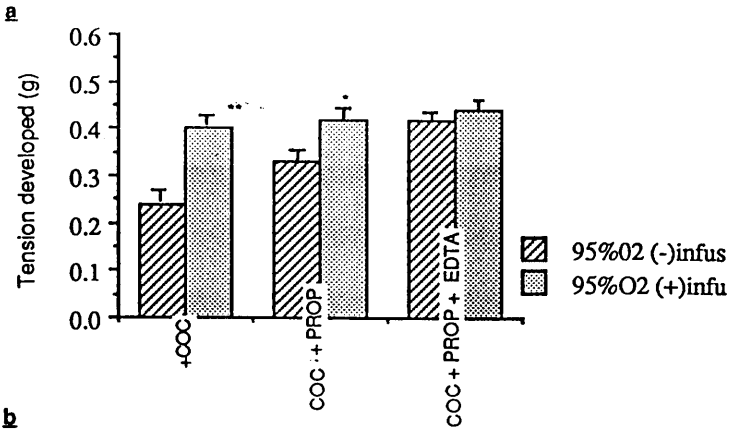
Responses obtained during infusion of NA matched those during bolus NA in the presence of EDTA.

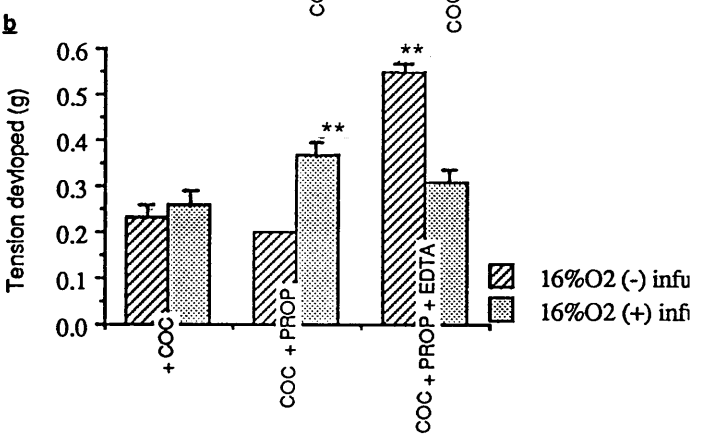
Points are means $\pm$ s.e.m, n = 4. *, ** represent $P < 0.05$ and < 0.01 respectively.

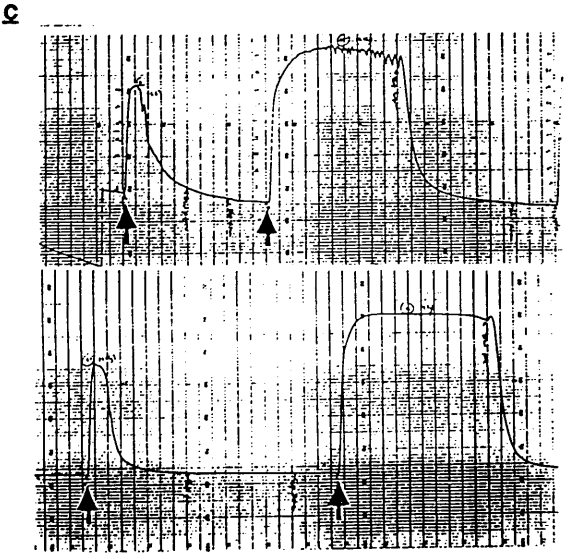
(c) shows results of a similar experiment carried out in the rat anococcygeus muscle.

The upper panel shows response to bolus NA and then to infused NA. The bolus NA produced a response that was not maintained. On the other hand, infused NA produced a response that was well maintained.

The lower panel shows response to bolus NA without and with EDTA. It was only in the presence of EDTA that a well maintained response was obtained.


a
Tension developed (g)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
*
+COC
COC + PROP
COC + PROP + EDTA
95%02 (-)infus
95%O2 (+)infu


b
Tension devloped (g)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
**
+ COC
COC + PROP
COC + PROP + EDTA
16%O2 (-) infu
16%O2 (+) infi


c

administration of NA (fig 2.5a & b). This is presumably because constant infusion gives a maintained NA concentration in the bath by 1.5min, while bolus administration of NA without additives undergoes oxidation. When cocaine or propranolol was added to the bath and NA responses were taken, ressponses to infusion were higher.

However when EDTA (23uM) was included along with the other additives, NA - induced response by infusion matched bolus administration of NA at 95%. At 16%, the response to bolus NA in presence of EDTA was higher than to infusion. These results suggest that the infusion of NA without EDTA mimicked bolus NA in presence of EDTA. Therefore the marked potentiation of NA effects by EDTA is due to prevention of NA oxidation in the bath. See figs 2.5a & 2.5b.

(ii). <u>Rat anococcygeus experiment.</u>

The above infusion experiment was repeated using the rat anococcygeus muscle. This preparation is very sensitive to NA (Gillespie, 1972). In the absence of EDTA it gives a contraction to NA that rapidly wanes.

The preparation was set up under a 1g tension. The resting tension gradually declined over a period of 2-3min to a resting tension of 0.3-0.6g, which thereafter remained constant.

Contraction to a submaximal concentration of NA 30nM was taken. This gave a contraction of 3.2±0.04g tension (n =

4). The contraction peaked within 30s and by 3min had completely returned to the baseline. Responses to NA were taken several times until reproducible.

Infusion of NA first at a low rate of perfusion and infusion, did not produce a well sustained response. However, by increasing the rate of infusion and perfusion to give a nominal bath concentration of 30nM NA a well sustained contraction to NA was obtained which declined by only 5% of the initial response. Once the infusion was stopped, the NA -induced contraction waned rapidly and returned to the baseline by 1.5min.

With inclusion of EDTA bolus administration of NA caused a contraction of similar magnitude to that by infusion (fig 2.5c). This experiment with anococcygeus allows the experiment to be conducted at even lower NA than in RPV. It shows that even constant infusion of NA is not sufficient to prevent the net loss of NA due to its rapid oxidation. Inclusion of cocaine plus EDTA in the perfusate took about 45min before any potentiation was seen. Subsequent contraction induced by bolus administration of NA caused a well sustained contraction which declined by only 2.5% by 5min.

This experiment further lends support to the observation that in the portal vein, the potentiation induced by EDTA was mainly due to its ability to prevent oxidation of NA and not to some other effects of EDTA.

Since a combination of EDTA with propranalol and cocaine did not increase the sensitivity of the RPV to NA more than EDTA alone did subsequent experiments were

Fig 2.6.

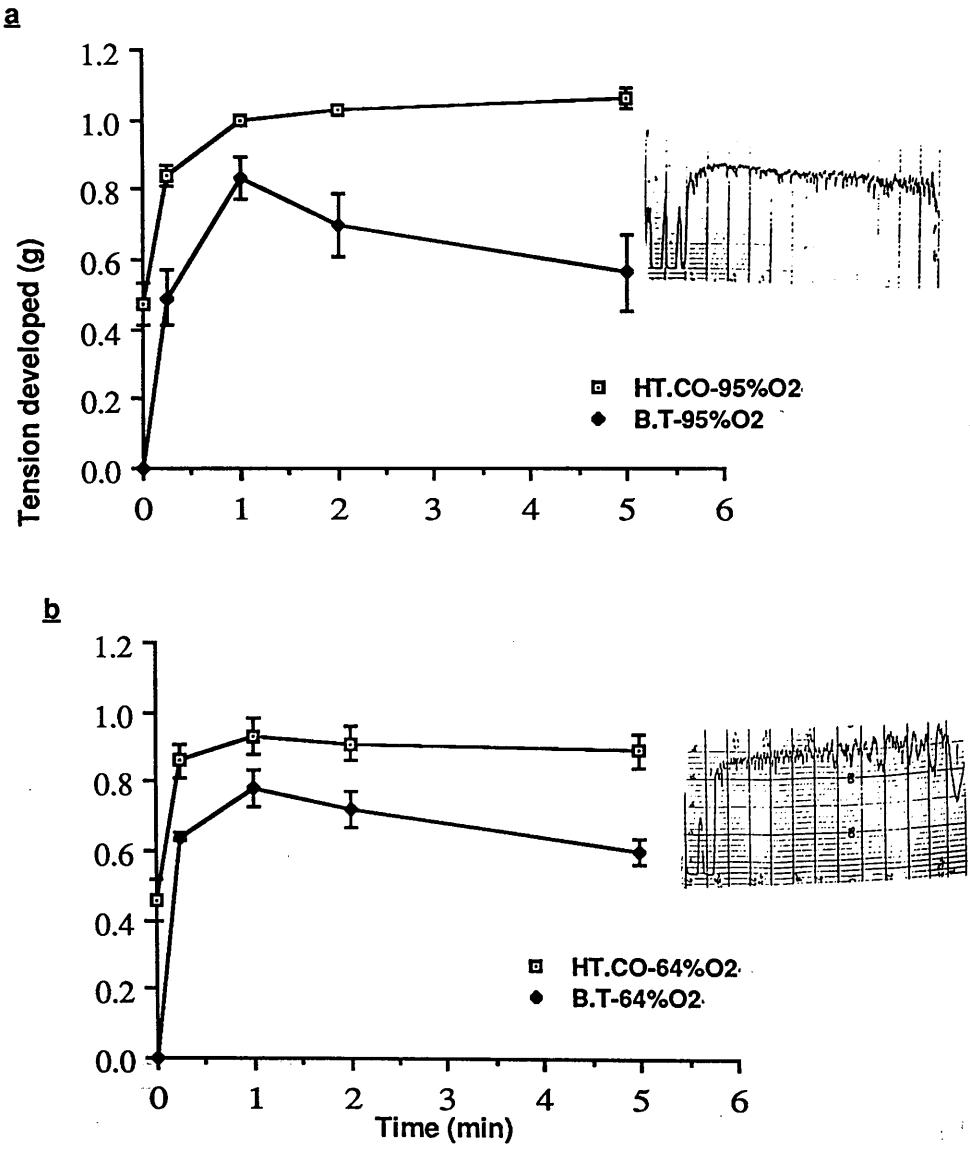
Time course of RPV response to NA 1μM at different O_2 tensions at a chart speed of 20mm/min. (a) - (e) show responses at 95%O_2 (600 mmHg), 64%O_2 (380 mmHg), 32%O_2 (230 mmHg), 16%O_2 (110mm Hg) and 8%O_2 (76 mmHg) respectively.

Represented in the figures are HT. CO = increase in height of response and B.T = increase in baseline tension.

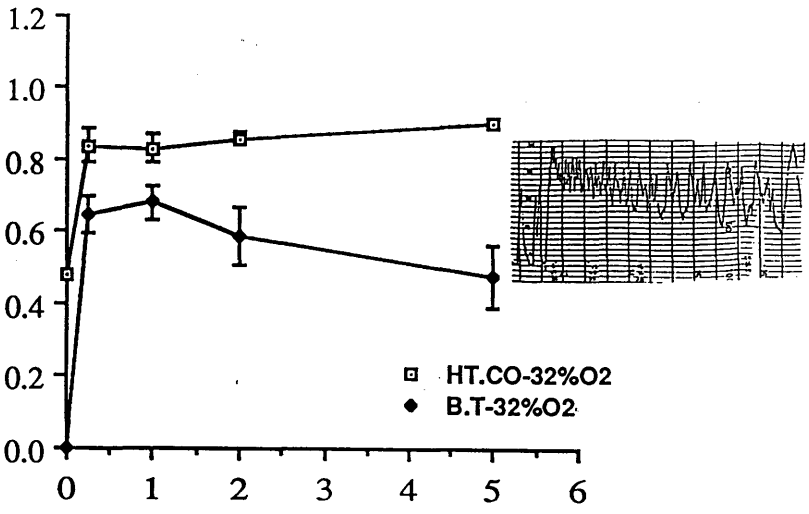
As O_2 tension is lowered the monophasic response at 95%O_2 becomes biphasic by 32%O_2. The phasic component of this response becomes increasingly prominent as O_2 is lowered. On the other hand, the tonic component of the response becomes progressively smaller as O_2 is lowered from 32%O_2.

Points on the 16%O_2 curve represent means ± s.e.m, n=18, while those on the other curves show means ± s.e.m, n=6.

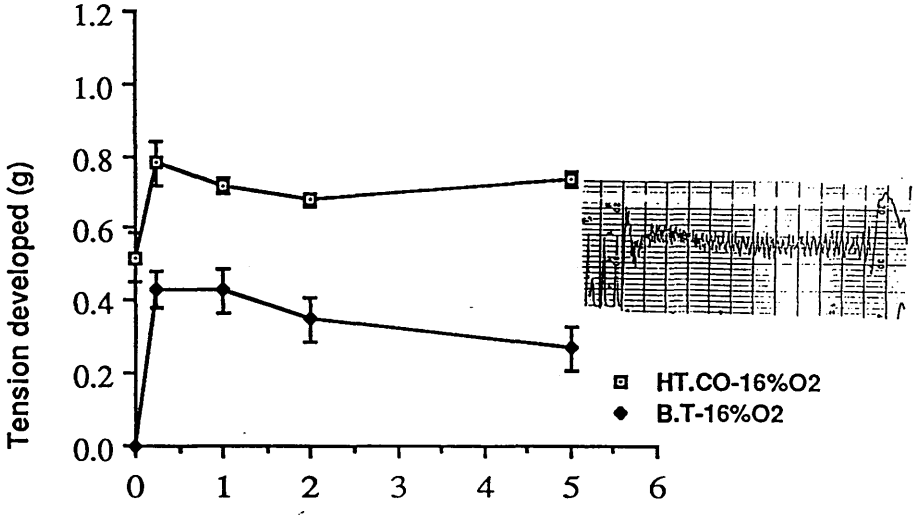
Insets show representative traces of NA-1μM induced contractions at the different O_2 tensions.



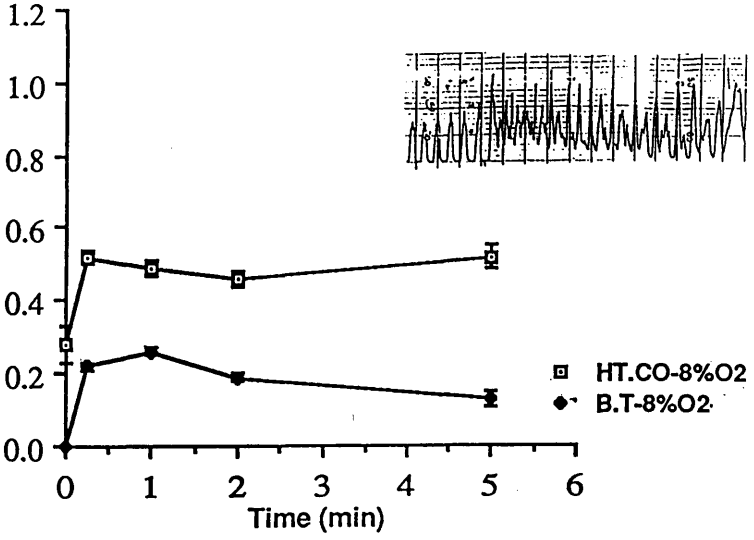
c


1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
HT.CO-32%O2
B.T-32%O2

d


Tension developed (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
HT.CO-16%O2
B.T-16%O2

e


1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Time (min)
HT.CO-8%O2
B.T-8%O2

determined using EDTA alone.

Having established the influence of these various factors the influence the O_2 tensions the effects of 95% and 16% on the responses to various agonists were examined.

Before determining the influence of the two main O_2 tensions, 95%O_2 and 16%O_2 which was the standards for testing most agonists effects of oxygen tensions intermediate between 95 and 16%O_2 were examined on responses to NA. Also an O_2 tension lower than 16%, i.e 8%, was examined.

(c). Intermediate O_2 tensions (64 and 32%O_2) and a lower O_2 tension (8%)

At 64%O_2, the responses were monophasic and of similar magnitude to those at 95%. On the other hand, when the O_2 was reduced to 32%, the response was biphasic. The magnitude of the phasic and the tonic contraction was similar, and the size of the tonic contraction was not statistically significantly different from the 95%O_2 response. At 16% , the phasic contraction was no bigger than it was at 32% ($P > 0.05$), but the tonic contraction was smaller, see figs 2.6 a -e. As the O_2 tension decreased the phasic contraction became more prominent and the tonic contraction decreased further. The phasic contraction is not represented in the mean data which took responses at fixed times. However, the

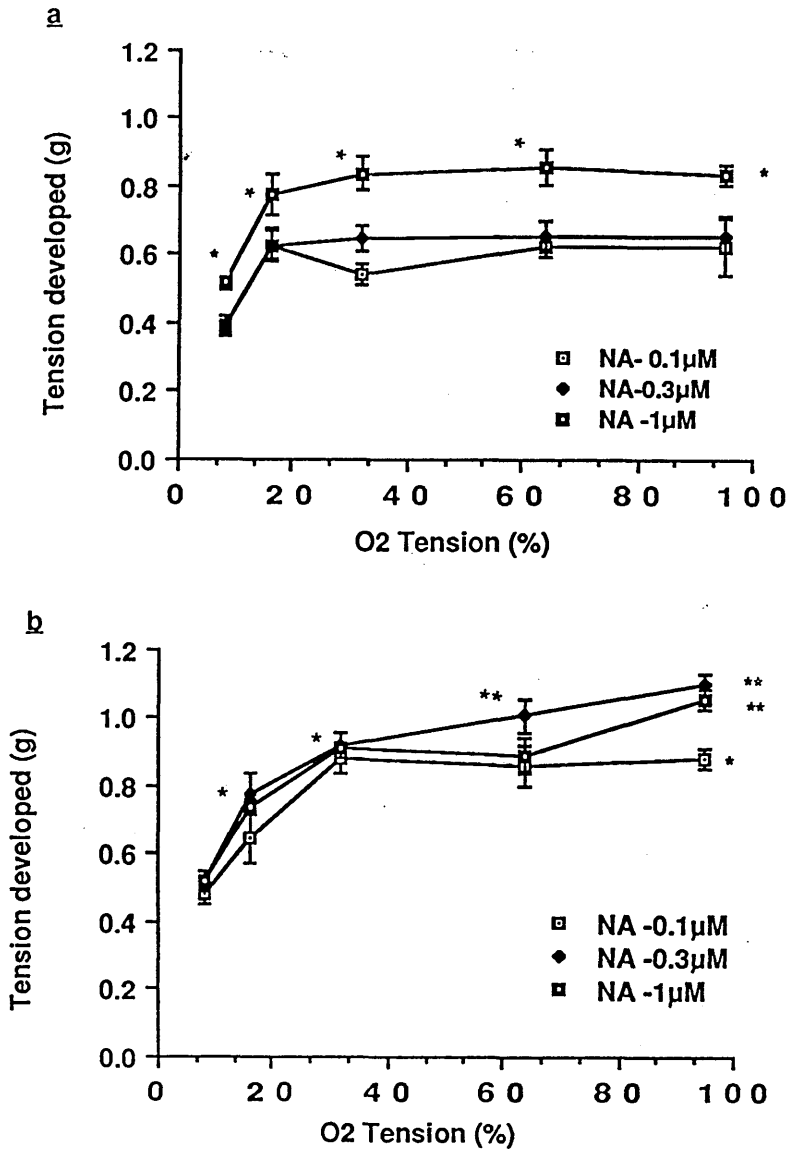


Fig 2.7.

RPV response to different concentrations (0.1µM, 0.3µM and 1µM) of NA at different O_2 tensions at (a) 15s and (b) 5min.

The fig shows that the phasic response (15s) at 16%O_2 and above hardly changed, while the response at 8%O_2 was smaller than those at higher O_2 tensions.

On the other hand the tonic response (5min) became increasingly smaller as O_2 was lowered from 32%O_2.

Points represent means ± s.e.m, n = 6.

*, ** represent $P < 0.05$ and 0.01 respectively, when responses at 8%O_2 were compared with those at other O_2 tensions.

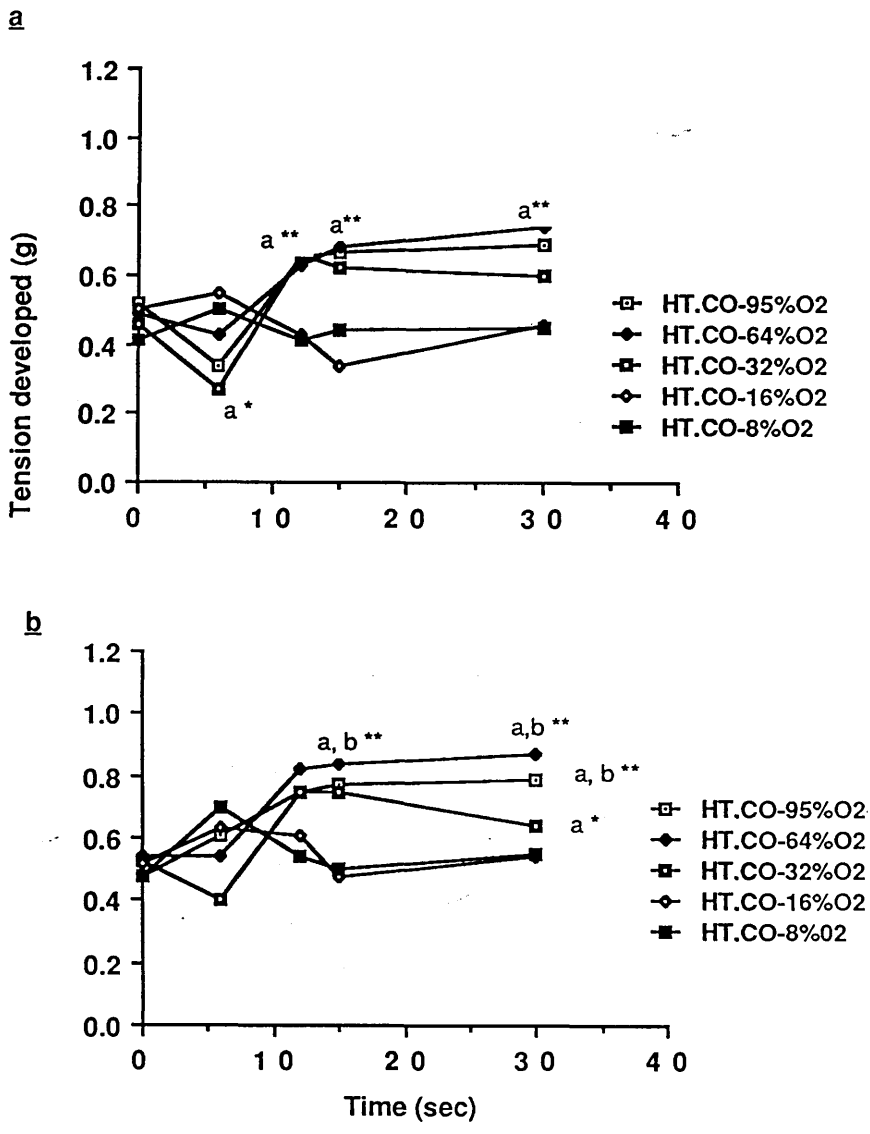


Fig 2.8.

Time course of RPV to NA (a) 0.3μM (b) 1.0μM at different O_2 tensions at a fast chart speed of 50mm/min. Points represent measurements of height of contraction made at 6s, 12s, 15s and 30s. Control spontaneous activity (control) prior to the addition of NA is shown.

Points represent means ± s.e.m, n = 6.

*, ** represent $P < 0.05$ and 0.01 respectively, when responses at $8\%O_2$ (^{a}) and $16\%O_2$ (^{b}) were compared with those at other O_2 tensions.

representative trace shows a prominent phasic contraction as O_2 was lowered (see figs 2.6c - e). Plotting the 15s and 5min response at three concentrations of NA versus O_2% (figs 2.7a & b) shows that the magnitude of the phasic contraction hardly changed from 16% - 95%O_2, but was statistically significantly ($P<0.05$) smaller at 8%O_2 than it was at 16%O_2 and at higher O_2 tensions, particularly at NA 1μM. On the other hand, the tonic contraction reduced as the O_2 was lowered from 32%O_2.

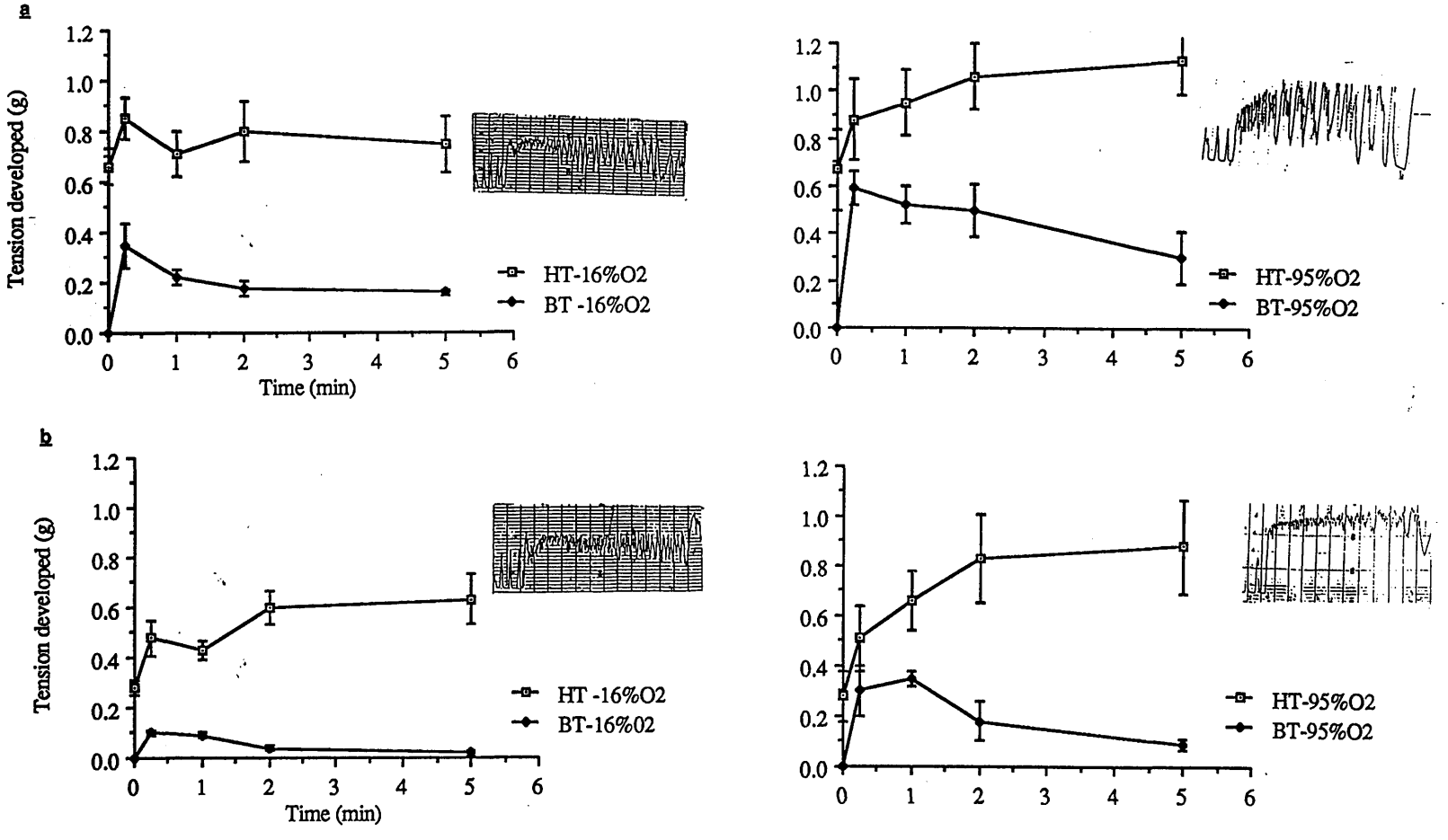
It was concluded from this study that there was an inverse relationship between the ability to distinguish the phasic contraction and the oxygen tension and that the threshold for distinguishing this phasic contraction was 32%O_2. The tonic contraction was more directly related to the oxygen tension < 8%O_2 to 32%O_2) and this was clear at the highest NA level of 1μM.

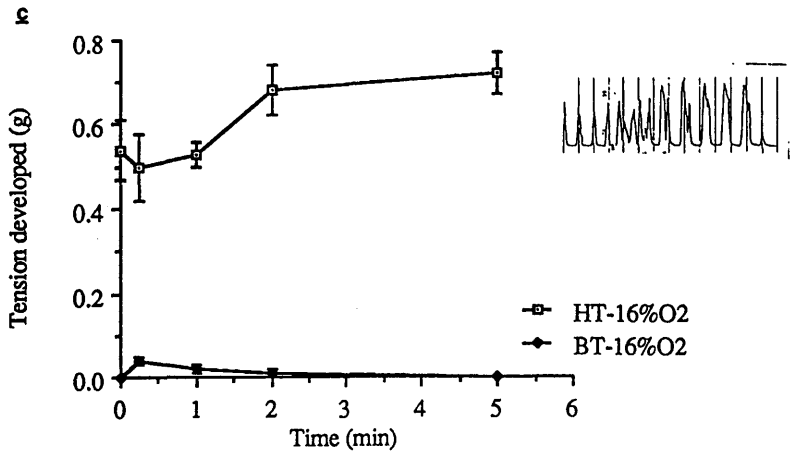
In order to determine the rate of rise of tension at each O_2 level, experiments were carried out at a faster speed of 50mm/min. Measurements at 6s did show that for NA 0.3μM the relative order for rate of rise was 16% > 8% > 95% > 64% > 32%O_2 (fig 2.8a). The 8% and 16%O_2 responses were statistically significantly higher ($P<0.05$) than the response at 32%O_2 but not significantly higher than that at either 16%O_2 or 95%O_2. A similar pattern of response was obtained with the higher concentration of NA 1μM, though the order was as

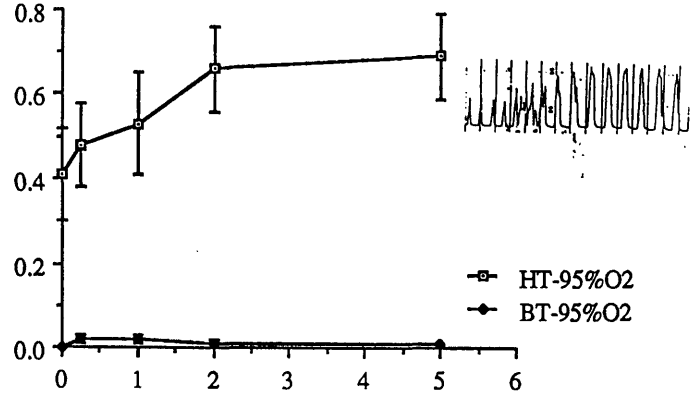
Fig 2.9.

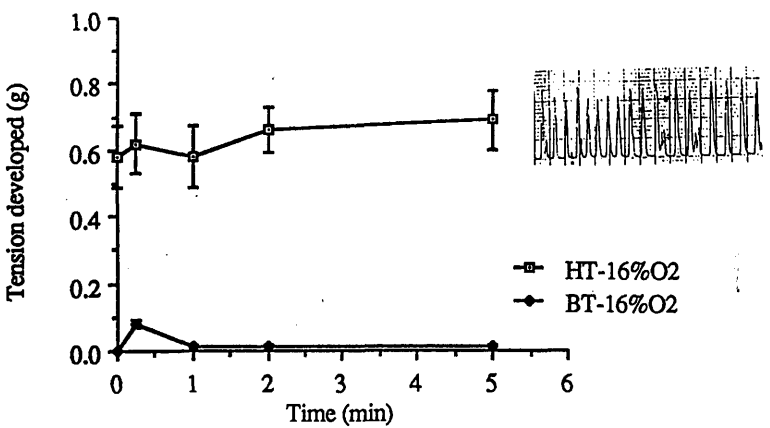
Representative traces of response to other agonists, (a) amidephrine 1.0μM, (b) phenylephrine 1.0μM, (c) xylazine 30μM (d) UK14304 10μM at 16%O_2 and 95%O_2.

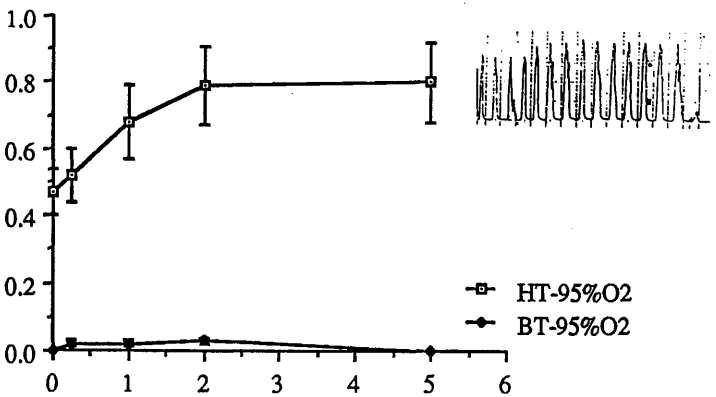
Note that while responses induced by amidephrine and phenylephrine decreased as O_2 tension was lowered from 95%O_2 to 16%O_2, those by UK14304 and xylazine hardly changed. Note also that UK14304 and xylazine hardly increased the baseline tension even at high concentrations.


a
Tension developed (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6
Time (min)
HT-16%O2
BT -16%O2
HT-95%O2
BT-95%O2
b
Tension developed (g)
Time (min)
HT -16%O2
BT-16%02
HT-95%O2
BT-95%O2


c
Tension developed (g)
0.0
0.2
0.4
0.6
0.8
0 1 2 3 4 5 6
Time (min)
HT-16%O2
BT-16%O2


0.0
0.2
0.4
0.6
0.8
0 1 2 3 4 5 6
HT-95%O2
BT-95%O2


d
Tension developed (g)
0.0
0.2
0.4
0.6
0.8
1.0
0 1 2 3 4 5 6
Time (min)
HT-16%O2
BT-16%O2


0.0
0.2
0.4
0.6
0.8
1.0
0 1 2 3 4 5 6
HT-95%O2
BT-95%O2

follows 8% = 95% = 16% > 64% > 32%O_2 (figs 2.8b.).
The measurements at 15's unlike those in the series of experiments above (at a lower chart speed) showed that NA induced reponses at 0.3µM or 1.0µM were statistically significantly smaller ($P<0.01$) at 16%O_2 than at 32%O_2 and above, but the results at 8%O_2 were consistent with the results above.

Results therefore show there is an inconsistent rate of rise between the different O_2 tensions. Perhaps it is not so much the depression of the phases but a change in their time courses that leads to separation at lower O_2.

3. Effects of oxygen tension on responses to other alpha-adrenoceptor agonists.

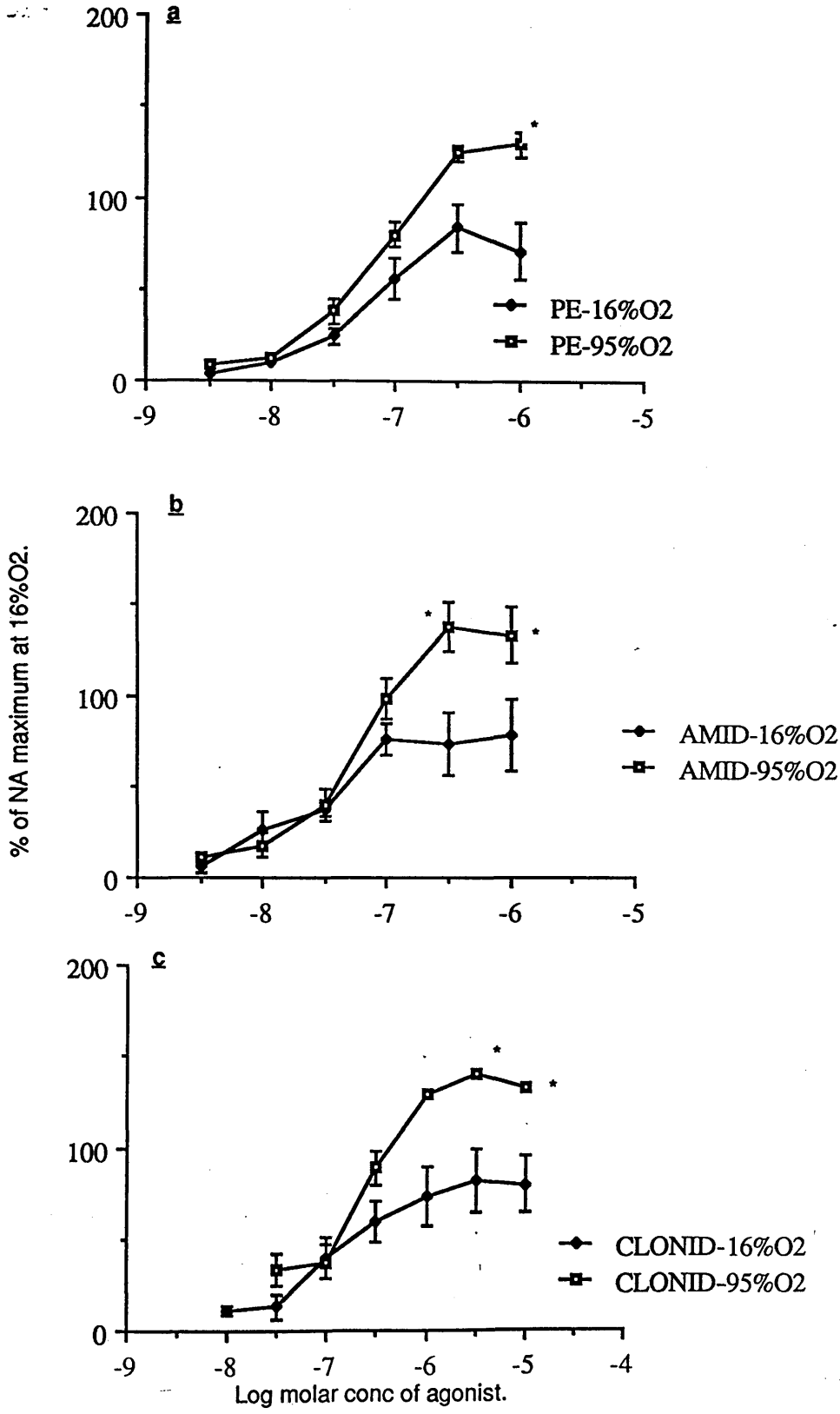
Hitherto, the effects of various experimental conditions were examined on NA -induced contraction. Having established these conditions, other alpha adrenoceptor agonists were examined first at 16%O_2 and then at 95%O_2. These agonists include phenylephrine, amidephrine, clonidine, (+)m-synephrine, UK14303 and xylazine.
In general, phenylephrine and amidephrine produced a similar pattern of response to noradrenaline- as decribed above. However the pattern of responses induced by clonidine, (+) m-synephrine, UK14304 and xylazine was different. This is described below.
See representative traces of the responses to these agonists in figs 2.9a-d.

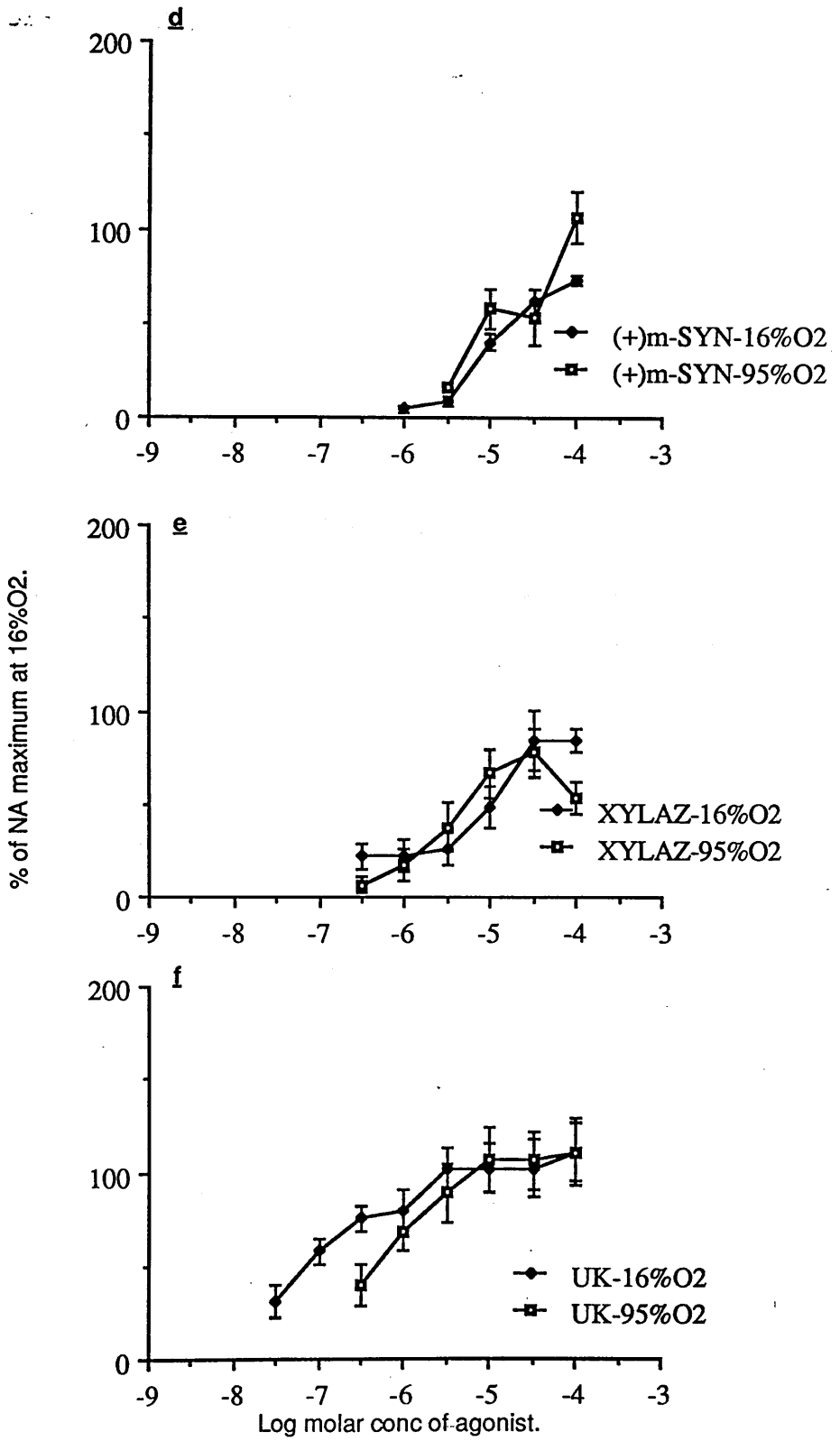
Fig 2.10.

Concentration/response curves to various agonists expressed as percentage of maximum response to NA at 16%O2. The data was compiled from change of tension by agonist expressed as % of maximum change of tension by NA.

Responses to NA, amidephrine and phenylephrine were reduced to approximately 60% at 16%O2 and they therefore behaved as if they were "partial agonists" at this O2 tension. On the other hand responses to UK1304, xylazine and (+)m-synephrine at 95%O2 were about 50% of NA maximum and therefore gave equivalent maxima to that induced by NA at 16%O2.

Points represent means $\pm$ s.e.m, n = 6, except for NA where n = 15- 20.
* represents statistically significantly ($P < 0.05$) different from response to NA at 16%O2.


a
200
100
0
-9
-8
-7
-6
-5
PE-16%O2
PE-95%O2
b
200
100
0
-9
-8
-7
-6
-5
% of NA maximum at 16%O2.
AMID-16%O2
AMID-95%O2
c
200
100
0
-9
-8
-7
-6
-5
-4
Log molar conc of agonist.
CLONID-16%O2
CLONID-95%O2


d
200
100
0
-9
-8
-7
-6
-5
-4
-3
(+)m-SYN-16%O2
(+)m-SYN-95%O2
e
200
100
0
-9
-8
-7
-6
-5
-4
-3
XYLAZ-16%O2
XYLAZ-95%O2
f
200
100
0
-9
-8
-7
-6
-5
-4
-3
UK-16%O2
UK-95%O2
% of NA maximum at 16%O2.
Log molar conc of agonist.

a. Phenylephrine

Contractions were obtained to phenylephrine (30nM - 10μM). The responses at 95% were monophasic, while at 16%O_2, they were smaller and biphasic. The -logEC_{50} values at 95% and at 16% were 7.40±0.08 and 7.30±0.09, n = 6, (P > 0.05) respectively. See fig 2.10a.

b. Amidephrine

Amidephrine (3nM - 10μM) contracted the tissue, with the pattern of response similar to that of NA or phenylephrine in 95% and 16%O_2. The -logEC_{50} values at 95% and 16%O_2 were 7.42±0.05 and 7.55±0.07, n = 6 (P > 0.05) see fig 2.10b.

c. Clonidine

Clonidine contracted the tissues for 10nM to 10μM without increasing the baseline tension . The -logEC_{50} values at 95% and at 16%O_2 were 6.91±0.1 and 6.93±0.08, (P > 0.05) n=6 respectively, see fig 2.10c.

d. (+)m-synephrine

(+)m-synephrine contracted the tissues from 1μM to 100μM, also without increasing the baseline tension. The -logEC_{50} values at 95% and at 16%O_2 were 5.07±0.01 and

5.24±0.1, n=4 (P > 0.05) respectively, see fig 2.10d.

e. Xylazine

Compared with NA, phenylephrine or amidepherine, xylazine had a relatively low potency in this tissue. The tissues contracted to xylazine only from 0.3μM, with the maximum reached at 10μM. The response to this concentration range was an increased amplitude of the contractile spikes sometimes with or without an increased frequency of the spikes. In all the concentrations examined no increased baseline tension was obtained. The $-\log EC_{50}$ values at 95%O_2 and at 16%O_2 were 5.65±0.11 and 5.45±0.2 (P > 0.05), n=5 respectively, (see fig 2.10e). There was no significant difference between the response at 16%O_2 and that at 95%O_2 at all concentrations of xylazine examined. This suggests that O_2 did not modify xylazine -induced contractions.

UK14304

Like xylazine, UK14304 showed a relatively low potency in the portal vein at 95%O_2. The tissues contracted to UK14304 from 0.1μM 100μM. The contraction was characterised by an increase in the contraction of the contractile spikes, accompanied by an increased frequency of the spikes. From 1μM - 100μM , the responses in 16% and 95% were of similar size. The -

$\log EC_{50}$ values at 95% and 16% O_2 were 6.33±0.1 (n = 6) and 6.29±0.13 (P> 0.05) (n=15) respectively. It was noted that the usually biphasic contractions at 16% obtained from other agonists were absent with xylazine or UK14304. Even though UK14304 caused an increase in the absolute height of contraction at higher concentrations, the change in tension determined by the S.A subtracted from the height of response in the presence of the drug was small vis a vis NA, phenylephrine or amidephrine, see fig 2.10f.

When the results in different O_2 tensions were plotted as percentage of mean maximum response to NA at 16% O_2 obtained from a seperate set of tissues, the picture that emerges is that the response at 16% O_2 behaved as though they were partial agonists since response to full agonists at 16% O_2 were reduced to approximately 60% of the maximum response to NA at 95%. On the other hand, drugs that were hardly affected by O_2 had their responses at 16% or 95% at approximately the same level as NA at 16% O_2. The maxima expressed as a percentage of NA maximum at 16% were: UK14304 111.1±17.8% and 111.1±15.6% (n=6) (P > 0.05) at 16% and 95% respectively; xylazine 77.7±13.3 and 84.4±15.6% (n=5) (P > 0.05) at 16% and 95% respectively, (+)m- synephrine: 73.3±6.7 and 106.7±13.3 (P > 0.05) (n=4) at 16% and 95% respectively; clonidine: 82.2±17.8 and 140±8.2 (P < 0.01) (n=5) at 16% and 95% O_2 respectively. Clonidine produced qualitatively similar responses to UK14304 or

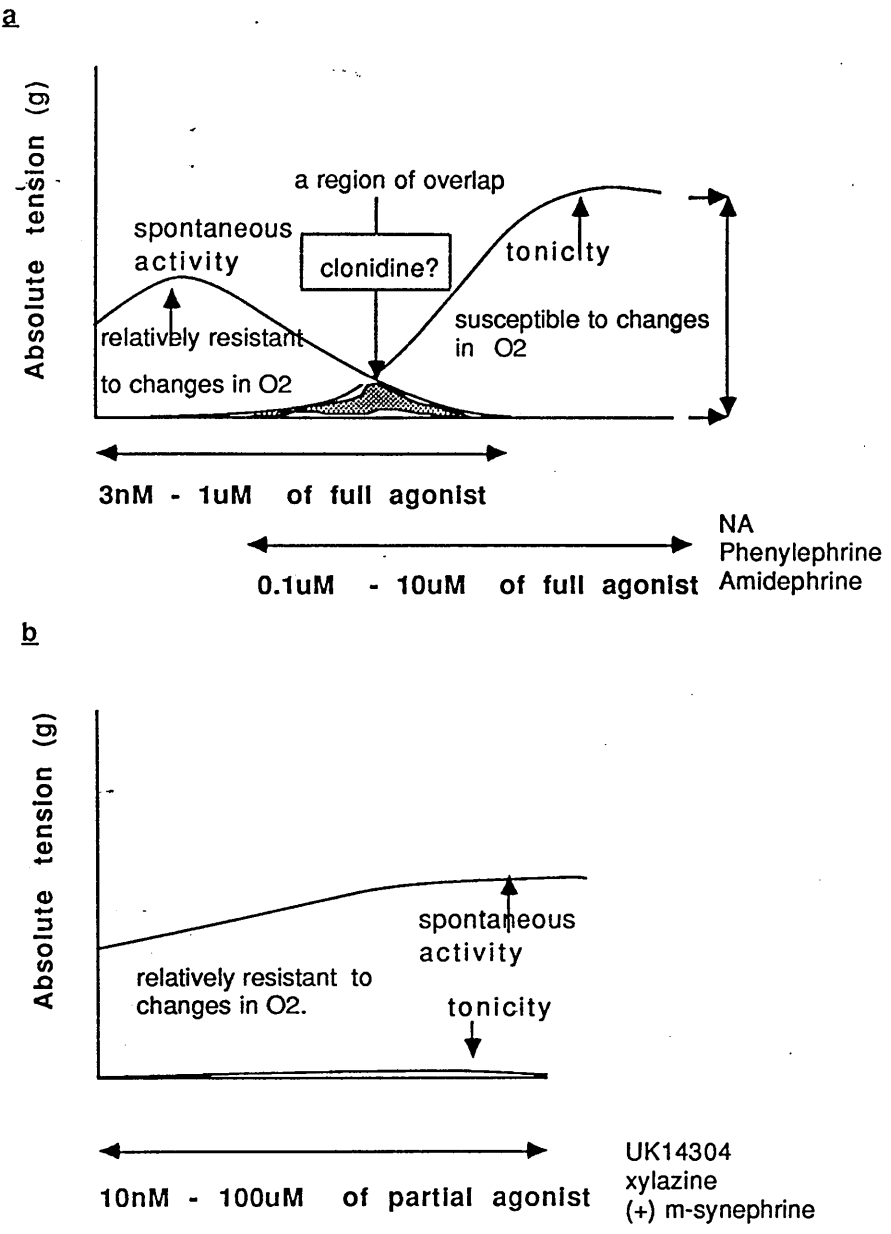


Fig 2.11.

Schematic representation of the pattern of responses induced by various agonists in the RPV and their susceptiblity to O_2.

(a). Responses to low concentrations of full agonists are characterised by an increase in the height of the contractile spikes (spontaneous activity) and as the concentration is increased there is a combination of an increase in the height of the contractile spikes and an increase in baseline tension (tonicity). From 1μM, there is usually a complete disappearance of the contractile spikes leaving a smooth tonic contraction. The tonic responses are relatively more sensitive to O_2 changes than the contractile spikes.

(b). Responses to low to high concentrations of partial agonists are characterised by an increase in contractile spikes without an increase in baseline tension. These responses are relatively more resistant to O_2 changes than drugs that induce both increase in contractile spikes as well as baseline tension (clonidine is the only exception to this rule).

xylazine, but its responses were suceptible to O_2 changes between 95% and 16%. At a lower O_2 of 4%, clonidine responses became lower still.

For full agonists the responses at low concentration were not much affected than the tonic response.

It was concluded from these studies that NA, phenylephrine and amidephrine are full agonists and are susceptible to oxygen tension changes, while xylazine and UK14304 are probably partial agonists at alpha-1 adrenoceptors and relatively less susceptible to O_2 changes.

See fig 2.11 for the schematic representation of the pattern of response of the RPV induced by full and partial agonists and their susceptibility to changes in O_2 tension.

4. Effect of alpha-1 adrenoceptor antagonist -prazosin on responses induced by either NA or UK14304 at 16%O_2.

a. NA

Prazosin at the three concentrations examined, 1nM, 30nM and 0.75μM caused a parallel shift of the concentration response curve to the right. The pA_2 value for the phasic component was 11.0 ± 0.12, slope 0.94± 0.06 (see fig 2.12a) and that of the tonic was 9.1±0.12, slope 1.12 ± 0.05, n= 5. (see fig 2.12b).

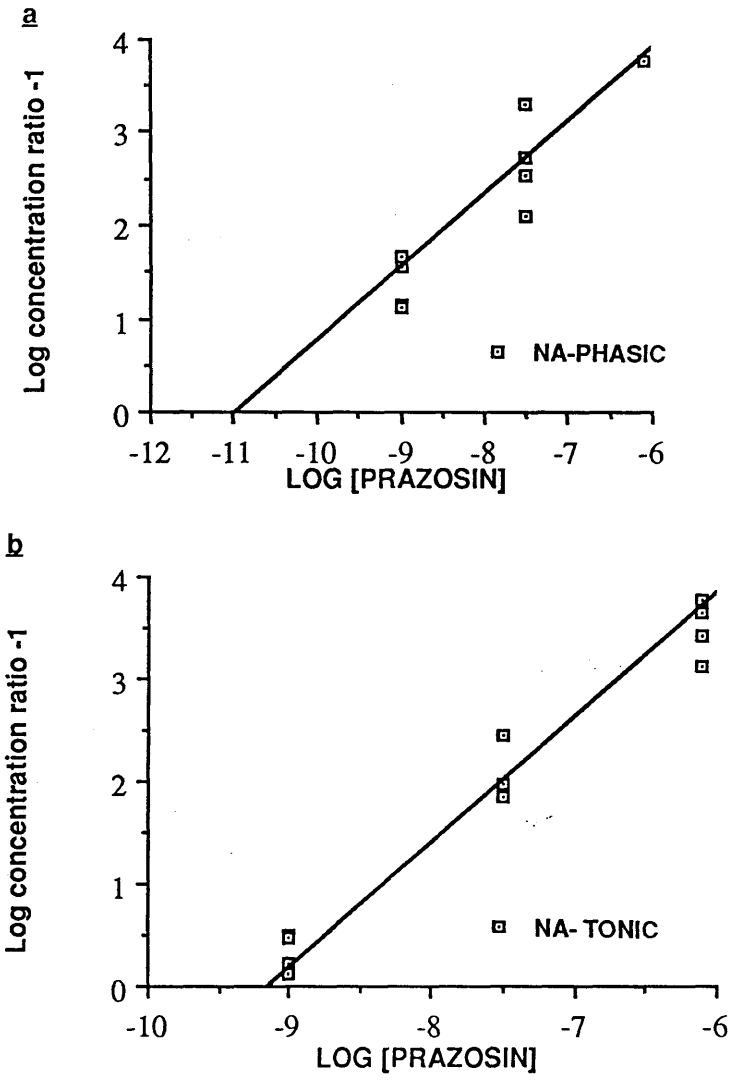


Fig 2.12.

The schild plot for prazosin against NA at 16%O_2.

(a) Prazosin against phasic response to NA. pA2 was 11.0 $\pm$ 0.12 and slope was 0.94 $\pm$ 0.06.

The pA2 value was 9.1 $\pm$ 0.12 and the slope was 1.12 $\pm$ 0.05.

The data was compiled from change of tension by NA without or with prazosin expressed as % of control maximum to NA.

Each antagonist concentration was tested against one tissue only.

Points represent means of 5 - 6 experiments (note that fewer points are shown where there is an overlap).

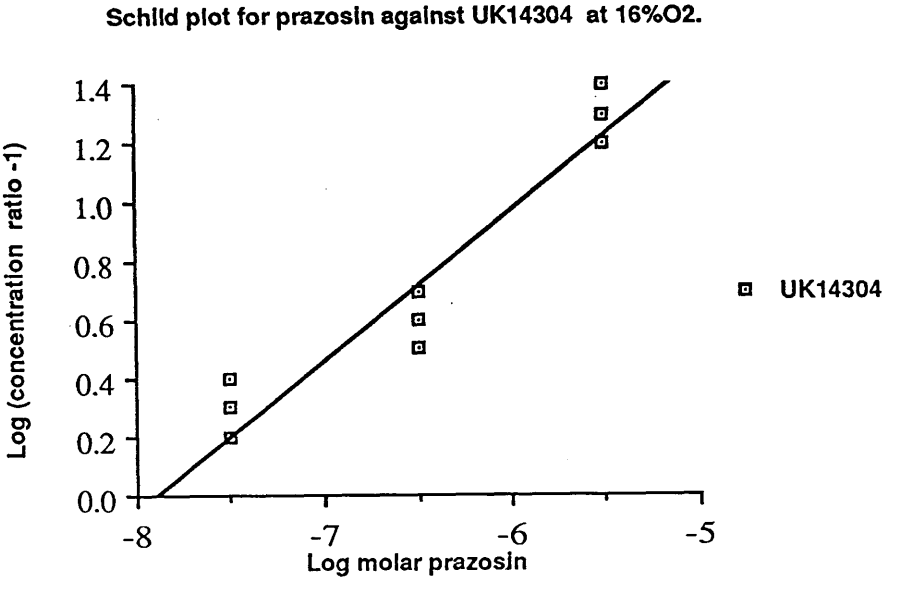


Fig 2.13.
The schild plot for prazosin against UK14304 at 16%O_2.
The pA2 value was 7.9 $\pm$ 0.26 and the slope was 0.55 $\pm$ 0.08.
The data was compiled from change of tension by UK4304 without or with prazosin expressed as % of control maximum to UK14304.
Each antagonist concentration was tested against one tissue only.
Points represent means of 4-5 experiments.

b. UK14304

Prazosin 1nM did not cause a shift of the UK14304 concentration response curve, but from 30nM a rightward shift of the log concentration response curve was obtained. Therefore a concentration response curve for UK14304 was constructed in the presence of prazosin, 30nM, 0.3μM and 3μM. The pA_2 value was 7.9±0.26 and the slope was 0.55± 0.08, n= 4. see fig 2.13.

5. **Effect of alpha-2 antagonist Wyeth 26703 on responses induced by UK14304 at 16%O_2**

The three concentrations (30nM, 0.3μM and 3μM) of Wyeth examined caused inconsistent shift in the UK14304 concentration response curves. This was taken to mean that the Wyeth compound did not have an effect on the UK14304 induced responses.

6. **Effects of mildly depolarising KCl 20mM on the increase in the contractile spikes induced by UK14304 and xylazine at 16%O_2.**

The object was to see whether in the presence of KCl 20mM, a maintained response similar to that obtained to that of NA could be obtained. That is, would it be similar to the contraction induced by Bay K 8644 in some vascular smooth muscle which occurs only when the

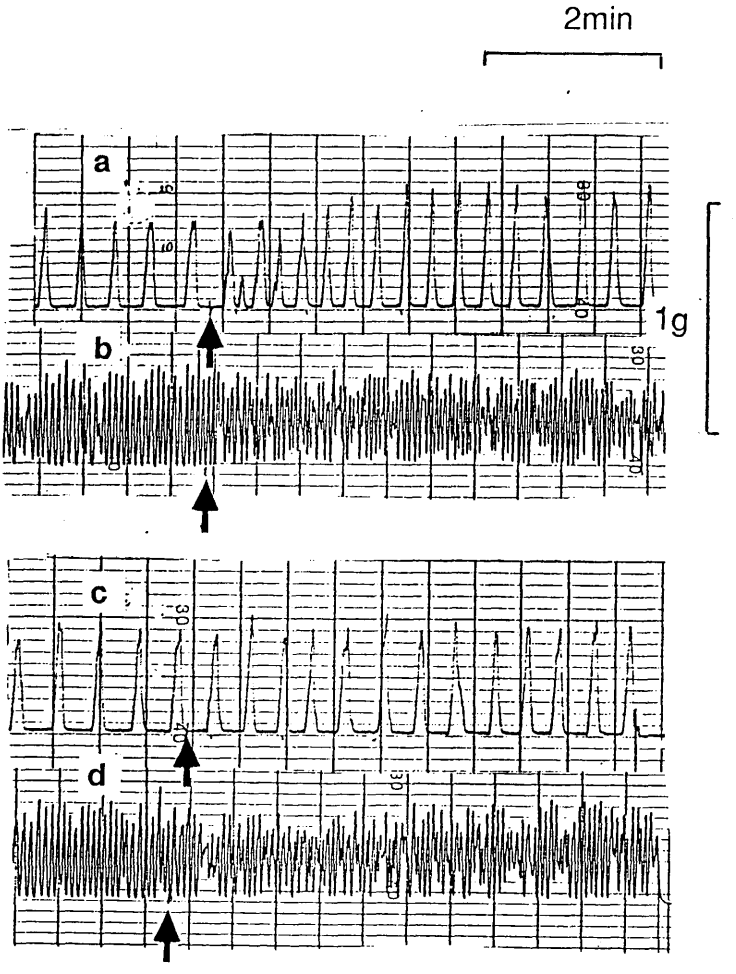


Fig 2.14.
Representative trace for UK14304 and xylazine induced effects in the presence of mildly depolarizing KCl 20mM.
(a) and (c) are control responses to UK14304 10μM and xylazine 10μM respectively in "normal Krebs" containing 4.7mM KCl. (b) and (d) are the responses to UK14304 and xylazine in the presence of 20mM KCl.
The frequency of spontaneous activity (SA) increased in the presence of KCl. When UK13404 or xylaxine was added there was no enhancement or additivity of contraction compared to the responses induced by these drugs without mild depolarization. A sightly increased frequency of SA was noted.
Arrows indicate where drugs were added.

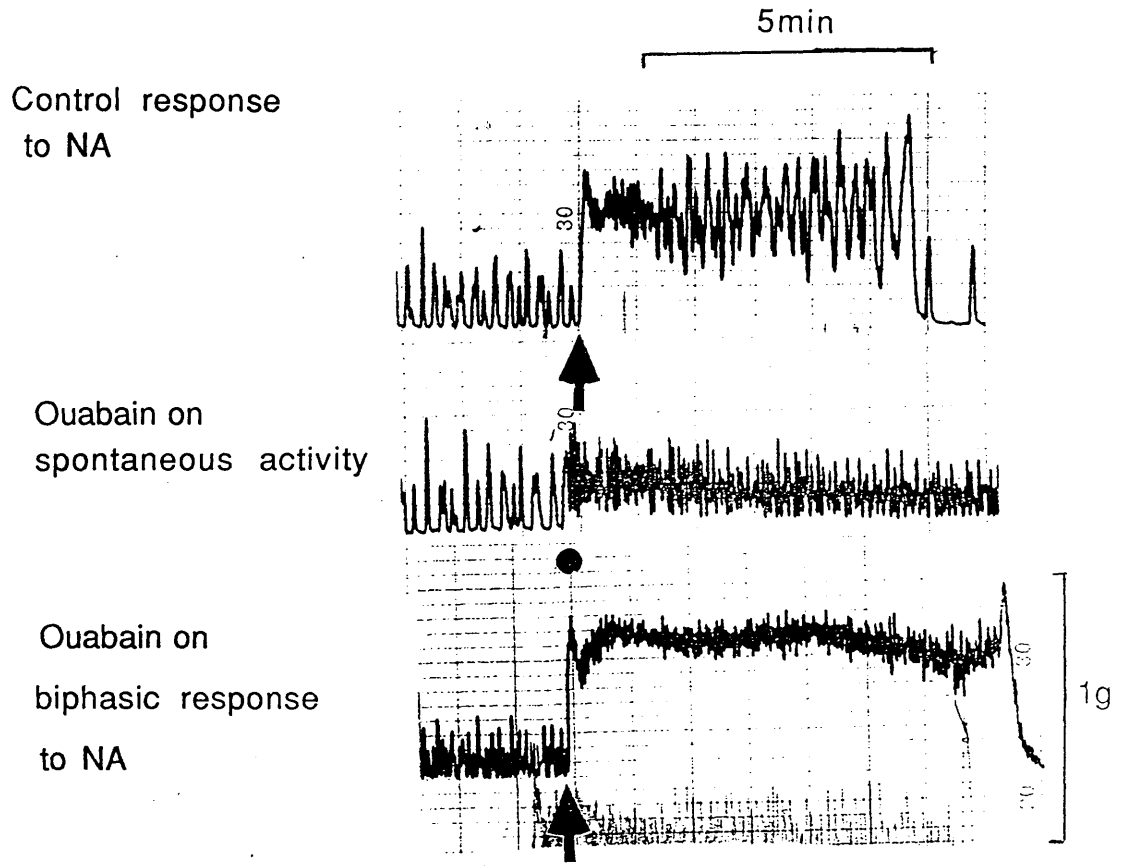


Fig 2.15.
Representative trace for effects of ouabain 0.1mM on the biphasic response to NA 1μM at 16%O_2.

Ouabain on its own caused a small contraction and greatly increased the spontaneous activity of the tissue. However, it did not modify the biphasic contraction induced by NA at 16%O_2.

tissue is mildly depolarized. KCl 20mM on its own greatly increased the frequency of the spontaneous activity with a slight increase in the baseline tension. UK14304 at 3μM or 10μM increased further the already increased frequency of spikes a small but statistically non-significant increase in the baseline tension. A similar response was obtained to xylazine 10μM, see fig 2.14.

It is therefore concluded these two compounds cannot behave like full agonists even under mildly depolarizing condition, though under this condition the spontaneous activity is less and could allow for less variability in the course of an experiment.

7. **Effect of ouabain 0.1mM on the biphasic response induced by noradrenaline 1μM at 16%O_2.**

During the 15min incubation with ouabain, the frequency of the spontaneous activity of the tissue was greatly increased and the tone of the tissue was slightly increased. The biphasic response induced by noradrenaline was not affected by ouabain, except for a generally less oscillatory tonic component due to the increased frequency of rhythmic activity (see fig 2.15).

8. **Effects of KCl or caffeine at 95% and 16%O_2**

a. KCl

Effects of two KCl concentrations (25mM or 50mM) were

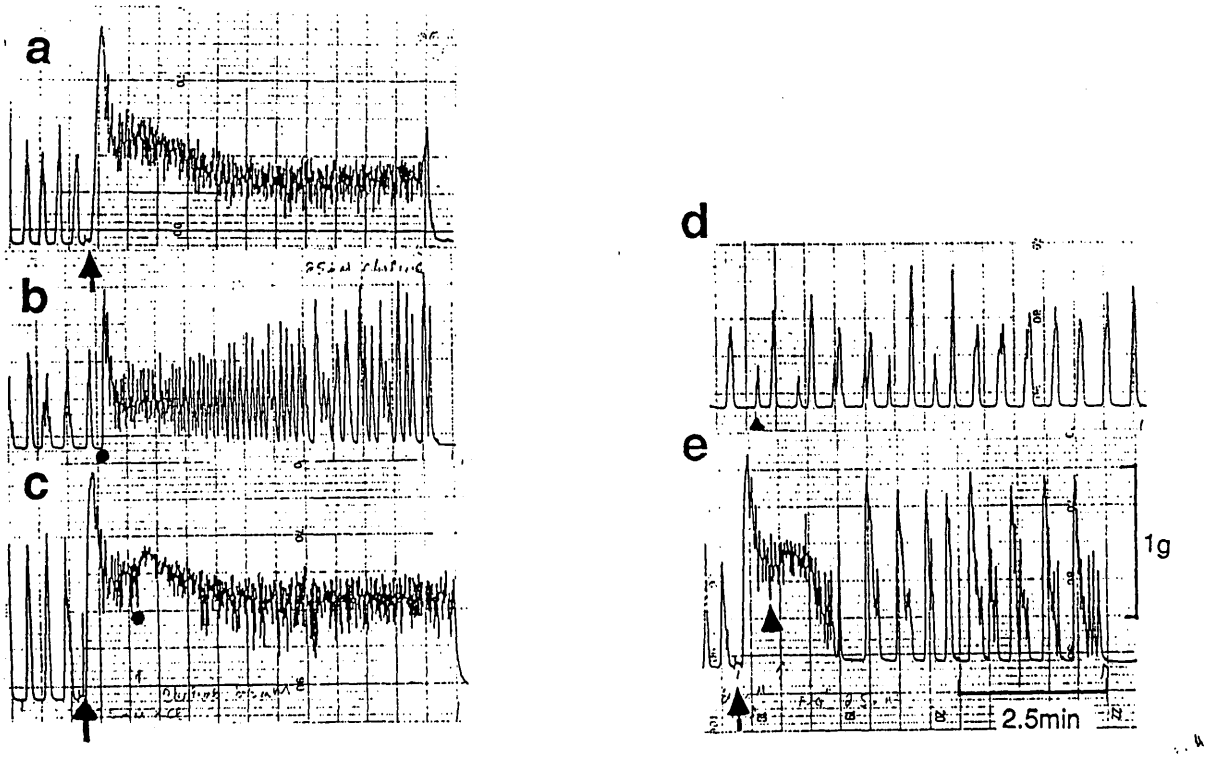


Fig 2.16.

Representative trace for KCl 25mM -induced response without or with either choline 25mM or NaCl 25mM at 16%O_2.

The left panel shows (a) control response to KCl (b) control response to choline (c) addition of choline after 2min response to KCl.

The right panel shows (d) control response to NaCl 25mM and (e) addition of NaCl after 2min response to KCl.

The arrows indicate where KCl was added. The dots show where choline was added, while the triangles show where NaCl was added.

examined. Responses induced by either concentration at 16% were clearly biphasic with the phasic contraction peaking after 15s- 30s. The phasic contraction was more variable with large values of standard error of the mean. The response at 95% was not clearly biphasic and the two phases when discernible were often of similar magnitude. The tonic contraction that followed was better maintained if KCl was not substituted for NaCl i.e high K high Na, than when Na^+ was substituted i.e high K low Na (particularly at 16%O_2).

The phasic component at 15s in high K (25mM) low Na at 16%O_2 was 0.91±0.08g and this declined to 0.47±0.04g by 5min ($P < 0.01$). In high K high Na on the other hand the phasic response did not decline by 5min. The 15s response was 0.79±0.13g, while it was still 0.79±0.14g. On the other hand the phasic component at 15s in high K low Na at 95%O_2 was 0.66±0.07 and this declined to 0.63±0.06g, n = 6 by 5min (not statistically significantly different from each other). In high K high Na the phasic response was 0.74± 0.14 and this rose to 0.97±0.16 n=6.

At a higher KCl concentration, 50mM, the trend was similar, with responses to high K in high Na being better maintained than they were at high K low Na.

The phasic component at 15s in high K (50mM) low Na at 16%O_2 was 0.91±0.04 and this declined to 0.45±0.03 by 5min ($P<0.001$). While in high K high Na, the phasic contraction was 0.78±0.28g and declined to 0.51±0.15g n=

4 by 5min ($P < 0.05$). At 95%O_2 the phasic response at high K low Na was 0.73 ± 0.06 and it declined to 0.68 ± 0.08 by 5min ($n = 4$) ($P > 0.05$). In high K high Na, the phasic esponse was 0.72 ± 0.25g and by 5min it increased slightly to 0.75 ± 0.27g ($P > 0.05$).

Could the less better maintained tonic contraction in NaCl-deficient solution indicate that this phase of the contraction was dependent on Na^+, or could the better maintained response to KCl- depolarization in NaCl-rich solution simply be due to osmotic effects?

In order to test the first possibility, NaCl 25mM was added to the tissue during the declining response of the tonic contraction to KCl depolarized tissue in Na^+ deficient Krebs to see whether a better maintained contraction would be obtained. With this procedure, NaCl 25mM caused the tonic contraction to rapidly decline to the baseline, with the onset of large contractile spikes which were slightly bigger than without added NaCl 25mM. The effects of NaCl on its own were also examined, see fig 2.16 . In both 95% and 16%O_2 tensions, NaCl caused a decreased frequency of contractile spikes, without a cessation of contraction, see fig 2. 16 .

The second possibility was examined by substituting choline 25mM for Na^+. Choline was added during an established waning of the tonic contraction, see fig 2.16. With this procedure, there was a better maintained tonic contraction in the presence of choline at either 16% or 95%. Choline alone contracted the

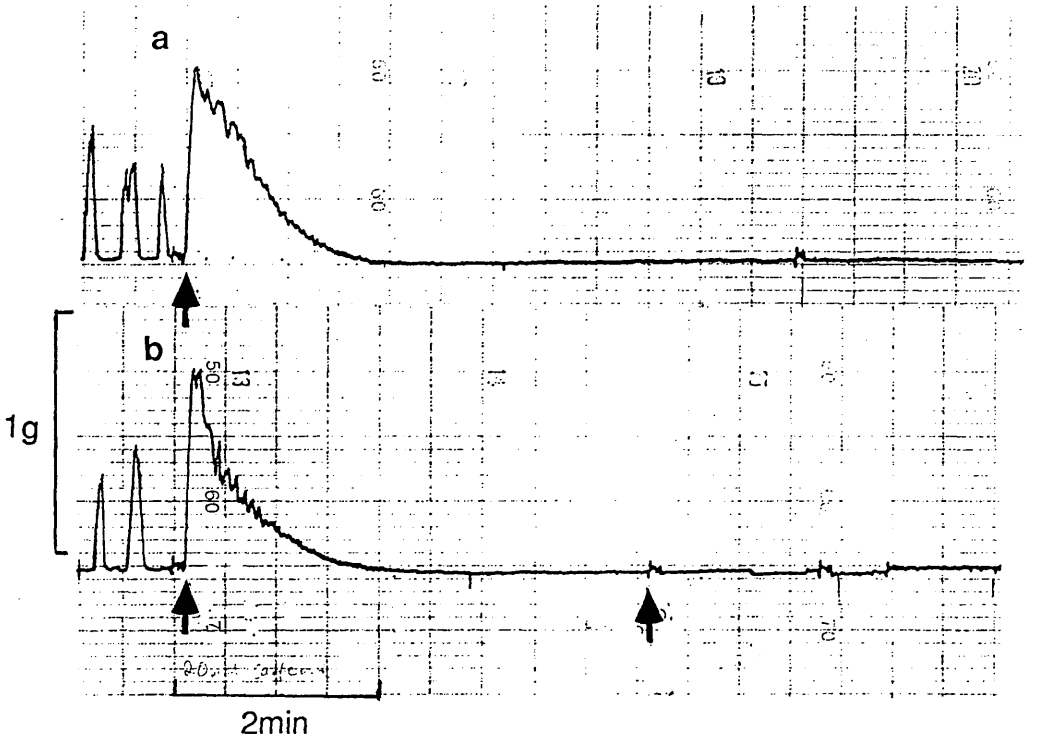


Fig 2.17.
Representative trace for caffeine 20mM -induced contration at (a) 95%O_2 and (b)16%O_2.

When caffeine was added directly to the tissue bath it caused no contraction. However, when it was added along with the Krebs it produced a transient contraction, as in this trace. The contraction was more transient at 16%O_2 than it was at 95%O_2. A second application of caffeine did not contract the tissues.
The arrows indicate where caffeine was added.

tissue, see fig 2.16. This better maintained tonic contraction in the presence of choline could be as a result of its agonistic effect, since it has a weak ACh-like action at high concentrations.

b. Caffeine

Caffeine depending on how it was administered into the bath could or could not cause a contraction.
When caffeine 20mM was added directly into the bath, there was a complete cessation of the tissue's spontaneous activty by 5min. The S.A was restored upon washing out caffeine. If however, caffeine was added to the bath along with Krebs there was a transient contraction (0.6 ± 0.05g at 16%O_2 0.55 ± 0.04 n=6, $P> 0.05$) which peaked within 15s and returned to the baseline by 1min and subsequently abolished the spontaneous activity of the tissues (fig 2.17a). Time matched controls without caffeine did not show this transient contraction suggesting it was not an artefact (n=6). The responses induced by caffeine were more transient at 16%O_2 than at 95%O_2, see fig 2.17b. A second response to caffeine 3-5min after the first could not be obtained. If however, the tissues were placed back in normal Krebs for 10 -15 min, a response similar to the initial transient contraction was obtained.

DISCUSSION

Quantitative as well as qualitative changes occurred in the responses to NA, amidephrine and phenylephrine when the O_2 tension was reduced from the hyperoxic level (95%) to the normoxic level (16%).

Quantitatively smaller responses to the full agonists NA, PE and amidephrine at 16%O_2 show how sensitive this preparation is to O_2 changes , confirming Ebeigbe , Pickard & Jennett's observation (1980). The aorta on the other hand is more resistant to O_2 changes in the range 650mmHg -120mmHg (i,e 95% - 16%O_2 (Shibata & Briggs 1967; Chang & Detar, 1980). The sensitivity of the rat portal vein to O_2 coupled with its inherent myogenic activity which simulates the vasomotion in arterioles and pre-capillary vessels (Mellander & Johannson, 1966) makes it a suitable vascular preparation for the study of the influence of O_2 tension in vitro.

Qualitatively, while the responses at 95%O_2 were monophasic (especially from NA 0.3μM) those at 16%O_2 were biphasic. The biphasic contraction was characterised by a phasic contraction peaking by 15s, followed by a tonic contraction. This tonic contraction was more susceptible to O_2 changes than the phasic contraction as it became progressively depressed from from 16%O_2 and below. Responses to UK14304 and xylazine were not much affected by change in O_2 tension from 95% to 16%. These two drugs showed low potency in this tissue. They only increased the height of the

contractile spikes, with very little increase in baseline tension.

The relative insensitivity of the spontaneous contractile activity to O_2 changes < 50mmHg (i.e < 8%); (Hellstrand et al, 1977 and this study), causes drugs which increase the spontaneous activity to be less affected by changes in O_2 from 95% - 16%O_2.
The difference between the effects of O_2 on the tonic component and on contractile spikes suggest that a different element in excitation-contraction coupling might be responsible.
Perhaps, the contracture to high concentrations of full agonists might be regarded in this tissue as an unphysiological artefact induced by high O_2 tension, and so the physiological mechanism could be obscured by the use of high O_2 tension

A comparison of the concentration/ response curves for "full agonists" with those of UK14304 and xylazine at either 16% or 95%O_2 suggests that the full agonists behaved like partial agonists at the lower O_2 tension. Since the maximal reponses to the full agonists at 16%O_2 matched those of the partial agonists at 95% or 16%O_2. This observation might suggest that at reduced O_2 tension the alpha-1 receptor reserve in the rat portal vein is diminished. If this argument were to hold, assuming that UK14304 and xylazine acted as partial agonists on alpha-1 adrenoceptors, then reduction of O_2

would be expected to have a more drastic effect on responses induced by them. For example, Digges & Summers (1983) showed that since guanfacine, which is a full agonist in the rat aorta, a preparation that has a large receptor reserve for alpha-1 (Ruffolo et al, 1980), is only a weak partial agonist in the rat portal vein. They therefore concluded that there must be fewer spare receptors in the portal vein than there are in the aorta.

(+)m-Synephrine is the optical isomer of phenylephrine and (-)m-synephrine has been shown to be a partial agonist at the alpha-1 adrenoceptor in the rat aorta (McGrath et al's unpublished observation). In the portal vein this compound was less potent, but behaved like xylazine and UK1404 in increasing the contractile spikes without an accompanying increase of baseline tension. Like UK14304 and xylazine responses induced by (+)m-synephrine were less susceptible to O_2 change between 95% and 16%.

Clonidine was however the only exception to the rule regarding drugs that increase the contractile phasic activity without an increase in baseline tension, in that, its responses were sensitive to changes in O_2 tension. Although its induced responses were similar to those of UK14304, xylazine or (+)m-synephrine, they were more sensitive to oxygen tension changes between 95% and 16%O_2. A further confirmation of this came from results obtained at an even lower O_2 tension, 4%. The responses

were smaller than they were at either 95% or 16%O_2.
The pD_2 value, 6.93±0.08 for clonidine at 95% was higher than the value obtained by Digges and Summers (1983) which was 5.18±0.05 n=6, but comparable with the value obtained by Ruffolo et al (1980) which was 7.03±0.15 n=6. Clonidine produced about 70% (Ruffolo et al, 1980) and 50% (Digges & Summers' data 1983) of phenylephrine's maximum. In this study clonidine produced about 90% of NA maximum which is quite suprising as it is generally believed to be a partial agonist in this tissue.
The sensitivity of clonidine to O_2 changes vis-a-vs UK14304 or xylazine might have to do with its relatively higher potency. However, if this argument were to hold such similar sensitivity to O_2 tension might be expected from equivalent responses to NA, phenylephrine or amidephrine.

Does 16%O_2 alter the drug-receptor interaction of NA?

Although a qualitatively as well as a quantitatively different response to 95%O_2 was obtained at 16%O_2, the pD_2 values of NA were similar at the two O_2 tensions, when the responses to NA were expressed as percentage of their individual maxima. pA_2 values for prazosin against the tonic component of the response correspond to the equilibrium response that has been measured in cumulative concentration response studies, e.g. Digges & Summers (1983). The pA_2 at 16%O_2, 9.1, obtained from this study is higher than those for NA at 95%O_2

rat:8.39± 0.13 (Digges & Summers, 1983); and in canine: 8.54 (Shoji et al, 1983). However similar values were found in other studies in rat: 8.99±0.11 n=6 (Ruffolo et al, 1980), 9.3 (Hicks, 1982) and 9.43 (Wilson, 1983).

The pA_2 of 11 for prazosin against the phasic component of NA contraction suggests that this component was more susceptible to prazosin than was the tonic component. However, this relatively high pA_2 for prazosin against the phasic even though competitive (slope 0.96) does not represent equilibrium conditions. Since equilibrium condition is one of the criteria for pA_2 determination, it is suggested that the pA_2 value for prazosin against the phasic component may not be a realistic value.

The pD_2 values (6.40) for NA in the presence of NA and cocaine (but not EDTA) were slightly higher than those obtained by Digges & Summers (1983). The difference in methodology might have accounted for this difference. The additives used in their physiological saline were a combination of desmethylimipramine (uptake -1 blocker), normetanephrine (extra-neuronal blocker) and propranolol (B-blocker). It was noted that other workers did not include EDTA in their physiological saline. Ruffolo et al, 1980 used cocaine and propranolol as their additives while Hicks (1983) used propranolol and ascorbate. Harris et al (1983) on the other hand used sodium metabisulphite to prevent NA oxidation. However the use of EDTA in this study increased the potency of NA

greatly and the pD_2 value obtained was 7.96±0.05 (95%O_2). The big difference which EDTA makes to the potency of NA underscores the importance of eliminating heavy metals which catalyse the oxidation of NA in the bath. It was however noted in Wilson's study (1983) that he used a cocktail of EDTA 10μM (an amount lower than 23uM used in this study), ascorbic acid, propranolol and cocaine. The pD_2 he obtained to NA was 6.81±0.04, which is lower than the value obtained in this study.

Are there any alpha-2 adrenoceptors in the rat portal vein?

UK14304 and xylazine had low potency in this tissue. They produced about 60% and 50% respectively of NA maximum. pA_2 for prazosin against UK14304 was 7.9 and for Wyeth was indeterminable. Most workers have shown that alpha-2 adrenoceptors are not present on the rat portal vein (Digges & Summers, 1983; Wilson, 1983). However, Hicks (1983) showed that alpha-2 mediated responses to the increase in phasic activity induced by low concentrations of NA and high concentrations of UK14304. According to Hicks the increase in phasic response to NA was relatively resistant to prazosin vis a vis the tonic response. In the present study such resistance to prazosin was not observed. However the use of a single concentration of prazosin (0.1uM) makes his finding less convincing. Hicks showed that increase in

phasic activity induced by UK14303 was more susceptible to yohimbine than it was to prazosin. This was not corroborated in the present experiments since antagonism by Wyeth 26703 a relatively selective- the alpha-2 antagonist, was weak and inconsistent, whereas a pA_2 value of 7.9 prazosin against UK14304 was found. Thus versus UK14304, the alpha-1 antagonist was considerably less potent while the alpha-2 antagonist had little effect. Taken together, this suggests that the response to UK14304 is mediated by neither alpha-1 nor alpha-2 adrenoceptors, as they are currently defined, but the data are consistent with Hick's. He did not test Wy 26703 and we did not test yohimbine, but the effects of prazosin are consistent. A mixture of presence of post-junctional alpha-1 and alpha-2 as well as pre-junctional alpha-2 adrenoceptor in the portal vein of another species i.e rabbit has been made by Docherty & Starke, (1982). However, differences in results due to species variation cannot be ruled out.

KCl on UK14304 and xylazine-induced contraction

The fact that contractions to UK14303 or xylazine were neither additive nor enhanced by the presence of mildly depolarising KCl suggests these compounds cannot behave as full agonists under normal and mildly depolarized conditions of the RPV. They in this manner differ from

drugs like Bay K 8644 that need, in most tissues, depolarized conditions before they can elicit agonist effects.

The biphasic response at low oxygen tension

The biphasic response at low O_2 tension was only elicited by full agonists. The threshold for the biphasic contraction was 32%O_2. Lower O_2 tensions showed more prominent phasic contraction with a corresponding reduction of the tonic component. This also holds true for KCl-induced contraction. However, in the case of KCl, biphasic contractions at 95%O_2 are not unusual, although the phasic contraction was more prominent at lower O_2 tension. Previous explanation of this biphasic response are that it could be due to (i) a difference in the sources of activator Ca^{2+} , with the rapid phasic contraction being due to intracellular Ca^{2+} and the slower maintained tonic response being due to extracellular Ca^{2+} (van Breemen, 1977; Liejten, 1979); (ii) alternatively this difference could be due to a membrane effect via Na-K ATPase (Detar & Bohr, 1980).

The possibility of two sources of activator calcium was tested by using caffeine, a substance that causes contraction of the vascular smooth muscle through the release of intracellular Ca^{2+}. Wilson (1983) showed that caffeine 20mM did not cause a contration of the RPV. However, from results obtained in this study it was

shown that caffeine could contract this tissue, depending on how it was administered to the tissue. If it was added directly into the bath caffeine would not contract the tissue, rather it would abolish the spontaneous activity within minutes. On the other hand, if it was added to the bath together with the physiological saline after draining, the tissue gave a transient contraction. This condition of observing contraction to Ca^{2+} came about accidentally. It was shown that this contraction was not an artefat (see fig 2.17). What is however puzzling is why one mode of caffeine administration leads to contraction while the other does not?

Assuming that this contraction to caffeine was derived from an intracellular source, the fact that the response at 16%O_2 was more transient than it was at 95%O_2 would suggest that there is more dependence (at 16%O_2) of the tissue response on superfically bound Ca^{2+}.

In a very recent paper by Dacquet et al (1987), it was shown that caffeine could contract the rat portal vein, an effect attributed to utilisation of intracellular Ca^{2+}. A similar result has been reported in the guinea-pig portal vein (Bond et al, 1984 and Nanjo, 1984)

KCl non-sustained response in Na-substituted Krebs.

In order to maintain the osmolarity of high KCl solution, the usually practice is to replace the NaCl by KCl on a molar basis. However, a survey of the use of

KCl in the portal vein showed that in most studies bolus injections of KCl are added directly into the tissue bath without compensating for osmolarity (Fiol de Cuneo et al, 1983; Campbell et al, 1986). The present study showed that while well maintained responses were obtained to high KCl in non-compensated physiological saline, this was not the case in Na substituted high KCl solution. We suggest that the Na substituted high KCl solution is "more physiological", avoiding osmotic effects. Although, Andersson et al (1974) showed that change osmolarity by high KCl does not affect the response of this tissue, our experiments suggest that it does. We were however unable to show in a simple way whether the better maintained response in the Na non-substituted saline was due to the extra Na or not because the responses when we added NaCl during KCl-induced contraction in Na substituted saline this caused a less maintained contraction even though the contractile spikes were enhanced. Thus the effect of increasing osmolarity after initial activation was quite different even though the saline composition was now identical to that obtained when extra KCl was added without substitution. To test whether this better maintained response was due to osmolarity or not, choline was tested on the KCl induced response in a Na non-substituted saline. Choline on its own, contracted the tissue and when tested on KCl induced contraction caused more contraction than osmolarity would have accounted for, if indeed this was the reason for a

better maintained response to KCl in Na non-substituted saline. However, these contractions were well maintained so, leaving aside the contractile effect of choline, this would support the hypothesis that increased osmolarity helps to maintain the response.

In conclusion, from the results in this section it seems that the use of the physiological O_2 tension (16%), does not radically alter the agonist - receptor interaction. We suggest that the differences that exist between the responses at supra-physiological (95%O_2) and physiological O_2 tension lie beyond the receptor. This might have to do with the excitation- contraction coupling mechanism. This possibility has been explored in the subsequent sections. We suggest that the use of supra-physiological O_2 tension might introduce extra and unwarranted effects which might not be a true reflection of in vivo conditions. Indeed, if in vitro experiments are designed to simulate in vivo conditions, the use of 95%O_2 in most pharmacological experiments is highly unjustified. Toxic effects at high O_2 tension cannot be ruled out (Bowman & McGrath, 1982).

CHAPTER 3

ALPHA - ADRENOCEPTOR AGONISTS AND Ca^{2+} DEPENDENCE OF THE ISOLATED RAT PORTAL VEIN.

SUMMARY

1. Ca^{2+}-induced responses in NA-stimulated RPV have been compared in unbuffered Krebs and in E.G.T.A + N.T.A buffered Krebs.

2. Though spontaneous activity was depressed by Ca^{2+} buffers in non-stimulated tissues, responses in the presence of NA were greater than in unbuffered. Consequently, higher $CapD_2$ values were obtained using the buffers.

3. Qualitatively, similar responses were obtained in both buffered and unbuffered. The $CapD_2$ values were unaffected by changing the O_2 from 95% to 16%, if allowance was made for a time dependent decrease between the 1st concentration/response curve and the 2nd.

4. Bay K gave a $CapD_2$ value of 4 in the buffers but gave a lower value of about 3.7 in the unbuffered saline.

5. Comparison of the Ca^{2+}-sensitivity of the tissue to various alpha-adrenoceptor agonists was made. NA and amidephrine at 0.3µM or 1µM gave $CapD_2$ values of 3.5 and above. On the other hand, UK14304 and xylazine at 10uM gave $CapD_2$ values less than 3, only the high

concentration of xylazine 100μM approaching 3.5. The maximum tension developed at 95% O_2 was significantly higher than at 16% for NA and amidephrine. In the case of UK14304 and xylazine the maximum tension at 95% was significantly higher than at 16% at high concentrations only, suggesting that the responses of these two agonists were less sensitive to oxygen tension changes than were NA and amidephrine.

6. An attempt was made at isolating the biphasic response to NA at low oxygen tension.

INTRODUCTION

Ca^{2+} is necessary for contraction in smooth muscle as well as in other contractile systems. The dependence of the response of the portal vein of various species: rabbit (Collins et al, 1972); rat (Sigurdsson et al, 1975; Ebeigbe et al, 1980; Ebeigbe 1982; Arner et al, 1983); guinea-pig (Hertog et al, 1984; Inoue et al, 1985) on extracellular calcium has long been recognised.

In the isolated rat portal vein, however, experiments carried out in 95% O_2 showed that this tissue's responses to NA and K^+ are particularly highly dependent on extracellular Ca. Evidence for this was that within a few minutes exposure to a $Ca2^+$ free medium the rat portal vein loses its spontaneous activity as well as responsiveness to drugs (Hellstrand et al, 1975) .These same authors were able to show that another source of Ca, i.e superficially bound Ca^{2+} could be

involved in K^+ -induced contraction and lately Ebeigbe (1982) has shown that this source of Ca could participate in NA -induced contractions.

It has been shown in Chapter 2 that at 95% O_2 ($\underline{P}O_2$ = 580±12mmHg), responses to agonists are monophasic, and bigger than at a lower, more physiological $\underline{P}O_2$ (16% O_2: 112±10mmHg), in which responses are biphasic. The biphasic response obtained at low O_2 tension is reminiscent of the response of the rat aorta to NA first described by Bohr 1963. According to Bohr such a biphasic response involves different sources of activator Ca^{2+}. He showed that the isotonic contraction of the rat aorta to adrenaline could be differentiated into fast and slow components. The fast component was completed within 45-60 sec of the initial stimulation and the slow component was the response over and above the fast component during the remainder of the observation period. The fast component was due to release of intracellular Ca, while the slow component was a result of the entry of extracellular Ca (Sitrin and Bohr,1971; Triggle and Swarmy 1980; Swarmy and Triggle 1980; Bose and Innes, 1975; Turlapaty, Hester and Carrier, 1976).

Recently it has been shown that contractions of the rat portal vein to NA (Ebeigbe et al, 1980) and indeed other full alpha adrenoceptor agonists (see Chapter 2) are dependent on oxygen. Ebeigbe et al (1980) have shown

a causal relationship between O_2 and Ca^{2+} in their study of hypoxic vasodilatation of different vascular smooth muscle preparations.

We have now examined the $[Ca^{2+}]_o$ dependence of contraction in different O_2 tensions to determine whether $[Ca^{2+}]_o$ dependence and the effects of drugs which alter this are altered by Po_2.
Secondly the biphasic nature of the response at lower Ca was further examined using the Ca^{2+} buffers-EGTA + NTA (i.e Ca loading and Ca washing method). The EGTA + NTA Ca^{2+} buffer has been recently used by McGrath et al (1984) to isolate the different components of the response of the rat anococcygeus and to identify these components with different levels of $[Ca^{2+}]_o$. A similar method has been adopted in this series of experiments to separate the fast contraction at 16%O_2 from the slow component at the low O_2 tension.

METHODS

Isometric contractions of longitudinal strips of portal vein were recorded (male Wistar rats 245-255g; Krebs' bicarbonate saline, Ca^{++} 1.25mM, 16%O_2: 79%N_2: 5%CO_2. $[Ca^{2+}]_o$ sensitivities of spontaneous activity and contraction to NA 0.3μM were determined and were repeated in 95% O_2 and 5% CO_2.

Responses were obtained employing 3 different protocols:

Protocol (1) Discrete responses (5 min) at each $[Ca^{2+}]_o$ level (non-cumulative), illustrating effects on the time course of contraction.

Protocol (2) NA was added when $[Ca^{2+}]_o$ was low and $[Ca^{2+}]_o$ was increased in steps (cumulative), allowing more rapid estimation of $[Ca^{2+}]_o$ sensitivity (i.e a even shorter period).

Protocols 1 and 2 were repeated :

(i) with $[Ca^{2+}]_o$ unbuffered, i.e. total Ca = $[Ca^{2+}]_o$ = 44uM to 5mM,

(ii) buffered with NTA (nitrilotriacetic acid) and EGTA (2.5mM of each), wherein total Ca = 2.5mM to 10mM but $[Ca^{2+}]_o$ = 1μM to 5mM.

The buffers allow accurate determination of $[Ca^{2+}]_o$ (otherwise impossible with $[Ca^{2+}]$ <0.1mM. Buffering is particularly necessary when normal sensitivity to $[Ca^{2+}]_o$ is increased by some experimental manoeuvre, e.g. drugs.

Sensitivity to $[Ca^{2+}]_o$ is expressed as $-\log(EC_{50})$ where EC_{50} is the $[Ca^{2+}]_o$ producing a response 50% of the

maximum obtained within the range of $[Ca^{2+}]_o$ tested.

Protocol 2 above was repeated in the presence of drugs that influence Ca^{2+} i.e. Bay K and nifedipine, which are a Ca^{2+} facilitator and a Ca^{2+} antagonist respectively.

When determining the effects of O_2, 1st and 2nd concentration response curves were determined in 16%O_2 in time matched control tissues while the 2nd CRC was run in 95%O_2 in the test tissues.

Protocol (3): Attempt to isolate the elements within the biphasic contraction induced by NA at 16%O_2.

Protocol 1 was modified as follows: 10min before discrete responses to NA 1µM were taken tissues were incubated in EGTA + NTA Krebs containing 1.25mM $[Ca^{2+}]_{free}$. The tissues were then washed into each $[Ca^{2+}]_o$ level for 5min before producing a response to NA 1uM for 5min. This procedure was repeated for the next higher Ca^{2+} level.

DRUGS USED:

Amidephrine hydrochloride, noradrenaline (Sigma), xylazine (Bayer), UK14304 (Pfizer), Bay K 8644, nifedipine (Bayer). (Except where otherwise stated, in experiments with NA , cocaine 1µM was added).

RESULTS

1. Cumulative $[Ca^{2+}]$ / response in non Ca^{2+} buffered saline

a. Effect on spontaneous activity

In control experiments, without agonists added, upon washing with zero Ca^{2+} three times over 30s, the spontaneous activity disappeared within a further 2 min. Upon the stepwise addition of Ca: the $[Ca^{2+}]$ threshold for the spontaneous activity was 0.63mM in either O_2 tension, the maximum phasic spontaneous activity was reached at 2.5mM Ca^{2+} in 5 out of 6 tissues and in 5mM Ca^{2+} in the remaining tissue.
See Table 3.1 for the $CapD_2$ values.

The time controls showed a log shift of 0.05 ± 0.01, n=4, which was not statistically significant . The responses to Ca^{2+} re-addition up to 5mM did not differ significantly $P > 0.05$ in the two O_2 tensions. See Fig 3.1a. The contractile spikes showed an inverse relationship between the frequency of the spikes and the $[Ca^{2+}]$.

b. Ca^{2+} response in NA stimulated tissues

In this experiment, two periods of exposing the tissues

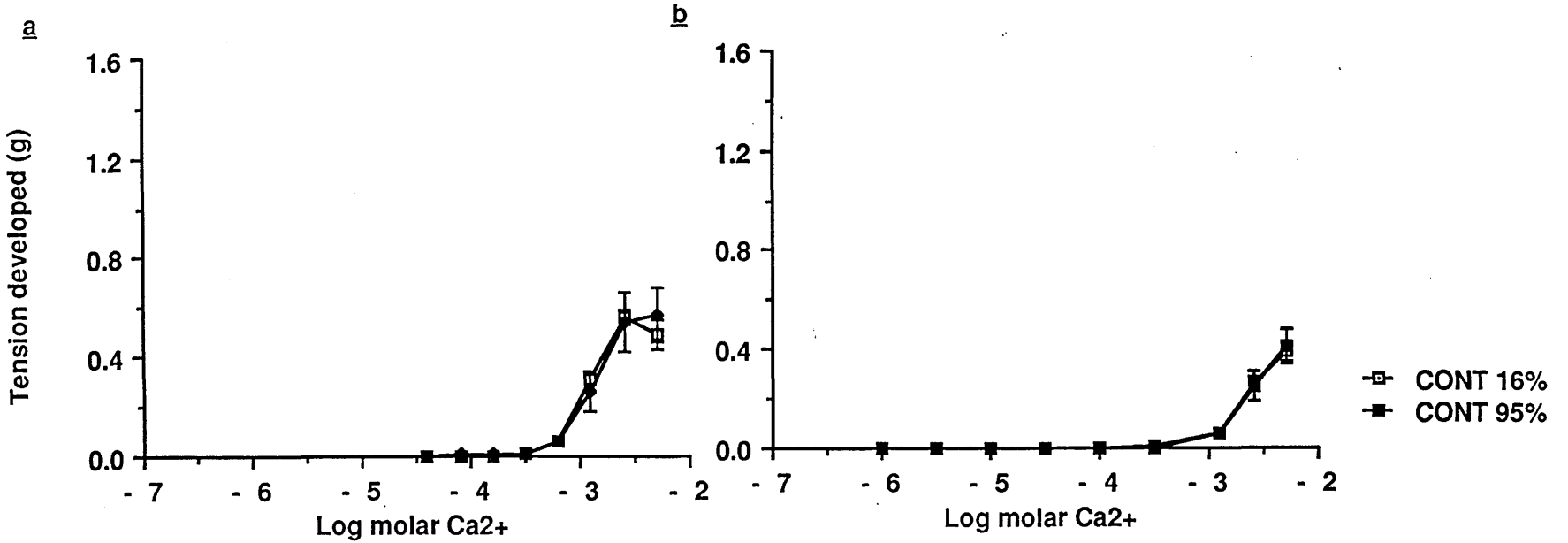


Fig 3.1

The response of the non-stimulated rat portal vein to Ca^{2+} cumulative re-addition from (a) unbuffered (b) 5.0mM EGTA + NTA buffered. This " spontaneous activity" was smaller in buffered than in unbuffered Ca^{2+}. The data at 16%O_2 and 95%O_2 was from paired experiments.

Points represent means ± s.e.m., n = 6.

to zero Ca^{2+} prior to Ca^{2+} re-addition were examined. Tissues were activated with either of two concentrations of NA (0.3μM and 1μM). See figs 3.2a-d.

The results show that there was no difference in responses between exposing the tissues to zero Ca^{2+} for 5min and 10min (a similar observation was made by Hicks (1983).

Addition of NA 0.3uM to tissues in "nominally" zero Ca^{2+} caused a small increase in tone of 0.03g±0.01 (n=6). This was well maintained and increased to 0.08g ±0.01 (n= 6) when a higher concentration of NA i.e. 10^{-6}M was used. Addition of 40μM Ca^{2+} caused a further increase in the maintained tone but few spikes occurred. From 75uM Ca^{2+}, the contractile spikes appeared with much increased frequency. Increasing Ca^{2+} concentration caused increased maintained response. However at 2.5mM Ca^{2+} the contractions were no longer maintained. The maximum response was usually reached at 2.5mM in 4 out of 6 of preparations while it was reached at 1.25mM in the remaining tissues.

Table 3.1 shows $CapD_2$ in control and NA treated tissues. It also shows values obtained during two periods of tissue exposure to zero Ca^{2+}.

The $CapD_2$ values were higher at the higher NA concentration (table 3.1). Also there was a slightly higher time control shift between the 1st and 2nd CRC at NA 1μM.

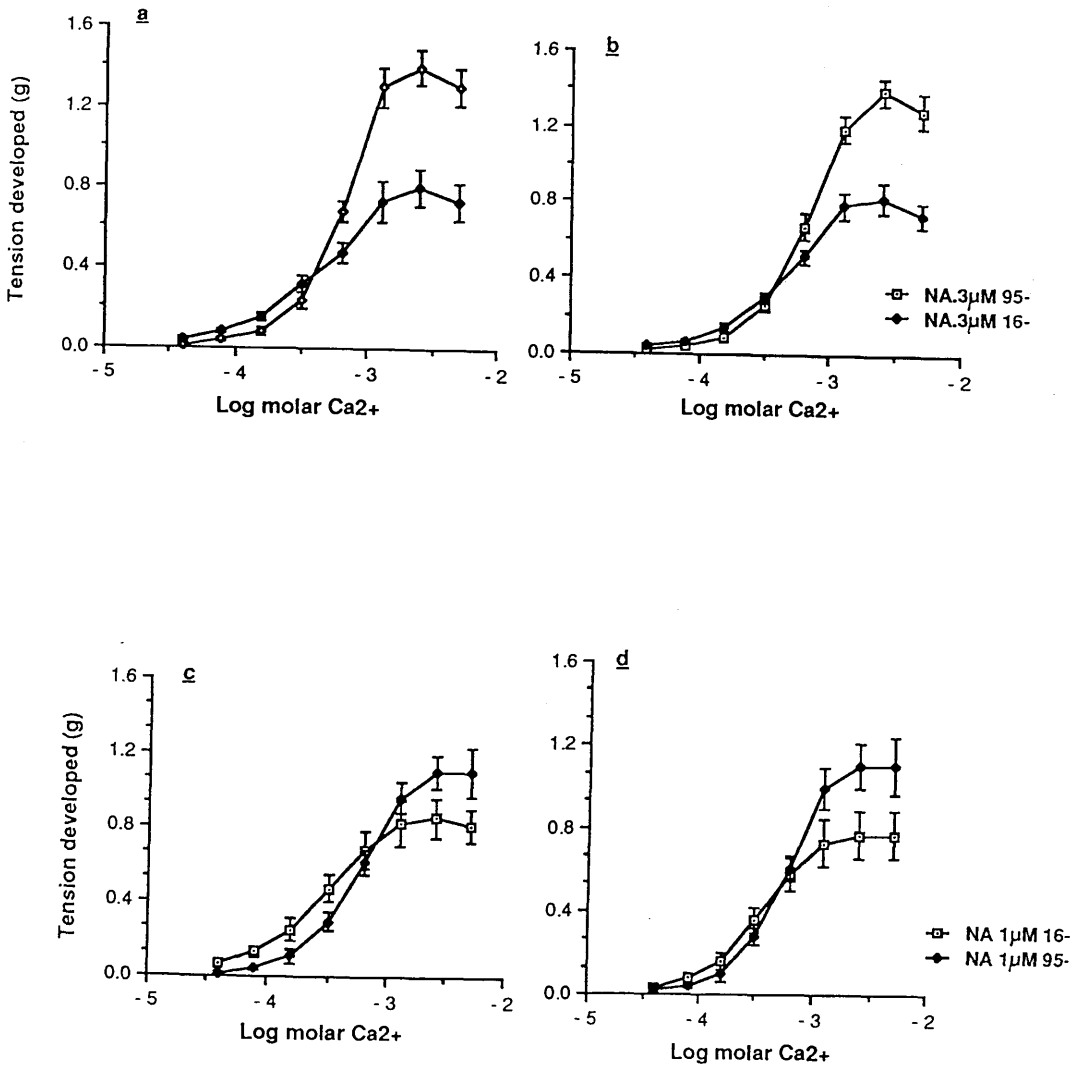


Fig 3.2.
Effect of time of incubation in zero Ca^{2+} on subsequent response of RPV to unbuffered Ca^{2+} cumulative re-addition at either 16%O_2 or 95%O_2 in paired experiments. a & b show the Ca^{2+} response in NA 0.3μM stimulated tissues with prior exposure to zero Ca^{2+} saline for 5 and 10min respectively, while c & d show the Ca^{2+} response under conditions similar to a & b but in the presence of a higher NA concentration, 1μM.

The $CapD_2$ was similar at both physiological Po_2 level i.e. 16% and hyperoxic level, i.e. 95%, in NA stimulated preparations. Therefore the $CapD_2$ was unaffected by the O_2 tensions (table 3.1).

The difference in Ca^{2+} response in the two O_2 tensions occurred at $[Ca^{2+}]$ above the $CapD_2$. From 1.25mM to 5mM Ca^{2+}, the responses were significantly higher in 95% than in 16%O_2 (see fig 3.2).

At lower levels of Ca^{2+} the Ca^{2+} responses remain qualitatively similar in the two O_2 tensions i.e from 35uM to 0.15mM. The response was characterised by a slow contraction which peaked between 1 and 2min did not show the biphasic response which is characteristic of the response in 16% at higher $[Ca^{2+}]$. From 0.63mM Ca^{2+}, responses in 16%O_2 were slightly biphasic, though the slow rise was still evident. However, because this series of experiments employed cumulative Ca2+ re-addition, it would be difficult to discern the two phases. As usual, responses in the higher O_2 tension were monophasic.

The data presented in figs 3.2 a-d and table 3.1 represent the peaks of maintained responses occurring by 5min.

Irrespective of the O_2 tension at the end of the re-addition there was a post-wash contraction on returning to zero Ca^{2+}. This contraction waned gradually with

Ca2+/ response to NA in unbuffered experiments (cumulative Ca2+ re-addition).

	-logEC50	
	16%O2	95%O2
NA 0.3μM		
5min	3.37 ± 0.06	3.32 ± 0.08
10min	3.38 ± 0.1 (NS)	3.35 ± 0.05 (NS)
NA 1μM		
5min	3.56 ± 0.05	3.34 ± 0.08
10min	3.46 ± 0.06 *	3.34 ± 0.06 (NS)

Table 3.1 shows the -log EC50 values for Ca2+ when two periods of exposure to zero Ca2+ prior to cumulative re-addition of Ca2+ were examined on NA stimulated RPV. Values are means± s.e.m., n=6.
T- tests show comparison between values at 5min and those at 10min. * and NS represent P <0.05 and not statistically significant respectively.

spontaneous activity, ceasing by 1min in 16%O_2 and 3min in 95%O_2. It was observed that the rate of disappearance of the spontaneous activity in zero Ca^{2+} was dependent on the level of the control spontaneous activity prior to addition of drugs (i.e more activity, slower disappearance).

2. Cumulative Ca^{2+}/ response in EGTA + NTA buffered saline

a. Effect on spontaneouus activity

In absolute size, control responses, i.e. spontaneous activity, were smaller in the Ca^{2+} buffered Krebs than they were in unbuffered. The Ca^{2+} threshold in this control experiment was higher in the buffers; it was 1.25mM in 5 out of 6 preparations and 0.31mM in the remaining preparation. From 1.25mM Ca^{2+} there was an increasing response to Ca^{2+}. See Table 3.2 for the $CapD_2$ values in unbuffered and buffered Ca^{2+}. Like the result in unbuffered Ca^{2+}, the Ca^{2+} control responses were similar in the two O_2 tensions, though the $CapD_2$ values in buffered Ca^{2+} were lower than in unbuffered Ca^{2+}.

b. Ca^{2+} response in NA stimulated tissues

In examining the responses to NA 0.3μM, both protocol 1 & 2 (see under methods) were used. Since protocol 1-

Characteristics of [Ca2+]o/response relationship (cumulative Ca2+).

		Unbuffered		Buffered		
	O2	16%	95%	16%	95%	
-logEC50	spontaneous	2.92 ± .02	2.84 ± .06	2.70 ± .20	2.65 ± 0.6	NS
	NA control	3.38 ± .06	3.35 ± .10	3.53 ± 0.13	3.23 ±0.08	NS, (Pa ***)
Max. tension (g)	NA control	0.83 ± .08	1.37 ± .07	0.59 ± 0.5	0.87 ± .06	
		***	*	**		
+Bay K 0.3μM						
	NA control	3.32 ± 0.02	3.14 ± 0.03	3.7 ± 0.04	3.43 ± 0.06	
	+ Bay K	3.77 ± 0.05	3.60 ± 0.05	4.1 ± 0.05	3.90 ± 0.05	(Pb **)
	Log shift	0.45 ±0.05	0.47 ± 0.05	0.33 ±0.09	0.47 ± 0.04	
			NS		NS	
+Nifedipine						
	NA control			3.37 +0.11		
	+ Nif 1nM			2.99 + 0.03 (NS)		
	NA control			3.7 + 0.03		
	+ Nif 10nM			2.80 +0.05 *		

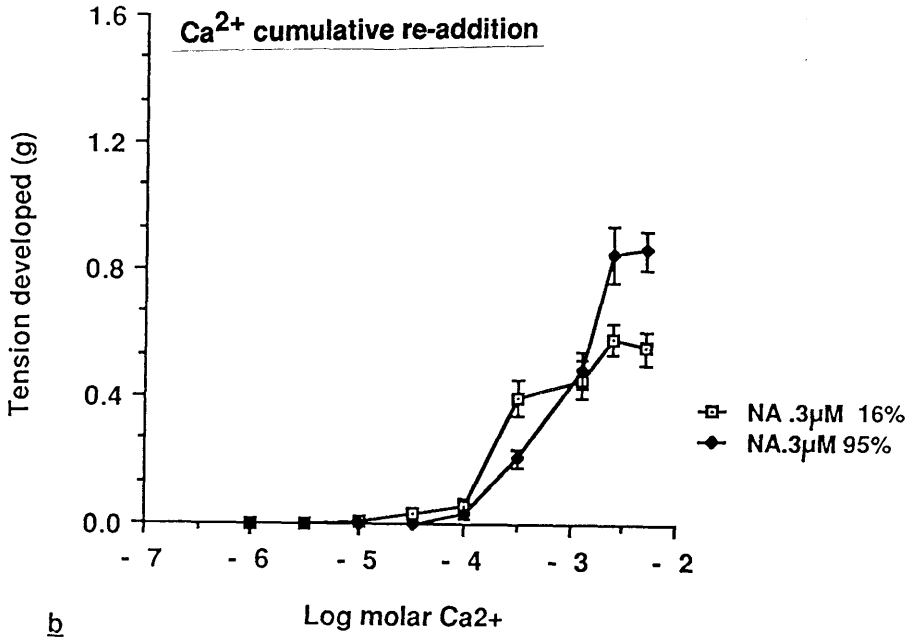
Table 3.2 shows the CapD2 values obtained in unbuffered and N.TA and EGTA buffered Ca2+ in control tissues and NA 0.3μM contracted tissues at either 95%O2 or 16%O2. Effects of Bay K or nifedipine were determined in separate series of experiments.

The values shown are means ± s.e.m in 4-6 preparations.

*, **, *** and NS represent $\overline{P}$ < 0.05, 0.01, 0.001 and not statistically significant respectively. The values compared are indicated by lines. In addition Pa is the comparison of CapD2 values of spontaneous activity wth those obtained in the presence of NA (NA control). This was statistically significant (P< 0.001) at both O2 tensions whether buffered or unbuffered.

Similarly Pb is the comparison of the CapD2 for NA control with NA in the presence of Bay K . This was statistically significant (P < 0.01) at both O2 tensions whether buffered or unbuffered.

a



b

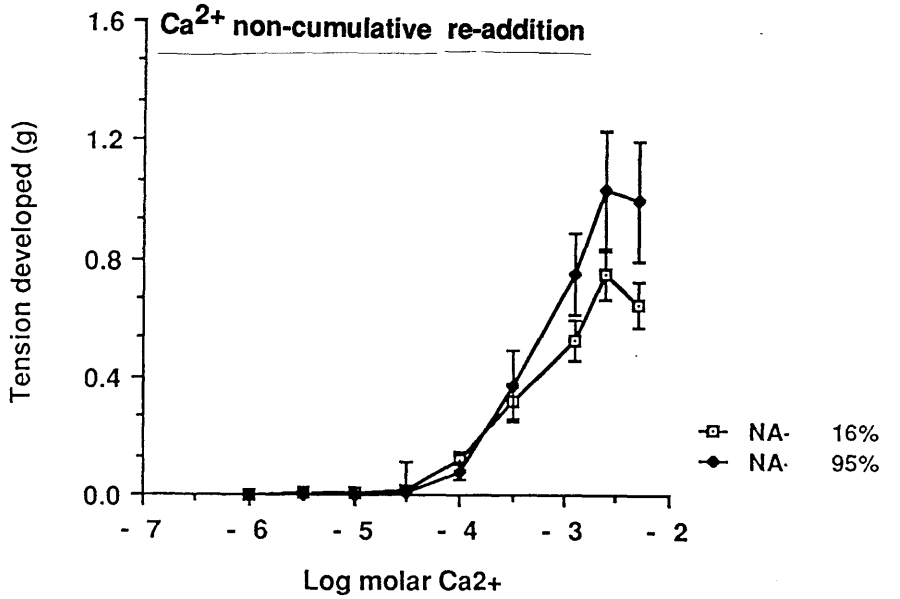


Fig 3.3

The response of NA 0.3µM stimulated RPV to either Ca^{2+} cumulative or non-cumulative re-addition in buffered Ca2+. Each procedure was carried out in paired experiments first at 16%O_2 and then 95%O_2. Points represent means ± s.e.m., n = 6.

which is non- cumulative, it allows the two phases in the response to NA at low O_2 to be discernible. The Ca^{2+} threshold was 30uM in 2 tissues and was 0.1mM in the remaining 4 tissues. The Ca^{2+} threshold for the appearance of the spontaneous activity however was 0.3mM which was much higher than the value in unbuffered Ca^{2+} (75μM).

Table 3.1 shows the $CapD_2$ obtained using protocol 2, see also fig 3.3a and stimulating with NA 0.3μM, which produced a log shift of 0.16. The slight decrease in sensitivity, using protocol 2, was due to loss of tissue sensitivity with time. This was confirmed by reversing the order of gassing in separate experiments, i.e. gassing first with 95%O_2 and then 16%O_2. The following $CapD_2$ values were obtained: 3.71±0.2 and 3.56±0.09 in 95% and 16% respectively. A log shift of 0.15 between the Ca response at 95% and 16% was obtained; this shift corresponds to that obtained when the tissues were gassed first with 16%.

The $CapD_2$ values 3.2±0.14 and 3.38 ± 0.10 at 16%O_2 and 95%O_2 respectively, using protocol 1 were lower than the values obtained by protocol 2, fig 3.3b.
Using protocols 1 & 2, it was found that differences in response obtained to NA in 16% and 95%O_2 occurred at 2.5mM and 5.0mM with the maximum response in both O_2 tensions attained at 2.5mM Ca^{2+}. The responses were significantly higher in 95% at there two above highest Ca^{2+} concentrations.

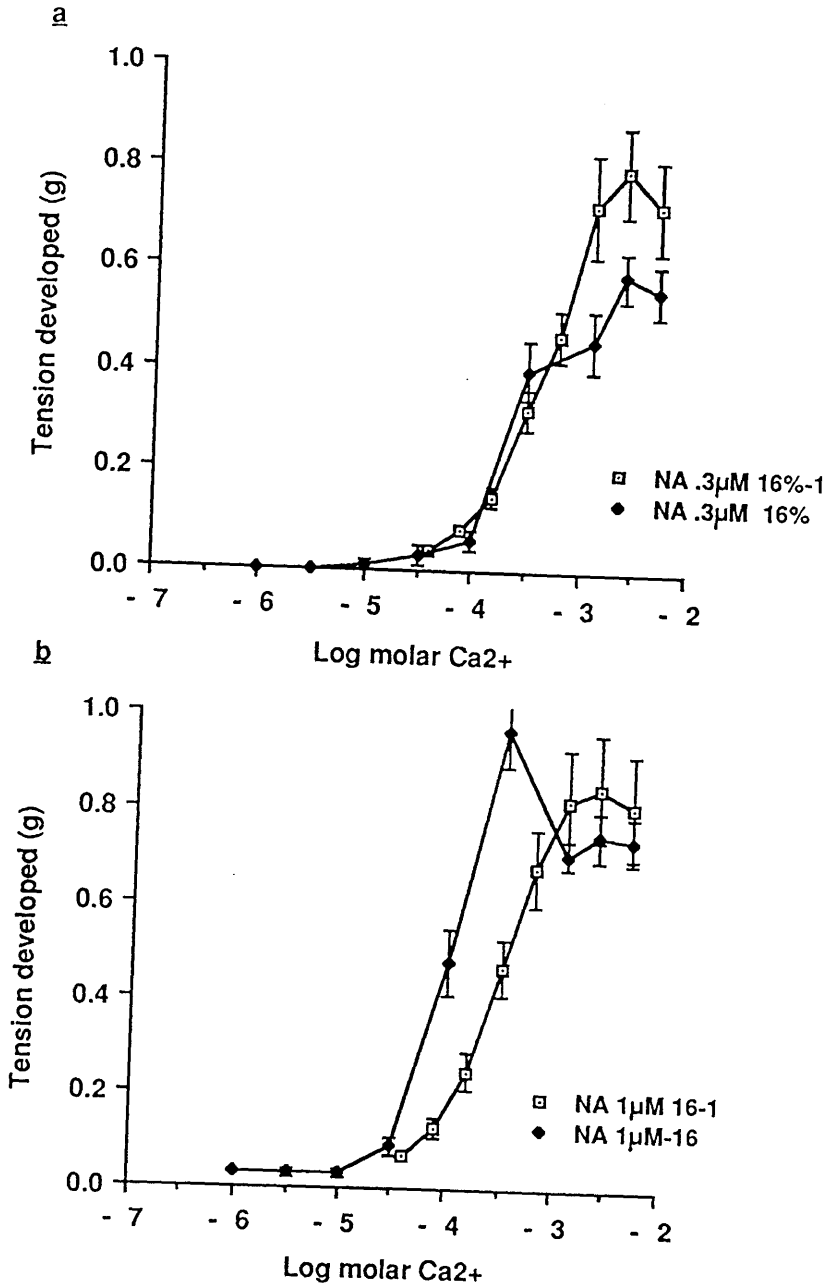


Fig 3.4

The response of RPV to Ca^{2+} cumulative re-addition when the tissues are stimulated with (a) NA 0.3μM and (b) NA 1μM in unbuffered and in buffered Ca^{2+}.

Points represent means ± s.e.m., n = 6.

With protocol 1 the biphasic response to NA was evident from 1.25mM Ca^{2+} and above. It was noted that noradrenaline - induced contraction waned faster using protocol 1 than protocol 2.

Comparing the results in buffered Ca^{2+} with those in unbuffered Ca^{2+}, the $CapD_2$ in the former were higher than they were in unbuffered Ca^{2+} Krebs, with protocol 1 but not with protocol 2.
The absolute sizes of the responses in the buffered and unbuffered Ca^{2+} Krebs at low Ca^{2+} were comparable though the responses at high Ca^{2+} were significantly higher ($P< 0.05$) in the unbuffered. See figs 3.4 a & b.

3. <u>'Ca^{2+}-free contractions'</u>

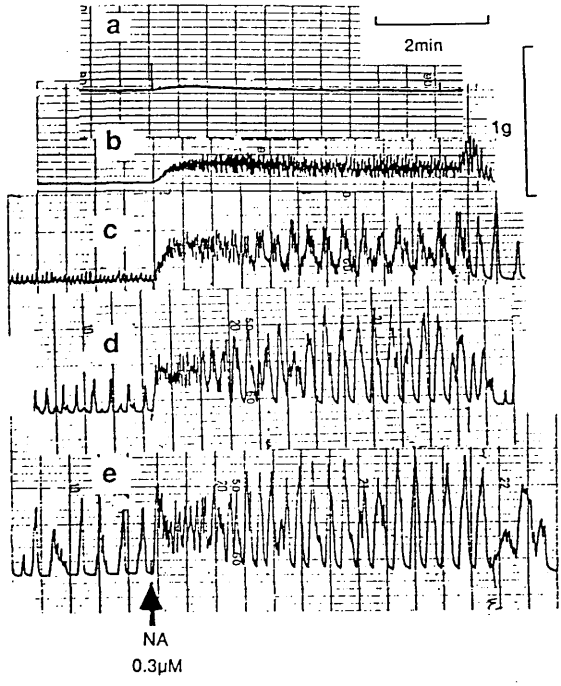
The rat portal vein has been shown previously to be unable to cause a contraction in 'Ca free Krebs' (Sigurdsson et al, 1975; Ebeigbe et al, 1980; Marriot 1985). In the present experiments, NA 0.3μM or 1μM caused a contraction in nominally 'Ca^{2+} free Krebs. This contraction was characterised by a small increased tone without phasic activity and was maintained throughout the 5min observation. The time course of this response is shown in fig 3.5. This contraction matched the NA (1μM) -induced contraction at 30μM in buffered Ca^{2+}. This suggests a contamination Ca^{2+} level of 30μM in the nominal 'Ca-free Krebs' as there was no response to NA

Fig 3.5

Upper panel shows time course of the RPV responses to NA 0.3μM at various unbuffered $[Ca^{2+}]_o$: zero, 0.31, 0.63, 1.25 and 2.5mM Ca^{2+} in a, b, c, d, and e respectively in non-cumulative unbuffered Ca^{2+} re-addition at 16%O_2.

Lower panel (left) shows time course of responses to NA 0.3μM at various low buffered $[Ca2+]_{free}$(EGTA + NTA buffers) 30μM, 0.1mM and 0.3mM.

Lower panel (right) shows time course of response of the rat anococcygeus muscle (McGrath, 1985) to NA 3μM in EGTA or EGTA + NTA buffers. Note the transient contraction obtained at low $[Ca]_o$ (1nM - 1μM). This contrasts with the RPV in which no response is obtained below 30μM. Responses in RPV above this concentration are not transient. $[Ca^{2+}]$ 1.25mM is the threshold concentration for the biphasic response obtained in the RPV at 16%O_2 (see upper panel -d).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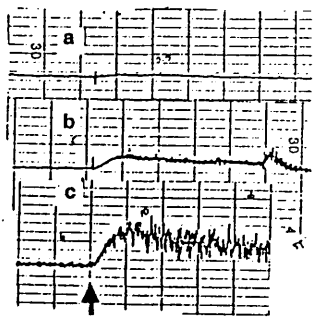
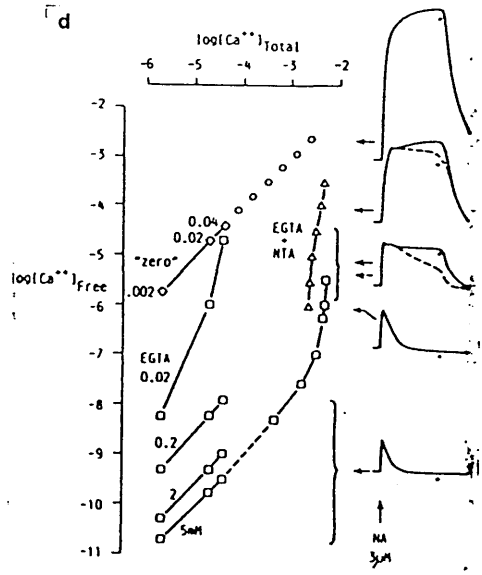
2min
b
1g
c
d
e
NA
0.3μM


a
b
c


d
log[Ca++]Total
-6
-5
-4
-3
-2
log[Ca++]Free
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
0.04
0.02
"zero"
.002
EGTA
0.02
0.2
2
5mM
EGTA
+
NTA
NA
3μM

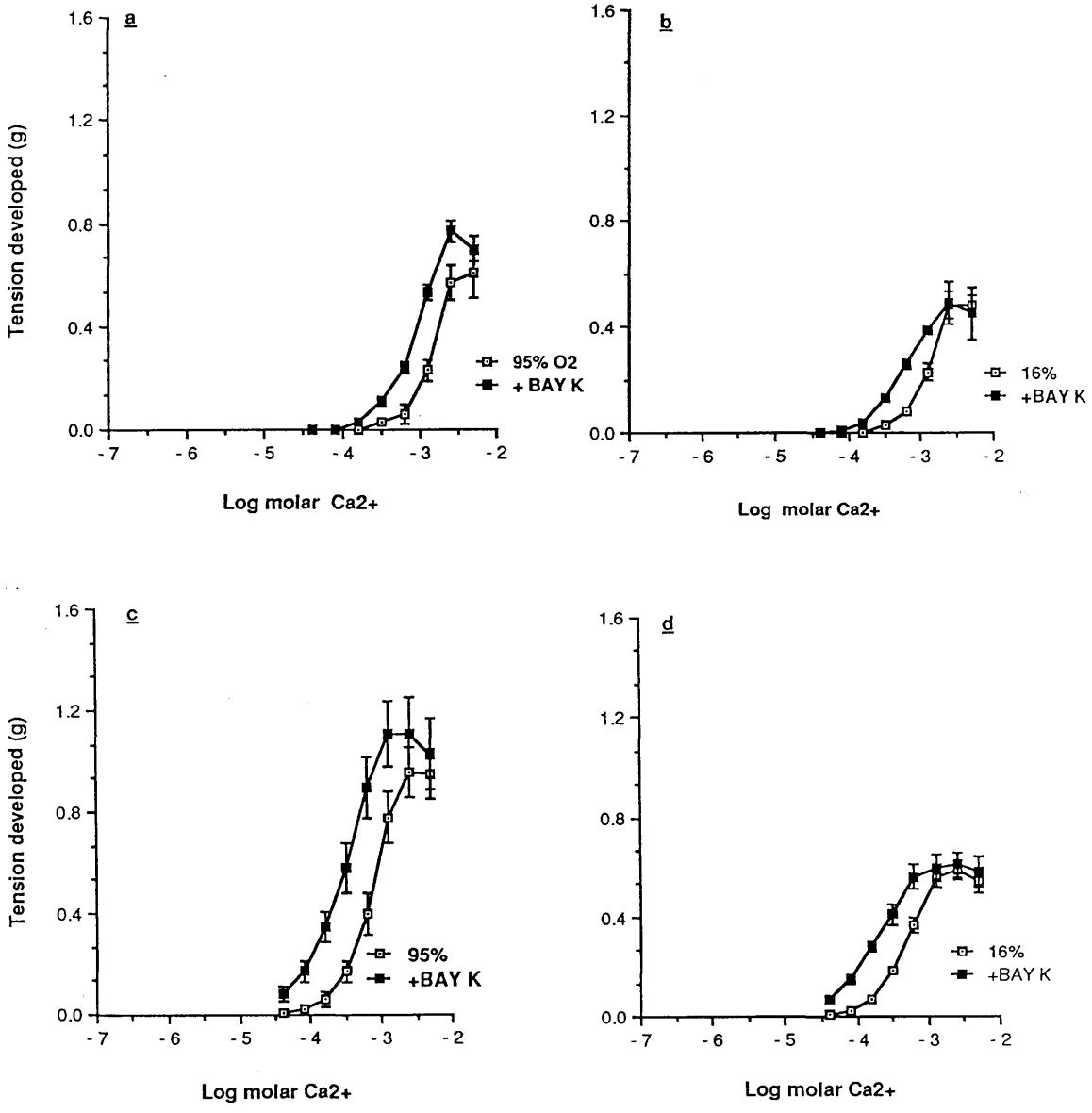


Fig 3.6

The effect of Bay K 8644 on the spontaneous activity during Ca^{2+} re-addition in unbuffered Ca^{2+} at (a) 95%O_2 and (b) 16%O_2 in separate experiments.

Also shown is the effect of Bay K 8644 on NA 0.3μM stimulated RPV during Ca^{2+} re-addition in (c) & (d) unbuffered Ca^{2+} at 95%O_2 and 16%O_2 respectively, in separate experiments, but before and after Bay K 8644 were carried out in the same tissues.

Points represent means ± s.e.m., n = 6.

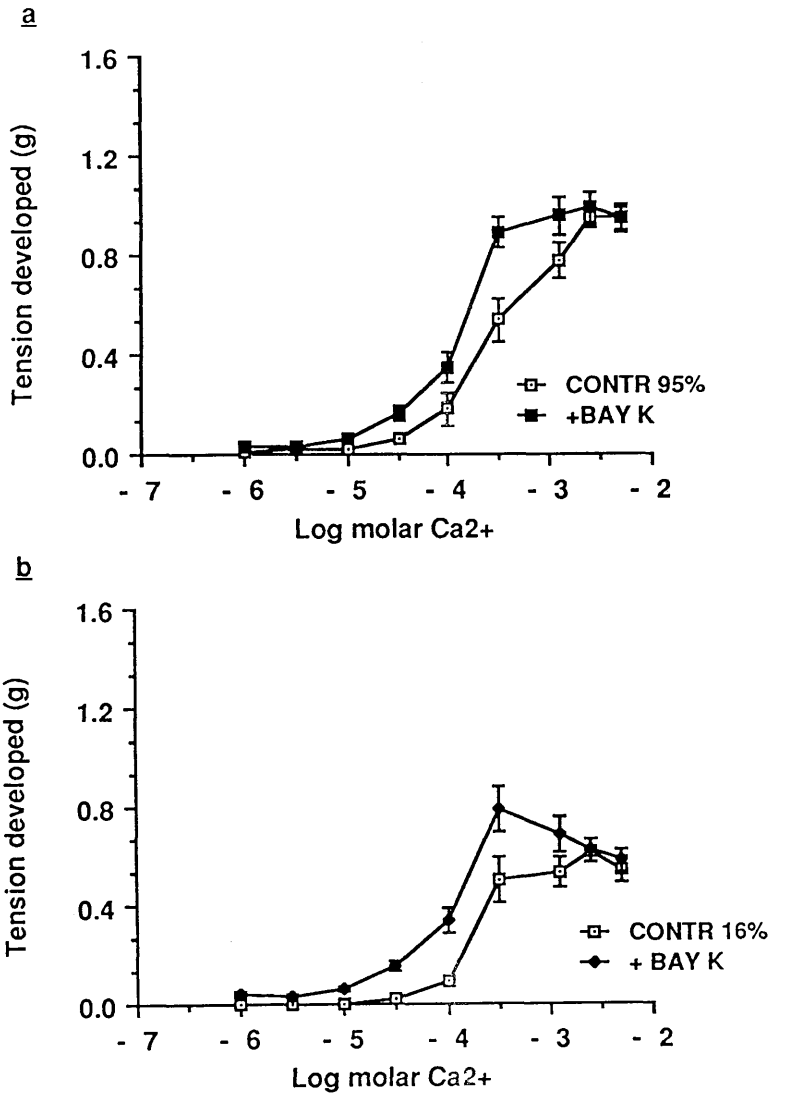


Fig 3.7

The effect of Bay K 8644 0.3μM on NA 0.3μM stimulated RPV during cumulative Ca^{2+} re-addition in buffered Ca^{2+} at (a) 95%O_2 and (b) 16%O_2 respectively. The experiments in each O_2 tension were carried out separately, but before and after Bay K 8644 were carried out in the same tissues.

Points represent means ± s.e.m., n =6.

in EGTA + NTA Ca^{2+}- free buffers as well as in low buffered Ca^{2+} level up to 10μM.
This response in "nominally" Ca-free Krebs was highly susceptible to nifedipine 1nM (see below).

4. Effect of Bay K and nifedipine on the Ca^{2+} response relationship of NA.

Experiments in unbuffered Ca^{2+} showed that the control $CapD_2$ in 16% was higher than it was in 95% , in unpaired experiments. This may arise because of a smaller absolute size of the maximum response in 16% and should not be over-emphasized.

In the presence of Bay K 0.3μM, the Ca^{2+}/ response curves in NA 0.3μM stimulated preparations were shifted significantly ($P < 0.01$ to the left in either unbuffered or buffered Ca^{2+}, see figs 3.6 and 3.7. The log shifts caused by Bay K at 50% maximum were not statistically different from each other at 95% O_2 and 16% O_2 whether in buffered or unbuffered experiments, see table 3.2. An interesting difference between the buffered and unbuffered Ca^{2+} experiments was that Bay K could produce a $CapD_2$ of approximately 4 in the buffered Ca^{2+} in either O_2 tension, while it was slightly lower than 4 in the unbuffered saline.

Nifedipine 1nM - 0.1μM caused a rightward shift in the Ca^{2+} response curves. These experiments were carried out

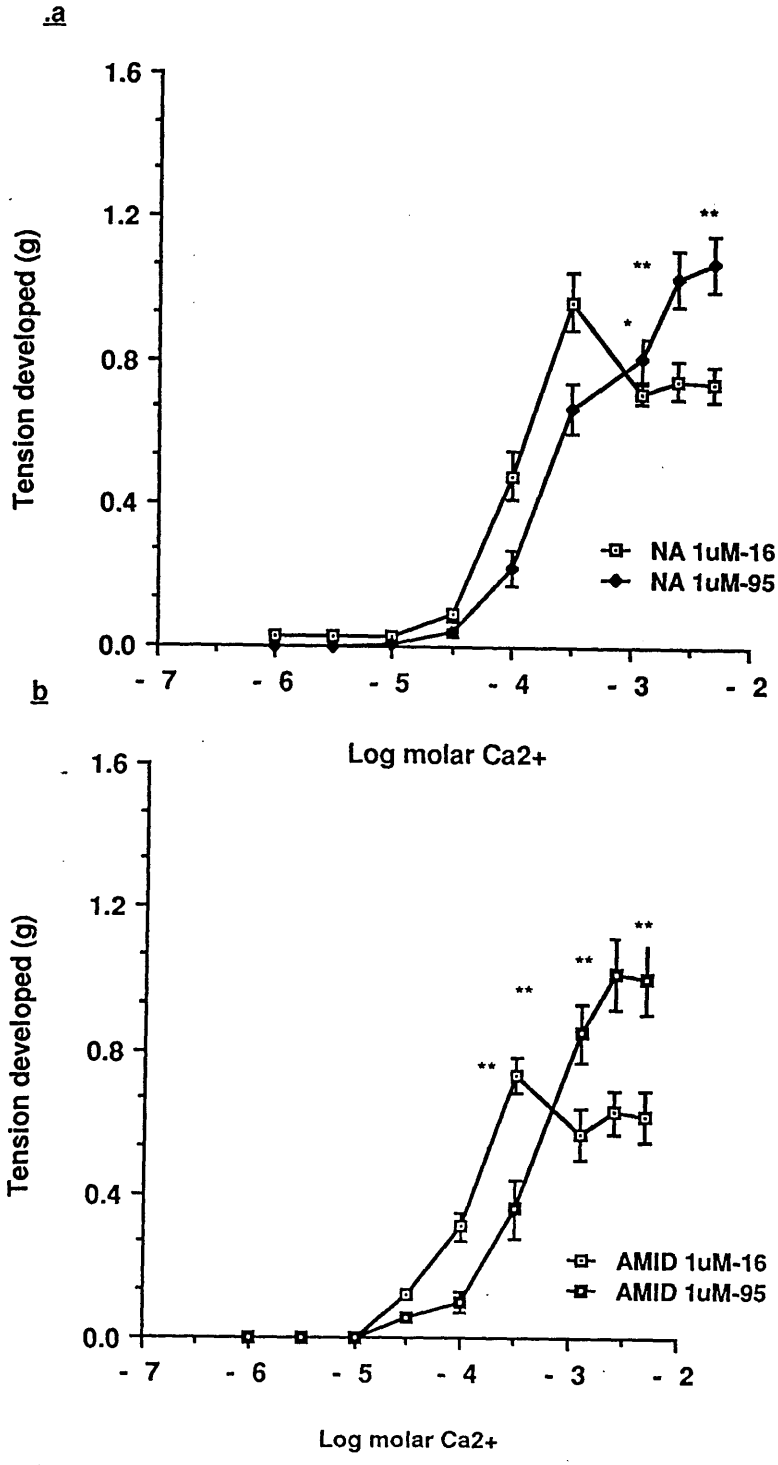


Fig 3.8

The response of RPV to Ca^{2+} cumulative re-addition when the tissues are stimulated with (a) NA 1μM and (b) amidephrine 1μM in buffered Ca^{2+} at 16%O_2 and 95%O_2. Each agonist was tested on separate tissues but the experiments at both O_2 tensions were paired.

Points represent means ± s.e.m., n =6.

*, ** show P < 0.05 and 0.01 respectively.

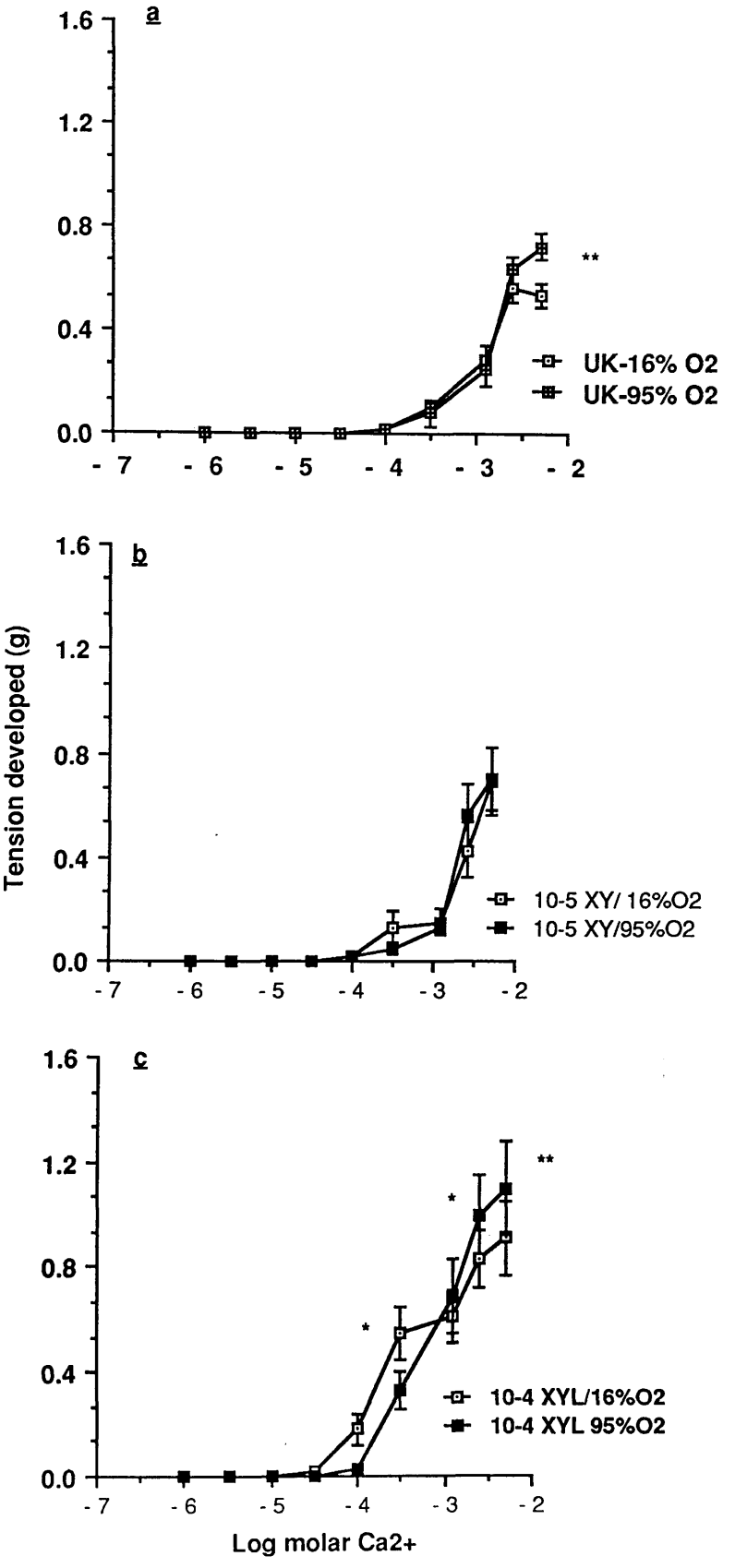
Fig 3.9

The response of RPV to Ca^{2+} re-addition when the tissues are stimulated with (a) UK14304 10μM and (b) & (c) xylazine 10μM and 100μM respectively, in buffered Ca^{2+} at 16%O_2 and 95%O_2.

Each agonist was tested on separate tissues but the experiments at both O_2 tensions were paired.

Points represent means ± s.e.m., n =6.

Note that response of UK14304 10μM were statistically significantly higher (** P< 0.01) and 95%O_2 than at 16%O_2 only at 5.0mM Ca^{2+}, while those for xylazine 100μM were significantly higher (** P < 0.01) at 95%O2 than at 16%O_2 from 2.5mM Ca^{2+}.


a
1.6
1.2
0.8
0.4
0.0
- 7
- 6
- 5
- 4
- 3
- 2
**
UK-16% O2
UK-95% O2
b
Tension developed (g)
10-5 XY/ 16%O2
10-5 XY/95%O2
c
*
*
**
10-4 XYL/16%O2
10-4 XYL 95%O2
Log molar Ca2+

only in buffered Ca^{2+} in 16%O_2. See Table 3.2 for the log shift caused by 1nM and 10nM nifedipine.

5. Cumulative Ca^{2+}/ response in buffered saline with other alpha-adrenoceptor agonists.

The Ca^{2+} response curves to other alpha-adrenoceptor agonists were examined, namely, amidephrine 0.3µM and 1uM; UK14304 3µM and 10µM; xylazine 10µM and 100µM. The $CapD_2$ values obtained are tabulated in Table 3.3. The protocol was to take the 1st CRC in 16% and then determine the 2nd CRC at 95%, after 15-30min in the latter. The table 3.3 shows that there was a drop in the mean $CapD_2$ at 95% irrespective of the agonist examined. However this drop was time dependent as there was a similar shift between the 1st and the 2nd CRC in 16%. This time dependent drop involved a loss of response at low Ca^{2+} levels. Most experiments were limited to two CRCs per tissue because of the time dependent loss in tissue sensitivity. However in one set of experiments a third CRC in NA stimulated tissue was examined. More of the tissue response was lost in the lower [Ca^{2+}] while the responses at the higher Ca were a little enhanced.

It was noted too that the higher the potency of the agonist the more was the time dependent loss. For example UK14304 and xylazine at 10µM caused a smaller time dependent shift than did NA or amidephrine (see figs 3.8 a & b and also fig 3.9 a, b & c)

Summary of the Ca2+/response characteristics of alpha-adrenoceptor agonists.

Agonist	16%O2 -log EC50	16%O2 Maximum tension (g)		95%O2 -log EC50	95%O2 Maximum tension (g)
Noradrenaline 0.3μM	3.70 ± 0.06 (6) NS (Pd)	0.59 + 0.05	NS (Pc)	3.50 + 0.10 (6) NS (Pb)	0.87 ±0.06 * (Pa)
" " " 1μM -	4.00 ± 0.03 (6) -	0.75 +0.05	-	3.69 +0.03 (6) * (Pb)	1.08 ±0.08 ** (Pa)
Amidephrine 0.3μM	3.72 ±0.15 (6) NS (Pd)	0.8 +0.11	*** (Pc)	3.06 +0.01 (6) ** (Pb)	0.99 ± 0.02 NS (Pa)
" " " 1μM	3.93 ±0.03 (6) (NS (Pd)	0.63 +0.06	NS (Pc)	3.71 + 0.03 (6) *** (Pb)	1.02 ±0.10 ** (Pa)
UK14304 3μM	2.99 ± 0.04 (4) *** (Pd)	0.61 + 0.1	*** (Pc)	2.84 + 0.01 (4) NS (Pb)	0.77 ± 0.1 NS (Pa)
" " " " 10μM	2.95 ± 0.05 (10) *** (Pd)	0.57 +0.05	*** (Pc)	2.91 +0.05 (10) NS (Pb)	0.73 ±0.05 ** (Pa)
Xylazine 10μM	2.68 ± 0.06 (6) *** (Pd)	0.69 +0.13	*** (Pc)	2.74 + 0.02 (6) NS (Pb)	0.70 ±0.12 NS (Pa)
" " " 100μM	3.56 ± 0.11 (6) ** (Pd)	0.91 +0.14		3.27 +0.06 (6) * (Pb)	1.10 ±0.18 ** (Pa)

Table 3.3 shows the -log EC50 values obtained for various agonists in buffered Ca2+ cumulative re-addition experiments.
The experiments in both O2 tensions were paired, with the responses first determined at 16%O2 and then 95%O2.. The values represent the means ± s. e.m of the -log EC50 values. The number of observations is indicated in brackets.
The maximum tension developed at 16%O2 has been compared with that at 95%O2, using student's paired 't' test.
The symbols *, ** and ***represent P values < 0.05, 0.01 and 0.001 respectively, while NS represents not statistically significant.
(Pa) statistical signficance test between the maximum at 16%O2 with that at 95%O2 for each agonist tested.
(Pb) statistical signficance test between the -log EC50 value at 16%O2 with that at 95%O2 for each agonist tested.
(Pc) statistical signficance test between the -log EC50 value for NA 1μM at 95%O2 with those at 95%O2 for other agonists tested.
(Pd) statistical signficance test between the -log EC50 value for NA 1μM at 16%O2 with those at 16%O2 for other agonists tested.

The $CapD_2$ values of the rat portal vein for the two concentrations of NA or amidephrine examined were 3.50 or above, while those for the UK14304 and xylazine were below 3, see table 3.3. The one exception was the very high concentration of xylazine, 100μM, where the $CapD_2$ moved nearer to that of NA.

The optimal $[Ca^{2+}]$ varied with the agonist and with agonist concentration. The optimal $[Ca^{2+}]$ ranged from 30μM to 2.5mM. Responses at 95% O_2 were significantly higher ($P < 0.01$) than at 16% at > 1.25mM $[Ca^{2+}]$ for noradrenaline and amidephrine. On the other hand responses were higher only at 5mM Ca^{2+}, for UK14304 and xylazine ($P < 0.01$). See table 3.3 for the maximum tension developed by each agonist at 95% O_2 and 16% O_2.

6. Biphasic response to NA at 16% O_2

The responses to NA 1μM at 16% and 95% were compared at various levels of $[Ca^{2+}]_o$ in buffered Krebs using protocol 3. The initial experiments were done using 2min equilibration in the Krebs containing varying $[Ca^{2+}]$ as described by Sigurdsson et al (1975), Ebeigbe, (1982) and Wilson, (1983).

There was $[Ca^{2+}]_o$ related response from $[Ca^{2+}]$, 1uM. From $[Ca^{2+}]_o$ 10uM, there was a maintained contraction to NA 1uM which peaked within 1 min. The time course of this response was unchanged up till Ca^{2+} 0.1μM. This maintained response was not the same as the transient

response described by McGrath (1985) using the same Ca^{2+} buffers in the rat anoccocygeus muscle. From Ca^{2+} 1.25mM, there was a biphasic response, see fig 3.5.
It therefore appeared that this method could not separate the different phases of the response. A similar experiment carried out at 95% O_2 did show a similar time course of monophasic response to NA 1μM across the range 1μM - 0.1μM Ca^{2+}. From 1.25 Ca^{2+} the responses to NA were bigger at 95% than were at 16% O_2, while the responses at 16% O_2 became biphasic those at 95% O_2 remained monophasic up to 5.0mM Ca^{2+}.

DISCUSSION

The aim of this Chapter was to determine the Ca^{2+} dependency of the smooth muscle contraction in rat portal vein when Ca^{2+} is unbuffered or buffered with E.G.T.A (2.5mM) and N.T.A (2.5mM). The responses to NA (0.3uM and 1uM) were dependent on extracellular Ca^{2+}. Even the small contraction in "nominally" zero Ca^{2+} unbuffered saline could be accounted for. The Ca^{2+} buffers allow more accurate determination of the free Ca^{2+} levels (McGrath et al, 1984) and therefore by comparing contractions under buffered and unbuffered conditions an estimation of the contamination level of Ca^{2+} could be determined. Thus Ca^{2+} contamination in nominally Ca^{2+} free Krebs, was estimated to be 30uM which is 1.5 times the value determined using a pH metric method (Silleu et al, 1974; Ashley & Moisescu,

1977) and 3 times the value estimated in similar experiments with the rat anococcygeus without E.G.T.A + N.T.A buffers (McGrath, 1985). The sources of contamination in nominally Ca^{2+}-free saline are glass, distilled H_2O and purest chemicals (Silleu et al, 1974; Ashley & Moisescu, 1977; Miller & Smith, 1984). This contraction in the nominally "Ca-free" was rapidly lost as it was not reproducible during the readdition of low Ca^{2+} concentrations up to 44µM . Unlike the I.T.C (initial transient contraction) found in other vascular preparations (van Breemen, 1972) and in rat anococcygeus (McGrath 1985, see fig 3.5) the response in the nominally"zero Ca^{2+}" unbuffered Ca^{2+} is abolished by reducing Ca^{2+} further with buffers and is therefore not "zero Ca^{2+}" resistant. The I.T.C is due to utilisation of intracellular Ca^{2+} in tissues that depend on intracellular as well as extracellular Ca^{2+} as the source of activation such as the vascular smooth muscle of the aorta and , rabbit ear artery, and in non vascular smooth muscle such as the rat anoccocygeus.

It may not be entirely valid to estimate the Ca^{2+} contamination level in nominally free Ca^{2+} using the responses in the buffers since , the $CapD_2$ values were slightly (higher in either 16% or 95%) than they were in the non-buffered Ca^{2+}. In fact allowing for this would reduce the estimate of the contamination level to the values found with other tests (see above). In the presence of Bay K the $CapD_2$ were approximately 4 with the buffers. Estimating this at > 0.1µM without

buffering is unreliable since accurate estimation is not possible without buffering since contamination and deliberately added Ca^{2+} are of the same order.

Although the responses in the buffered saline in NA stimulated isolated portal vein were higher than the equivalent responses in the unbuffered Krebs, the spontaneous activity of the tissue was depressed in the buffers so that any synergism would be reduced. This loss of spontaneous activity was unexpected, but such depression could be a property commmon to most buffers as discussed below.

The main drawback to the use of the 5.0mM E.G.T.A + N.T.A buffers is the fast deterioration of the tissue responses during the second and third concentration response curves, especially at the lower Ca^{2+} concentrations of 1μM - 30μM: and at the higher Ca^{2+} concentrations responses were not depressed and were sometimes slightly enhanced. The higher the concentration of activator, the bigger the shift of the 2nd CRC to the right of the 1st CRC. The time dependent shift was more pronounced with the potent activators NA and amidephrine than was with the less potent agonists, UK14304 and xylazine.

This time dependent shift to the right of subsequent CRC appears to be a property of the E.G.T.A + N.T.A buffers since in the unbuffered saline there were small but non-significant shifts to the right between the 1st, 2nd and

3rd CRCs. The absolute size of the spontaneous activity in the Ca^{2+} buffers was smaller than in unbuffered. Taken together these results suggest some depression of responsiveness by the buffers. It is not clear whether this is a consequence of buffering *per se* or is due to a further toxic effect. Altura et al (1982) reviewed artificial buffers such as tris(hydroxymethyl)aminomethane (Tris), 2-(N-2-hydroxypiperazin-N'-yl)ethanesulphonic acid (HEPES), morpholinopropanesulphonic acid (MOPS), N,N-bis(2-hydroxyethyylglycine) (Bicine), piperazine-N,N'-bis(2-ethanesulphonic acid) (PIPES), and imidazoles commonly used as buffering agents in physiological media for studies of the role of Ca^{2+} in excitation-contraction coupling events in muscle. These authors showed that some of these buffers such as Tris and other zwitterion buffers may exert toxic effects on numerous cell types (Brown & Groot 1986; Stinson & Spencer, 1968; Shipman 1969; Morris 1971; Medzon & Gedies, 1971; Goshima 1973; Daniel & Wolfe 1975; Brune,1980). However, it is not known whether similar toxic effects on cells are exerted by Ca^{2+} buffers.

Any differences obtained between the Ca^{2+} readdition in 16% and 95%?

The $CapD_2$ values were not different at 16% and 95% in the unbuffered saline. In the buffered saline however, the time dependent fall in sensitivity caused an

apparent fall in the 2nd gas tension tested whatever order was employed. The only real difference between the two oxygen tensions was the higher response in 95% at the higher Ca^{2+} levels. In the unbuffered saline more consistent responses were obtained, throughout the $[Ca^{2+}]_o$ range, responses being equal at lower levels and significantly higher at 95% than at 16% only at 2.5mM Ca^{2+} and 5.0mM Ca^{2+}. In the buffered saline, the responses were higher at 95% O_2 from 1.25mM Ca^{2+}, comparing the 1st CRC in the buffered saline with same in unbuffered saline.

This suggests that at the lower levels of Ca^{2+}, the rat portal vein cannot discriminate between hyperoxia and normoxia. The differential effect of oxygen at the higher levels of Ca^{2+} occurred only with the potent activators NA and amidephrine, being more difficult to demonstrate with the less potent UK14304 and xylazine, except at a high concentration, of xylazine i.e. 100μM. This confirms the report in Chapter 2 using 2.5mM Ca^{2+} that the increase in the height of contractile spikes caused by either UK14304 or xylazine is hardly affected by oxygen (Fasehun et al, 1986).

With the exception of xylazine, the $CapD_2$ values obtained in the rat portal vein are comparable with those in the rat anococcygeus which were 3.91 (3.69), 3.88 (3.71), 3.72 (3.27) for NA, amidephrine and xylazine respectively, [equivalent RPV values in bracket] (see table 3.3). The $CapD_2$ values for xylazine

were higher in the anococcygeus than in the portal vein.

Bay K 8644 caused a similar shift in the concentration/ response curve of Ca^{2+} suggesting that in both O_2 tension, Bay K could facilitate to a similar extent in the two O_2 tensions.
Nifedipine, a Ca^{2+} antagonist, caused a rightward shift in NA concentration/ response curve at 16%O_2.
The effects of Bay K and nifedipine have been more fully examined in the next three sections.

The biphasic nature of the response to NA at lower O_2 tension.

The biphasic contraction is quite different from those observed in tissues which utilise both extracellular as well as intracellular sources of Ca^{2+} since the Ca^{2+} threshold is relatively much higher at about 1.25mM and above. The biphasic contraction at lower O_2 tension which is not due to inhibition of the sodium pump as it is resistant to ouabain (see previous section), but could be due to the utilisation of two sources of calcium as the O_2 tension is lowered from hyperoxia to normoxia. Below normoxic levels the phasic component of the contraction to NA as well as KCl became more distinct as reported in the previous chapter and confirming Hellstrand, et al (1977).

The rat portal vein, as indeed the portal vein of other species: rabbit and guinea-pig (Collins et al 1972; Inoue et 1985; Hertog et al 1983) is dependent on extracellular Ca^{2+} for its contraction to drugs (Sigurdsson et al 1975; Ebeigbe et al 1980; Marriot 1985). However, Sigurdsson et al (1975) suggested that superficially bound Ca^{2+}, which is wholly dependent on the presence of extracellular Ca^{2+} does participate in the contraction of the rat portal vein, confirming Collins et al's (1972) observation in the rabbit portal vein. Sigurdsson et al demonstrated a rapid rate of rise of KCl-induced (122mM) contraction during simultaneous addition of high KCl and 2.5mM Ca to isolated rat portal vein, after prior exposure of such tissues to nominally zero Ca^{2+} for 6min up to 30min. They suggested that this was due to superficially bound Ca^{2+}. However these authors did not buffer the Ca^{2+} and the possibility of 'contamination Ca' contributing to the rapid rate of rise of the response cannot be ruled out. Ebeigbe (1982a) showed that it was possible obtain a transient contraction to NA which was susceptible to ryanodine, an intracellular Ca^{2+} antagonist. He suggested that this contraction was due to intracellular Ca^{2+}. The procedure employed in this experiment is similar to Ebeigbe's, yet the contraction at low buffered Ca level was not transient. A very recent paper by Dacquet et al (1987) demonstrated that in Ca^{2+} free, EGTA- containing solutions high concentrations of Ach (0.1mM), NA (10uM) and caffeine 25mM caused a transient contraction, which

they suggested was a measure of the amount of internal calcium present in a drug-sensitive calcium store. This intracellular Ca^{2+} was maximally filled after 10-12min loading in 2.1mM Ca^{2+} and was depletable after 4-6min in Ca^{2+}-free solution. The protocol employed by Dacquet et al is similar to the one employed in the present experiment except that they employed a supramaximal concentration of NA i.e. 10uM. Thus it seems that while higher concentrations of NA may produce some release of intracellular Ca^{2+}, the levels used in the present study did not.

From the results presented in this chapter it appears that different sources of activator Ca^{2+} could account for the biphasic response to NA and indeed that of the other full agonist- amidephrine, but perhaps because these two sources of Ca are in rapid equilibrium with each other it would be difficult to separate them. The phasic component is probably due the the utilisation of 'superficially bound Ca'. Its predominance as the oxygen tension is lowered could suggest the dominance of this source of Ca^{2+} over free extracellular Ca at lower O_2 tension. Indeed it has been shown by Marriot (1985) in the rat aorta that there is increased dependency of the vessel on intracellular Ca^{2+} in hypoxia as assessed by the effect of normoxia and hypoxia on the the EGTA-resistant component of NA-induced contraction.

However this second source of activator Ca^{2+} in the portal vein differs for the 'intracellular source' of Ca

activator in vessels such as the aorta in the following regard:

(1) The evidence for caffeine- induced contraction in this tissue is controversial. Caffeine, which causes contraction by releasing intracellular Ca^{2+} has been shown not to contract the rat portal vein (Anderson et al 1974; Wilson, 1983) while Dacquet et al (1987) suggested it could. Caffeine was shown to contract the RPV (Chapter 2), but because of the pecularity of the contraction, caution has been exercised in intrepreting the result.

(2) Ultrastructural observations of Ebeigbe (1979) indicated that intracellular structures associated with the storage of Ca^{2+} are poorly developed in the rat portal vein. Similar observations have been made in the portal vein from other species namely rabbit and guinea pig (Somlyo & Somlyo, 1971; Devine et al, 1972), which also show spontaneous activity.

(3) Contractions to NA in "Ca^{2+} free" saline are susceptible to low concentrations of nifedipine, i.e. lnM, and are potentiated by the Ca^{2+} facilitator Bay K 8644. In these regards it is different from the contraction due to intracellular Ca^{+} activation in the aorta.

CHAPTER 4

EFFECTS OF BAY K 8644 IN ISOLATED RAT PORTAL VEIN.

SUMMARY

1. In Krebs' containing "normal" Ca^{2+} (2.5mM), Bay K 8644 (1nM - 1μM) produced concentration-related enhancement of the spontaneous phasic activity of isolated portal vein , which was optimal at 0.3μM, but at [Bay K 8644] $\geq$ 3μM the phasic activity was attenuated.

2. In normal Ca^{2+} Krebs, Bay K 8644 (3nM - 10μM) did not potentiate responses induced by noradrenaline [NA] 0.3uM. Higher Bay K 8644 concentrations (3μM and 10uM) however caused small attenuation of the noradrenaline induced responses: these experiments were carried out in 4%O_2,16%O_2 and 95%O_2, with similar results.

3. In low $[Ca^{2+}]_o$ (< 40μM) however Bay K 0.3μM greatly enhanced responses to NA with maximum potentiation attained at [Ca]= 1.25mM. At $[Ca^{2+}]_o$ 2.5 and 5.0 mM responses to NA in the presence of Bay K were not different from the control. This was similar at 95% and 16% O_2 tensions.

4. In "Ca^{2+}-free" Krebs, KCl (50mM) produced no response: subsequent Ca^{2+} re-addition gave a sustained contracture. Bay K from 1nM potentiated these Ca^{2+}-induced responses in KCl -depolarised tissue in 16%O_2

but did not potentiate the corresponding responses in 95%O_2. However in tne presence of a mildly depolarising concentration of KCl (20mM), Bay K 8644 was able to facilitate Ca^{2+}-dependent responses at 16% and 95% to the same extent.

INTRODUCTION

Bay K 8644 (methyl-1-4 dihydro-2,6- dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate], a 1,4-dihydropyridine derivative, is an analogue of nifedipine. It has been reported to possess positive inotropic and vasoconstrictor effects opposite to those of nifedipine ,and to enhance KCl-induced but not noradrenaline -induced contractions of the rabbit aorta (Schramm et al, 1983). An attractive interpretation of these results is that Bay K 8644 promotes transmembrane influx of extracellular calcium through specific calcium channels in both cardiac and vascular smooth muscle (Schramm et al, 1983) possibly by acting as an allosteric effector of the channel to shift a pre-existing equilibrium between closed and open channels towards the open state or by increasing the channel open time (Triggle, 1984).

The rat portal vein is a tissue which has a myogenic activity similar to microvascular vasomotion (Wiedeman 1966), has been previously employed to evaluate possible Ca^{2+} related mechanisms involved in excitation-

contraction coupling (Sigurdsson, Uvelius & Johannson 1975; Jetley & Weston, 1980; Hermsmeyer, Trapani & Abel 1981; Hicks 1983 a,b), and is particularly sensitive to Ca^{2+} entry blocking drugs (Jetley & Weston, 1980). In the isolated rat portal vein Bay K 8644 in concentrations up to 1uM potentiated the spontaneous phasic contractions of the tissue (Mikkelsen, 1985; Mikkelsen, Nyborg & Jakobsen 1985).

In the present study we have examined the effects of Bay K 8644 on noradrenaline- and KCl-induced effects with a view to further elucidating the excitation-contraction coupling mechanism in the rat portal vein. We have also examined the possible modulatory role which O_2 may have on such a mechanism.

METHODS:

After equilibration with 16% O_2, the tissues were contracted for 5min with either NA 10µM or KCl 50mM, and then washed. This was repeated until reproducible responses were obtained. Tissues were then washed and allowed to rest for 15min. They were then washed 2-3 times with zero Ca^{2+} and, after 5-10min in the latter, test drugs were added. From this point two different protocols were followed. (i) In the Ca^{2+} cumulative re-addition experiments, 5min later, Ca^{2+} was re-added, cumulatively in 3 times steps of concentration. (ii) In the Ca^{2+} "non-cumulative" experiments, after the washout with zero Ca^{2+} and 5-10min incubation in the latter, a

response to the test activator was taken for 5 min followed by wash out. Thereafter the tissue was exposed to increasing Ca^{2+} concentrations, allowing 10 min in each before the activator drug was tested for 5 min. With each protocol, after completing this control Ca^{2+} concentration/response curve with NA or KCl, the tissues were incubated with Bay K 8644 for 15-30 min before repeating the curve. Parallel controls were run without activator and time controls were run without Bay K 8644 for the second curve.

In "zero Ca^{2+}" Krebs, $CaCl_2$ was omitted. In experiments that involve high KCl, NaCl was replaced on an equimolar basis by KCl.

EDTA 23uM was routinely included in the Krebs while cocaine 1uM was also added for all those involving NA. All experiments were carried out at 37°C.

The bath Po_2 tensions at 95%, 16% and 4% were 590 ± 12, 110 ± 10 and 30 ± 2 mm Hg respectively.

To avoid the breakdown of dihydropyridines by light, the solutions were protected from light and the experiments were carried out in organ baths wrapped with aluminium foil.

Analysis of results.

The spontaneous phasic activity and drug induced effects were analysed separately. Each component was expressed as % of its own control response or as absolute tension measurements. Paired and unpaired student's t-tests were

used to compare results in 16% with those in other O_2 tensions. P values less than 0.05 were considered to be significant.

pD_2 is the negative log molar concentration of a compound that produces 50% of the maximum response obtained across a range of concentrations tested. This was used to quantify either sensitivity to Ca^{2+} or Bay K 8644.

Drugs:

(-) Noradrenaline bitartrate (Sigma), Bay K 8644 (Bayer) and cocaine (McCarthys). Stock solutions of all drugs were dissolved in distilled water except for Bay K 8644 which was dissolved in 20% w/v of alcohol in water. The highest bath concentration of ethanol used (0.2%) did not affect the tissue responsiveness. All concentrations refer to the final bath concentrations of the drugs.

RESULTS

1. Effect of Bay K 8644 on the phasic activity of the portal vein in $[Ca^{2+}]_o$= 2.5mM.

a. Spontaneous activity in non-stimulated preparations:

examined at 95%O_2.

Bay K 8644 tested non-cumulatively (1nM - 10μM) increased the height of spontaneous phasic contraction of the portal vein with a bell-shaped concentration-response curve, without increasing baseline tension. At the optimal concentration of 0.3μM, Bay K 8644 caused 109.1 ± 10.5% (n=6) potentiation which was reduced to 34.2 ± 18.2% at 10μM. See Fig 4.1. The pD_2 value for Bay K 8644 was 7.72±0.08.

b. In NA or KCl- stimulated preparations.

There was a qualitative difference in contraction to noradrenaline between 95%O_2 and 16%O_2. The response to NA in the lower pO_2 tension was biphasic. A fast phasic component lasting 15s was followed by a maintained tonic component. On the other hand, the response in 95%O_2 in most of the tissues was monophasic except to high concentrations of (NA > 10μM), to which biphasic responses were obtained (Fasehun et al, 1985; Fiol de Cuneo et al, 1983).

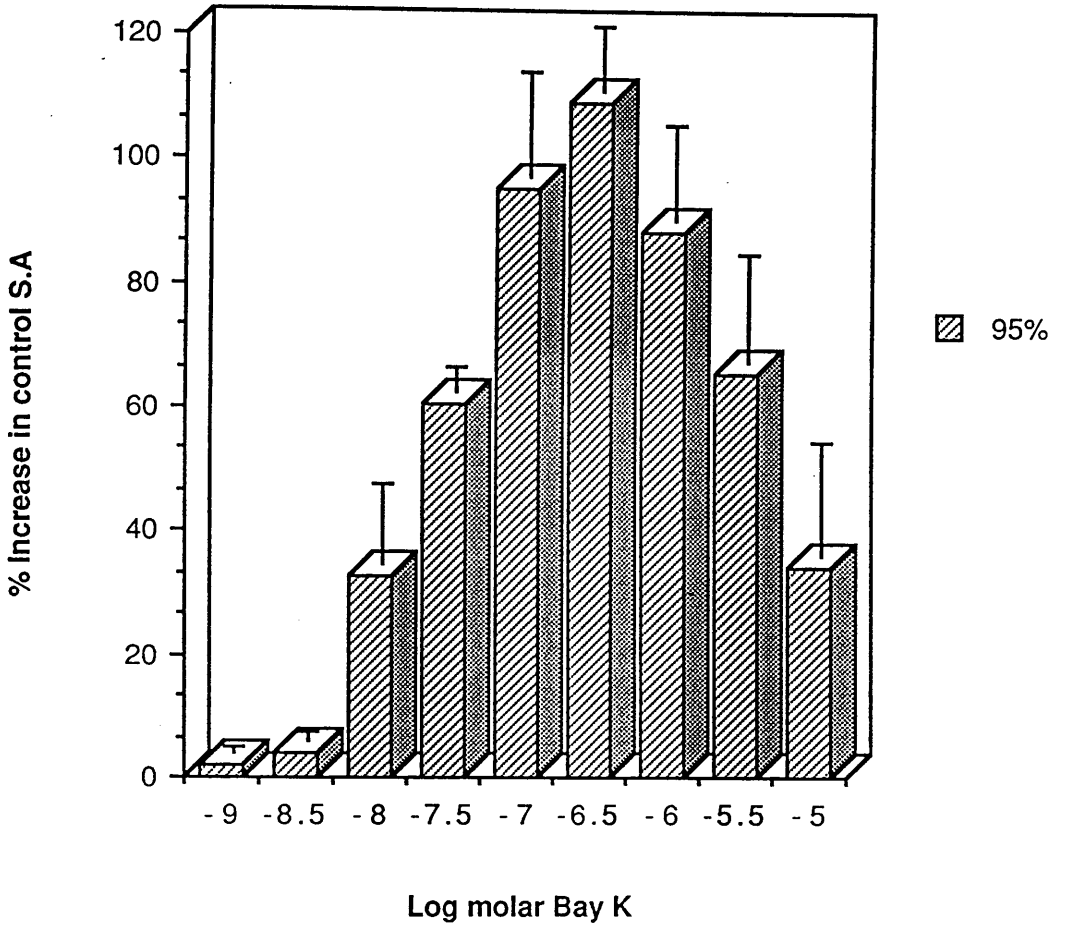


Fig 4.1

The effect of increasing concentrations of Bay K on the spontaneous phasic activity of the non-stimulated rat portal vein in Krebs containing 2.5mM Ca^{2+} at 95%O_2.

Bay K 8644 was added non-cumulatively to the bath. The mean spontaneous phasic activity by 5min was determined.

Bay K log concentration/ response curve was bell-shaped with high concentrations causing inhibition.

The points represent means ± s.e.m, n= 6.

The response of the isolated portal vein to KCl 50mM was biphasic in both oxygen tensions. The biphasic response to KCl consisted of a fast component 15s, followed by a tonic component which waned to 50% of the initial maximum response by 5min .

In $[Ca^{++}]$ = 2.5mM in either 95%O_2 or 16%O_2, Bay K (1nM - 1μM) caused an increased spontaneous phasic contraction of the portal vein, without affecting the contraction induced by NA or KCl see figs 4.2- 4.5.

2. Effect of Bay K on NA or KCl induced contractions in varying $[Ca^{++}]_o$: examined at 16% and 95%

Since Bay K could not facilitate contractions to either (KCl) or (NA) when $[Ca^{2+}]_o$ = 2.5mM we decided to examine the effect of Bay K on the non-cumulative response to NA and KCl, in different $[Ca^{2+}]_o$.

a. Spontaneous activity.

Spontaneous activity varied with $[Ca^{2+}]_o$. The phasic spontaneous activity of the tissue increased up to 5mM Ca^{++} at 95%, and to 2.5mM Ca^{2+} in 16%. However the height of contraction was not statistically significantly different at 16% and 95% at each $Ca2^{+}$ level examined. Bay K enhanced the spontaneous activity at the lower Ca^{2+} to a similar extent at 16% and 95%O_2,

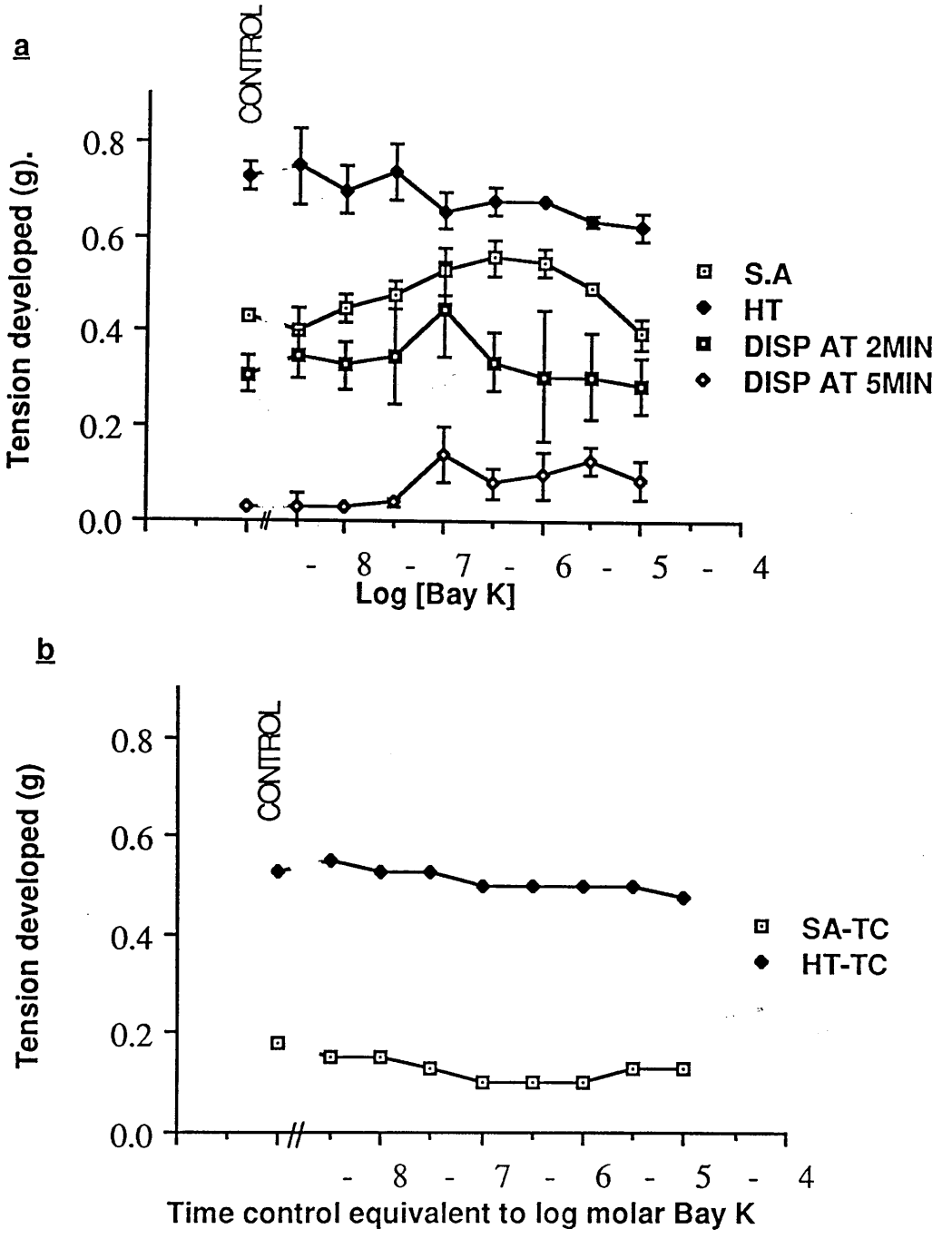


Fig 4.2

(a) Bay K concentration/ response relationship in tissues contracted with NA 0.3μM in Krebs containing 2.5mM Ca^{2+} at 95%O_2. After obtaining control response to NA 0.3uM, tissues were incubated 15 - 30min with Bay K 0.3uM. Then response to NA was taken for 5min. The tissues were then washed out and placed in a higher Bay K concentration.

(b) The time-matched control response of the tissue to NA without Bay K at the same oxygen tension.

Points represent means ± s.e.m, n=4.

S.A = spontaneous activity.

HT = height of contractile spike or tonic contraction.

DISP = baseline tension increase measured at 2min and 5min.

Note that control levels of the parameters measured are indicated in this figure and in figures 4.3 -4.5

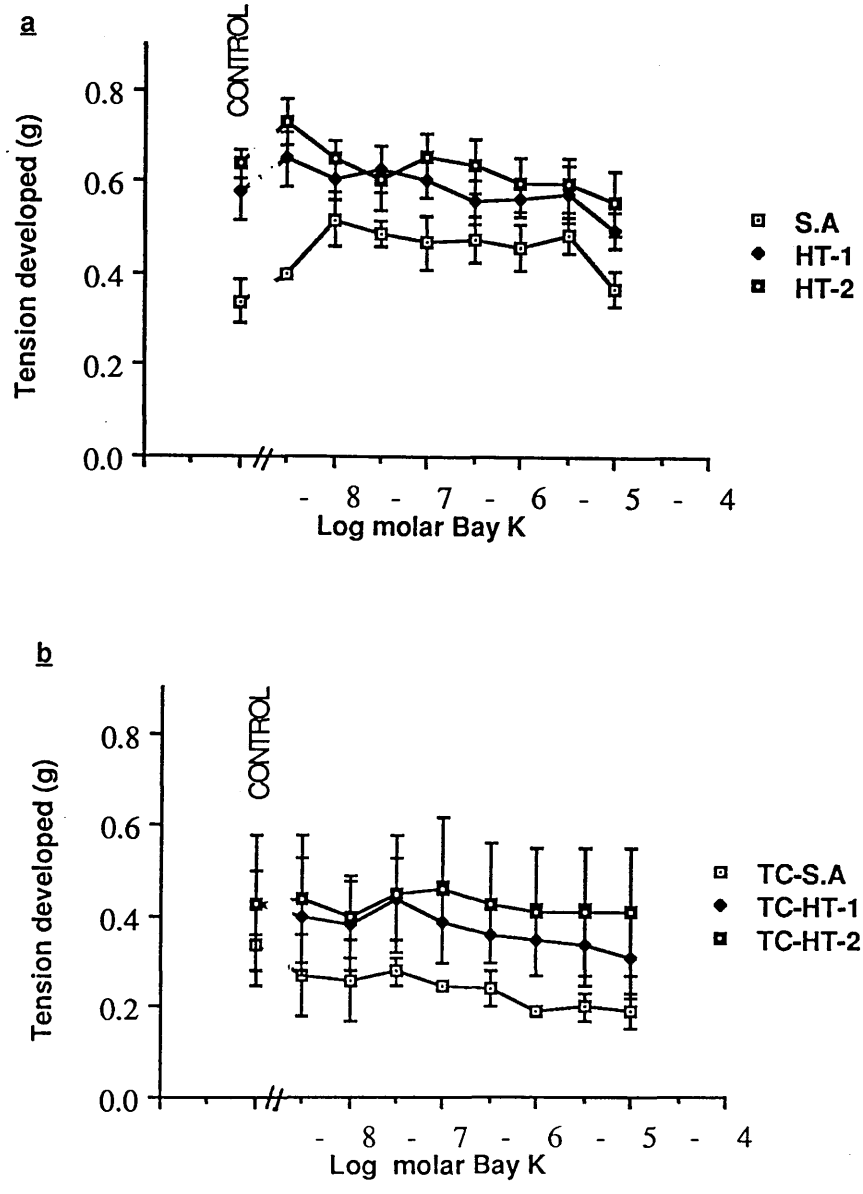


Fig 4.3

(a) Bay K concentration/ response relationship in tissues contracted with NA 0.3μM in Krebs containing 2.5mM Ca^{2+} at 16%O_2.

Protocol same as for fig 4.2.

(b) The time-matched control response of the tissue to NA without Bay K at the same oxygen tension.

HT-1 = height of contraction by 15s.

HT-2 = height of contraction by 5min.

Points represent means ± s.e.m, n=4.

TC-SA = Time control spontaneous activity.

TC-HT = Time control height of contraction.

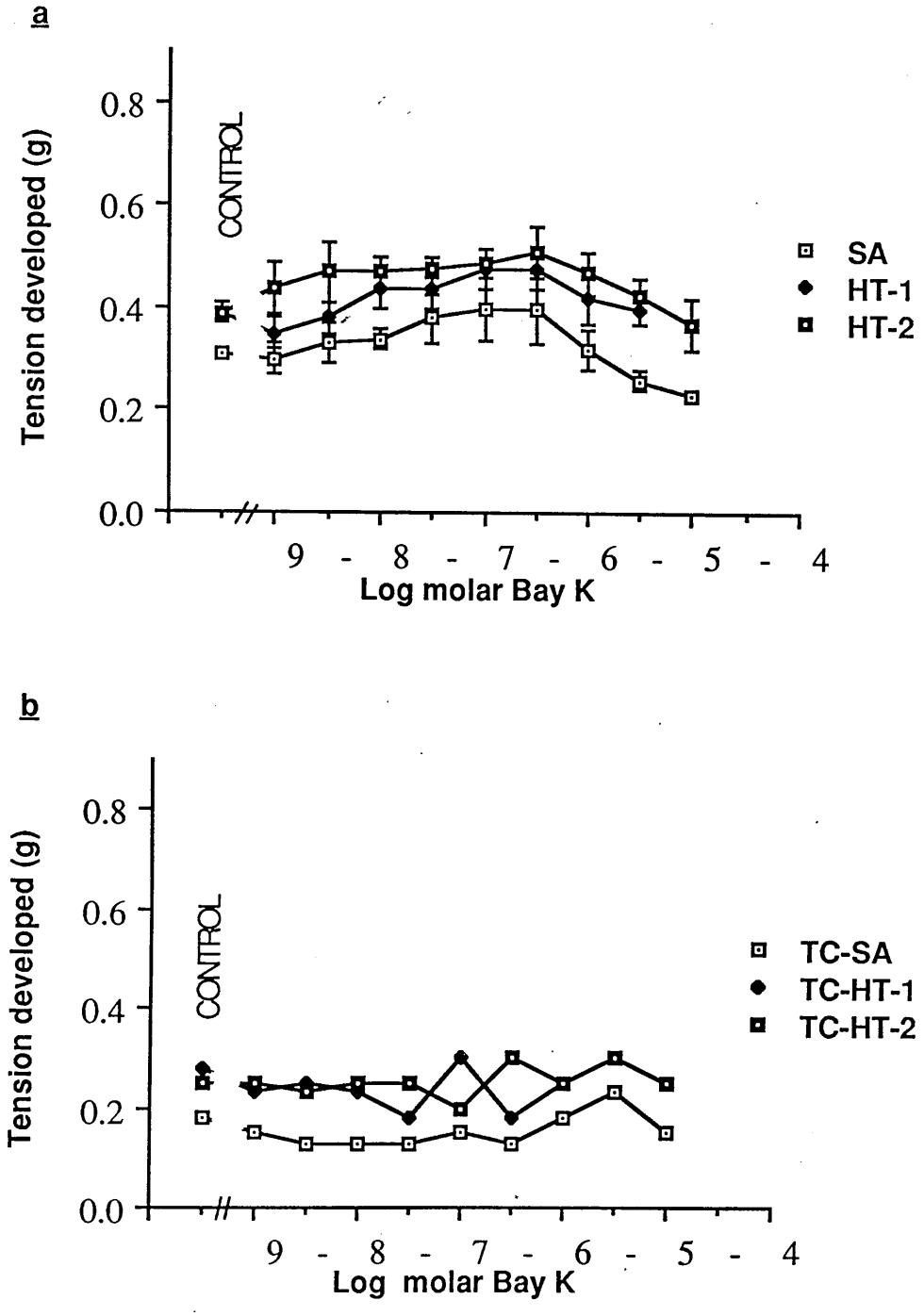


Fig 4.4

(a) Bay K concentration/ response relationship in tissues contracted with NA 0.3μM in Krebs containing 2.5mM Ca^{2+} at 4%O_2.

Protocol same as for fig 4.2.

(b) The time-matched control response of the tissue to NA without Bay K at the same oxygen tension.

HT-1 = height of contraction by 15s.

HT-2 = height of contraction by 5min.

Note that two separate heights of contraction were measured because NA response was biphasic.

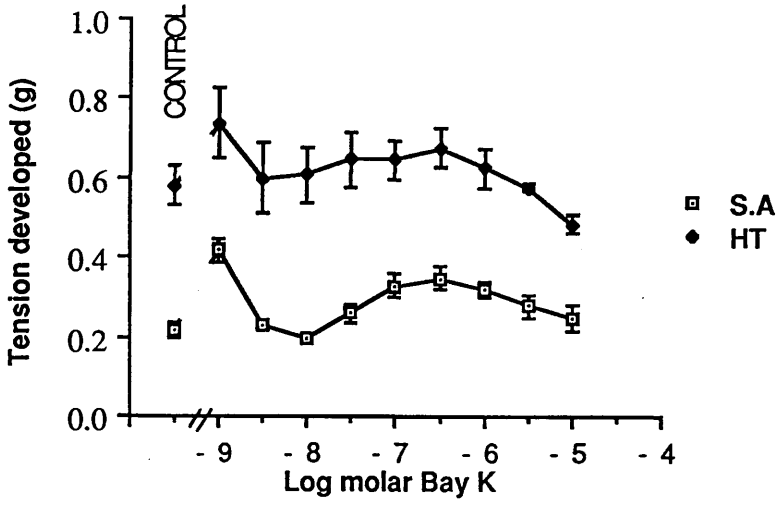
Points represent means ± s.e.m, n=4.

Fig 4.5

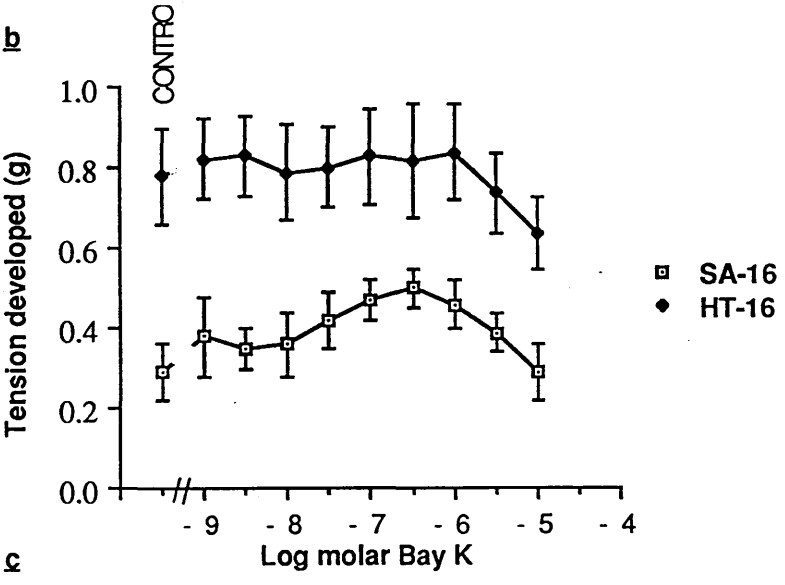
Bay K concentration response relationship in tissues contracted with KCl 50mM in Krebs containing 2.5mM Ca^{2+} at (a) 95%O_2, (b), 16%O_2 and 4%O_2 .

Protocol same as for fig 4.2. Experiments in different O2 tensions were carried out in separate experiments.
S.A = spontaneous activity.
HT-1 = height of contraction by 15s.
HT-2 = height of contraction by 5min.
Points represent means $\pm$ s.e.m, n=4.

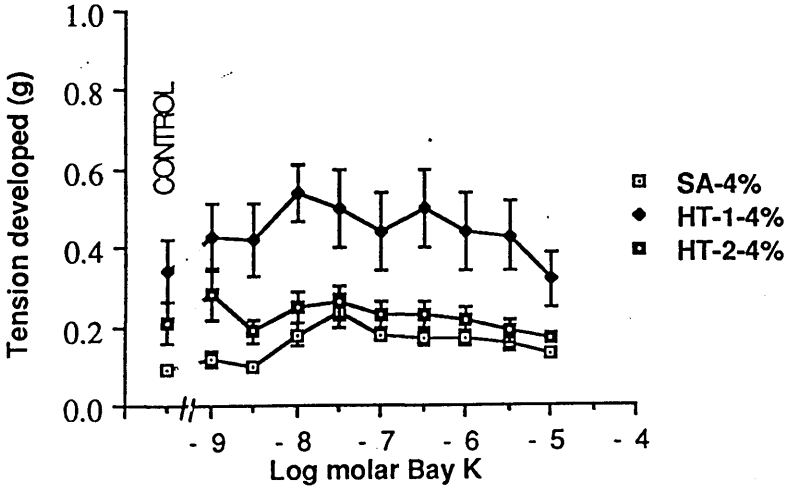
a


1.0
0.8
0.6
0.4
0.2
0.0
CONTROL
Tension developed (g)
- 9
- 8
- 7
- 6
- 5
- 4
Log molar Bay K
S.A
HT

b


1.0
0.8
0.6
0.4
0.2
0.0
CONTRO
Tension developed (g)
- 9
- 8
- 7
- 6
- 5
- 4
Log molar Bay K
SA-16
HT-16

c


1.0
0.8
0.6
0.4
0.2
0.0
CONTROL
Tension developed (g)
- 9
- 8
- 7
- 6
- 5
- 4
Log molar Bay K
SA-4%
HT-1-4%
HT-2-4%

at the lower Ca^{2+} to a similar extent at 16% and 95%O_2, but enhanced the responses from 2.5mM Ca^{2+} more at 95% than at 16%. See figs 4.6a & b.

b. Noradrenaline- induced contraction

(i). Non-cumulative variation of $[Ca^{2+}]$.

In the presence of noradrenaline 0.3μM, responses at 2.5mM and 5.0mM Ca^{2+}, were significantly higher ($P < 0.05$) in 95% than they were in 16%. At lower $[Ca^{2+}]_o$ the responses at the two different O_2 tensions were not significantly different from each other. Consequently, the $CapD_2$ values were not significantly different in the two O_2 tensions, see figs 4.6a & b. See also Table 4.1.

Bay K 8644 0.3μM, caused facilitation of NA-induced responses at low $[Ca^{2+}]_o$ in each of the two oxygen tensions. The sizes of the responses in Bay K 8644 were not significantly different from each other at the different O_2 tensions. See Table 4.1 for $CapD_2$ and log shift values.

In each O_2 tension Bay K 8644 caused a significant increase in contraction ($P<0.01$) across the $[Ca^{2+}]_o$ range 0.16 - 0.63mM but at $[Ca^{2+}]_o$ > 1.25mM the NA-induced contraction was not significantly different from the control. In the presence of Bay K 8644 0.3uM the NA-induced contraction in 0.31mM $[Ca^{2+}]$ matched the

Fig 4.6

Effects of Bay K 0.3μM on the response of RPV to NA in varying unbuffered $[Ca^{2+}]_o$ (non-cumulatively) at (a) 16%O_2 and (b) 95%O_2. Tissues were pretreated with Bay K for 15-30min at 2.5mM Ca^{2+}. Subsequently they were washed with zero Ca^{2+} and left in zero Ca^{2+} Krebs for 5min with Bay K added. NA was then added non-cumulatively for 5min, after which the tissues were washed and placed in Krebs with the next higher Ca^{2+} concentration.

Experiments at the two O_2 tensions were carried out in separate experiments.

Points represent means ± s.e.m, n = 6.

*, ** represent $P < 0.05$ and 0.01 respectively.

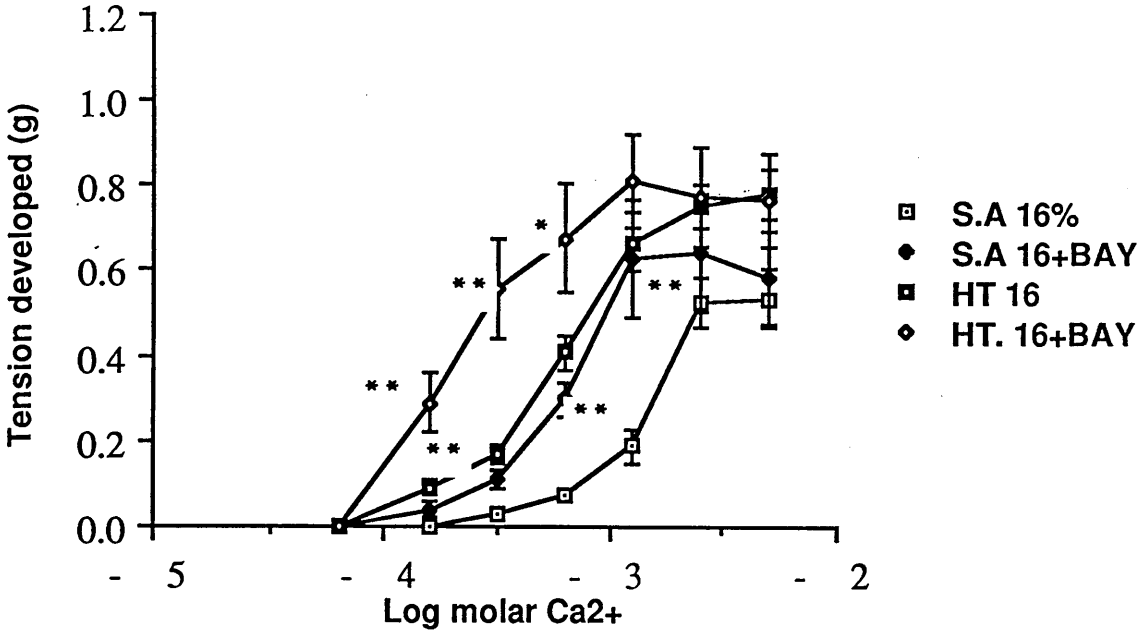
S .A = spontaneous activity.

SA + Bay K = SA in the presence of Bay K.

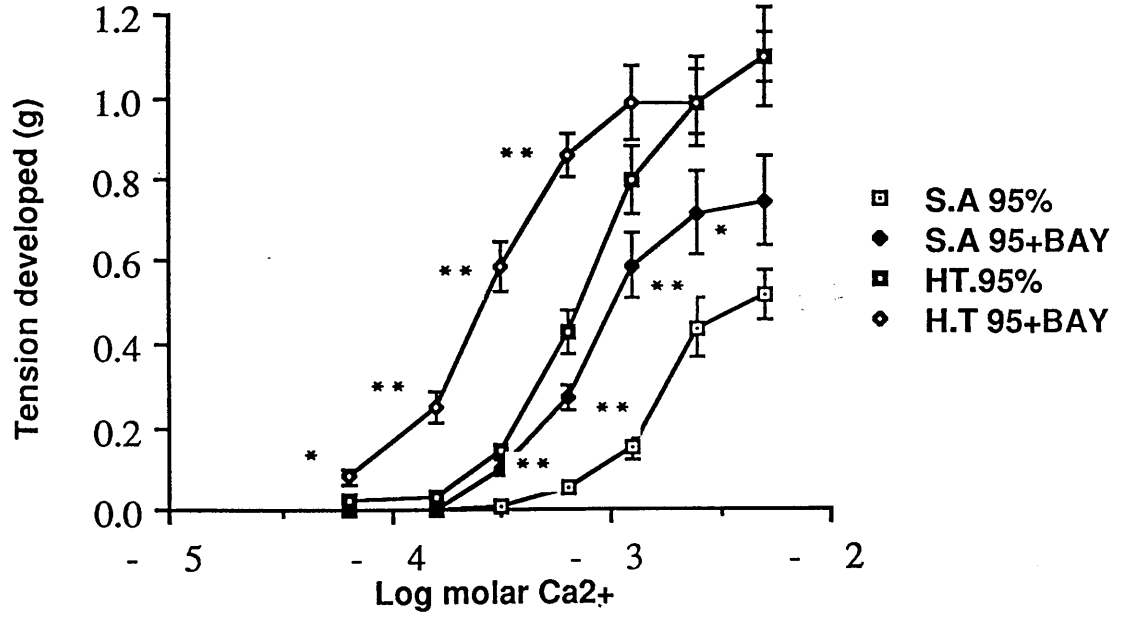
HT = height of contraction to NA

HT + Bay = HT in the presence of Bay K.

a


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Tension developed (g)
- 5
- 4
- 3
- 2
Log molar Ca2+
S.A 16%
S.A 16+BAY
HT 16
HT. 16+BAY

b


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Tension developed (g)
- 5
- 4
- 3
- 2
Log molar Ca2+
S.A 95%
S.A 95+BAY
HT.95%
H.T 95+BAY

Oxygen tension	16%O2		95%O2	
S.A				
S.A control	2.84 ± 0.03		2.82 ± 0.03	
+Bay K 0.3μM	3.23 ± 0.06	*	3.25 ± 0.04	* *
Log shift b/w 1 & 2 CRC	0.05 ± 0.01		0.05 ± 0.01	
Log shift b/w Control and Bay K treated	0.35 ± 0.09	* *	0.37 ± 0.04	* *
Ht of Contraction				
		NS		
NA control	3.32 ± 0.01		3.27 ± 0.05	
+ Bay K 0.3μM	3.78 ± 0.04	* *	3.65 ± 0.06	* *
Log shift b/w 1& 2 CRC	0.08 ± 0.02		0.04 ±0.01	
Log shift b/w Control and Bay K treated	0.32 ± 0.01	* *	0.34 ± 0.01	* *
		NS		

Table 4.1. Shows the CapD2 values of spontaneous activity and height of contraction of isolated rat portal vein during non-cumulative variation of Ca2+ in NA 0.3μM stimulated tissues.
The experiments at 16%O2 and 95%O2 were carried out in separately.
Values are means + s. e. m., n = 5 - 6. *, ** show P <0.05 and 0.01 respectively.

control response induced by the same concentration of NA in 0.63mM $[Ca^{2+}]_o$.

In the presence of Bay K 8644 the maximum response to NA was attained at 1.25mM Ca^{2+} (control 2.5mM Ca^{2+}). Thus Bay K allowed each response characteristic to be produced at approximately half the level of $[Ca^{2+}]$, i.e a 2 fold leftward shift in the $[Ca^{2+}]$/ response relationship. This is borne out by the shift in pD_2 values.

In 16%O_2 controls the biphasic responses obtained to NA in $[Ca^{2+}]_o$ =2.5mM, were not evident in lower $[Ca^{2+}]_o$ (< 0.63mM). In the presence of Bay K 8644 they were evident only in 2 out of 6 preparations: in these, the biphasic response was just detectable at 0.31mM Ca^{2+}, but as the Ca^{2+} level increased, the two phases of the contraction became more distinct.

Cumulative Ca^{2+} re-addition versus NA 0.3μM.

See section on buffer and non-buffer (Chapter 3).

3. K^+-induced contractions.

a. Bay K versus $K^{\pm}$ (50mM)-induced contraction: cumulative Ca^{2+} readdition.

Control responses of strips (starting in zero Ca^{2+}) to Ca^{2+} cumulative readdition were first taken without extra KCl and this produced the usual graded increases in spontaneous activity. Then the tissues were washed

Fig 4.7

The effect of Bay K 0.3μM on the Ca^{2+} response of the portal vein in KCl 50mM depolarized tissues during unbuffered Ca^{2+} cumulative re-addition from zero Ca^{2+} Krebs at (a) 16%O_2 and (b) 95%0_2 , in unpaired experiments.

Also shown is the effect of Bay K on the spontaneous activity during Ca^{2+} cumulative re-addition determined from a separate series of experiments.

Points show means ± s.e.m, n = 6-9 observations.

The Ca^{2+} response in K^+ -depolarized tissues were compared with K^+ -depolarized in the presence of Bay K, using paired student's t-test. *, ** represent $P < 0.05$ and 0.01 respectively.

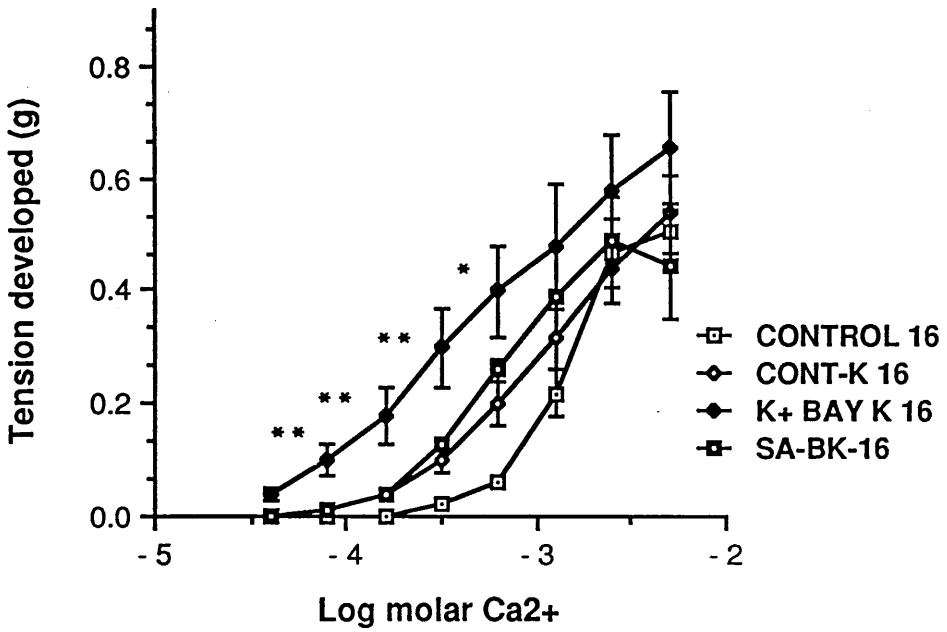
SA = Ca^{2+} response for spontaneous activity.

SA -BK = " " " " " in the presence of Bay K.

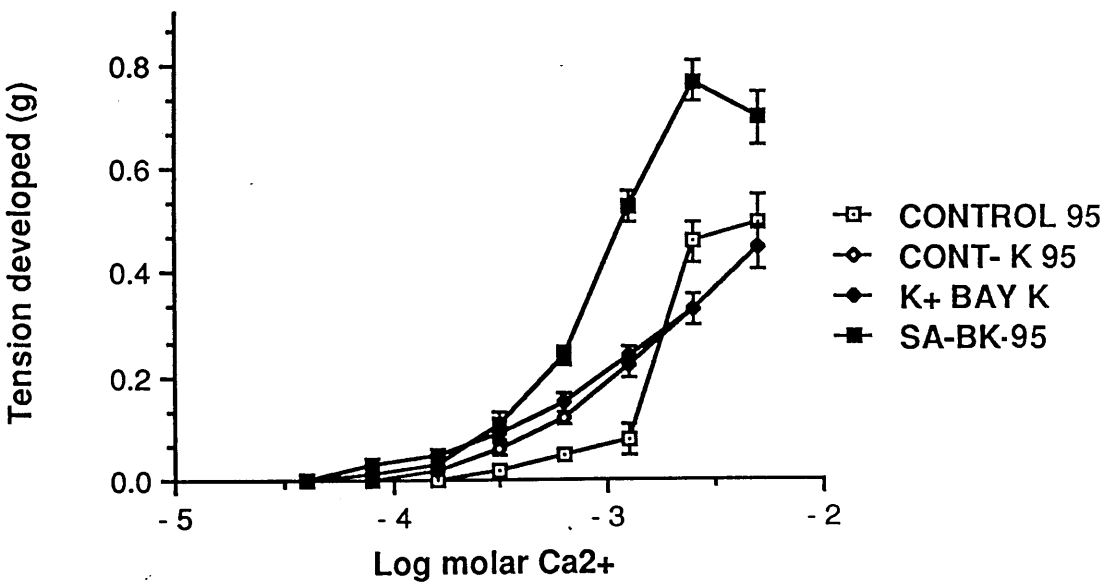
Cont-K = Ca^{2+} response in KCl depolarized tissue.

K + Bay K = " " " " in the presence of Bay K.

a


Tension developed (g)
0.8
0.6
0.4
0.2
0.0
- 5
- 4
- 3
- 2
Log molar Ca2+
* *
* *
*
CONTROL 16
CONT-K 16
K+ BAY K 16
SA-BK-16

b


Tension developed (g)
0.8
0.6
0.4
0.2
0.0
- 5
- 4
- 3
- 2
Log molar Ca2+
CONTROL 95
CONT- K 95
K+ BAY K
SA-BK-95

with zero Ca^{2+} Krebs and introduced into zero Ca^{2+} Krebs containing 50mM KCl. Subsequent re-addition of Ca^{2+} produced concentration-related sustained contraction which were devoid of spontaneous activity unlike NA-stimulated preparations. This abolition of spontaneous activity did not happen if the tissue was exposed to high $[Ca^{2+}]_o$ between KCl additions.

There was a stepwise increase in contraction as $[Ca^{2+}]$ increased from 40μM to 5.0mM. The control $CapD_2$ values were similar in both O_2 tensions when no extra KCl was added, but the $CapD_2$ was slightly higher in 16% when the KCl was increased to 50mM. On the other hand the control $CapD_2$ values with or without KCl 50mM were similar in 95% O_2 (Table 4.2a).

When the tissues were exposed to Bay K 8644 0.3μM there was potentiation of the Ca^{2+} response in KCl-depolarised tissues in 16%O_2 but not in 95%O_2 (Table 4.2a). In the presence of Bay K 8644 the $CapD_2$= 3.56 ± 0.14 with a log shift from control of 0.51 ± 0.08, see figs 4.7a & 4.8. In 95%O_2 however, Bay K 8644 hardly potentiated the Ca^{2+} response: in KCl 50mM depolarised tissues the $CapD_2$ value in the presence of Bay K was 2.85 ± 0.04 n=6. The mean log shift was 0.06 ± 0.02, see fig 4.7b & 4.8. Bay K 8644 significantly enhanced the Ca^{2+} response ($P<0.01$) in 16%O_2 only in the range of Ca^{2+} from 0.4μM to 0.63μM: from 1.25mM Ca^{2+} the responses in Bay K 8644 were not significantly different, from controls ($P>0.05$). In Bay K 8644, comparing 95%O_2 and

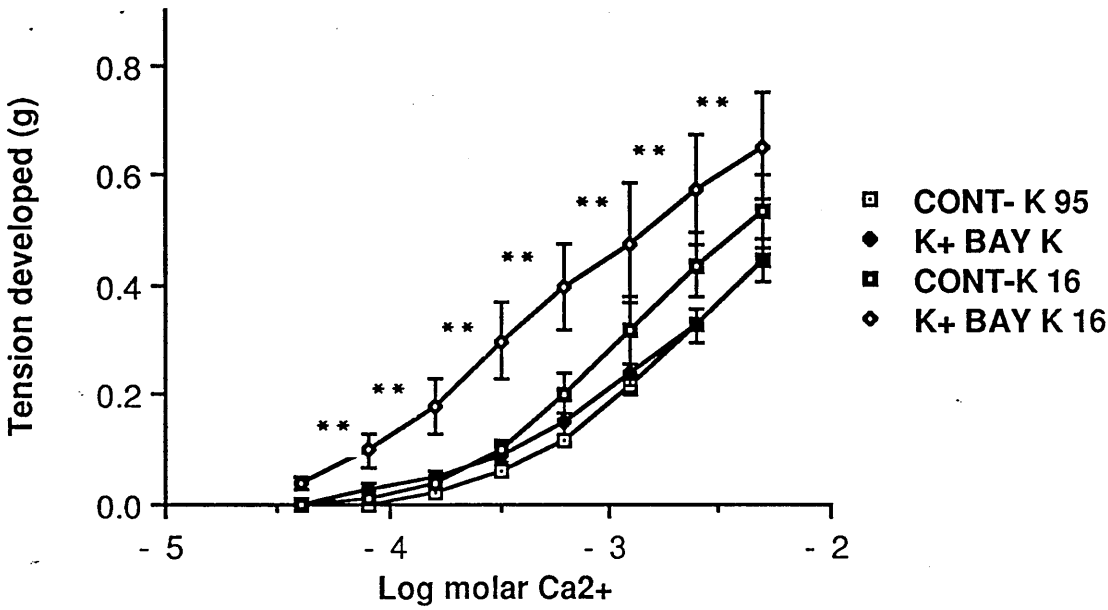


Fig 4.8

The effects of Bay K 0.3μM on the Ca^{2+} response of the portal vein in KCl 50mM -depolarized tissue during unbuffered Ca^{2+} cumulative re-addition from zero Ca^{2+} Krebs at 16%O_2 and 95%O_2, in unpaired experiments.

Bay K hardly shifted the Ca^{2+} CRC at 95%O_2, but caused a big shift at 16%O_2.

Points represent means ± s.e.m, n = 6-9. Ca^{2+} responses in K^+-depolarized tissues in the presence of Bay K at 16%O_2 and 95%O_2 have been compared using unpaired t-test. *, ** represent $P < 0.05$ and 0.01 respectively.

Oxygen tension	16% O2		95% O2	
Control (with no extra KCl) A	2.87 ± 0.03 (9)	NS	2.81 ± 0.06 (6)	
Control (with extra 50mM KCl) B	3.07 ± 0.11 (9)	*	2.79 ± 0.03 (6)	NS
In presence of Bay K 0.3μM C	3.56 ± 0.14 (5)	**	2.85 ± 0.04 (6)	*
Log shift b/w B & C	0.51 ± 0.08 (5)	***	0.06 ± 0.02 (6)	

Table 4.2a. Shows the CapD2 values obtained when Ca2+ is readded cumulatively in isolated rat portal vein stimulated with KCl 50mM in the presence of Bay K 0.3μM.
The numbers of observation are indicated in brackets. Values are means ± s.e.m. *, ** and *** are P < 0.05, 0.01 and 0.001 respectively.

Oxygen tension	16%O2		95%O2	
Control (with no extra KCl) A	2.84 ± 0.03 (4)	NS	2.81 ± 0.03 (4)	
Control (with extra 20mM KCl) B	2.93 ± 0.06 (4)	NS	3.07 ± 0.03 (4)	*
In presence of Bay K 0.3μM C	3.14 ± 0.06 (4)	NS	3.21 ± 0.08 (4)	*
Log shift b/w B & C	0.21 ± 0.05 (4)	NS	0.20 ± 0.06 (4)	

Table 4.2b. Shows the CapD2 values during cumulative Ca2+ re-addition using KCl 20mM in the presence of Bay K 0.3μM.
The numbers of observation are indicated in brackets. Values are means ± s.e.m. * indicates P < 0.05.

16%O_2 the Ca^{2+} response in 16% O_2 were significantly greater up till 2.5mM Ca^{2+}, but were not significantly different at 5.0mM Ca^{2+}.

The time matched control in 95% showed that there was no shift between the first and second concentration response curves. On the other hand the time control in 16% showed a loss in tissue sensitivity to Ca^{2+} of 0.2 log unit between the 1st and 2nd concentration response curves showing that the increase in Ca^{2+} sensitivity caused by Bay K in 16% O_2 is actually underestimated. See figs 4.7 & 4.8.

Since the effect of Bay K 8644 on contractions induced by KCl 50mM was negligible at 95% O_2, we examined the effect of Bay K 8644 on a milder KCl-induced depolarisation.

b. Effect of Bay K on Ca^{2+} response in KCl 20mM.

In unpaired experiments, Bay K 8644 potentiated the Ca^{2+}-induced response in K± 20mM depolarised tissues to a similar extent in 95%O_2 and 16%O_2. The mean log shifts by Bay K in 95%O_2 and 16%O_2 were 0.20±0.06, n=4 and 0.21±, n=4, respectively. Ca^{2+} responses were facilitated by Bay K 8644 up to 0.63mM Ca^{2+}. See Table 4.2 b.

In KCl 20mM depolarised tissues unlike 50mM KCl, contractile spikes were present, superimposed on the concentration-related contraction to increasing $[Ca^{2+}]_o$.

But unlike the control and NA stimulated tissues in which there was an inverse relationship between $[Ca^{2+}]_o$ and frequency of the spikes, there was no evidence of decreasing spike frequency to increasing Ca^{2+} in 20mM KCl depolarised portal vein; instead it increased.

Thus Bay K 8644 could enhance the calcium-induced response in KCl 50mM depolarised tissues in 16%O_2 only, while in a milder depolarising KCl solution, i.e 20mM Bay K , it could enhance the Ca^{2+} response to a similar extent at either O_2 tension.

DISCUSSION:

The portal vein shows spontaneous myogenic activity (Axelsson et al, 1967; Sigurdsson et al, 1975), which involves Ca^{2+} inward current (Kumamoto et al 1978; Mironneau & Gargouil, 1979; Jetley & Weston, 1980). These effects are triggered by slow depolarisation originating in pacemaker cells (Hermsmeyer, 1971; 1973), which give rise to spontaneous action potential firing. The rate of firing of action potential spikes can be increased by a number of stimulants including low concentrations of NA (Weston 1978; Jetley & Weston 1980) or angiotensin II (Hamon & Worcel 1982). Low concentrations of KCl 10 or 20mM increased the extracellular electrical and mechanical activity in rat portal vein (Hicks, 1983). In the present study Bay K in concentrations 1nM - 0.3μM increased the amplitude

and frequency of the contractile spikes of the rat portal vein, but at concentrations of 1μM and above it depressed the spontaneous activity. A similar observation has been made by Mikkelsen 1985, using 95%O_2. In our hands Bay K 8644 0.3μM caused 109% increase in spontaneous activity in 2.5mM Ca^{2+}. A higher increase of 500% was obtained by Mikkelsen, who used a lower Ca^{2+} 1.25mM, but would have a lower control value of spontaneous activity, since the spontaneous activity of the portal vein is highly dependent on extracellular Ca^{2+} (Sigurdsson, et al 1975, Weston 1978, Jetley & Weston, 1980; Hicks, 1983). Hence there was a bigger proportionate change in tension in Mikkelsen's experiment. This is clear also in figs 4.6a and 4.6b.

Only in a few other tissues has Bay K been known to contract the tissues on its own, e.g. femoral arteries of spontaneously hypertensive rats but not those of WKY normotensive rats (Aoki & Asano, 1986); feline cerebral arteries (Uski & Andersson, 1985).

In many vascular smooth muscle tissues e.g. rabbit aorta, Ca^{2+}- facilitators such as Bay K 8644 and CGP 28392 have little effect on the resting tone unless the concentration of the external KCl is raised to 10-15mM (Schramm et al, 1983a, b; Su et al, 1984; Loutzenhiser et al, 1984; Cheung, 1985). Bay K 's inability to contract the rabbit aorta was due to the vessel's quiescence and inactivity of the potential-sensitive Ca^{2+} channels in the resting conditions (Schramm et al,

1983a, b).

There is no single pattern of excitation-contraction coupling in smooth muscle (Bolton, 1979). Differences exist with respect to spontaneous activity and lack thereof, in Ca^{2+} sources and in the association of spike potentials and graded membrane potential changes with mechanical events. While it takes fairly high concentrations of KCl (> 100mM) to fully open all voltage operated channels in other vascular smooth muscles such as in aortae (Mikkelesen et al, 1985a, b) and feline arteries (Uski, 1985), the greatest sustained change in tension in the rat portal vein associated with depolarisation occurred at K^+ concentrations of 30 - 40mM. At these concentrations, repolarisation is prevented, voltage dependent Ca^{2+}-channels are opened and Ca^{2+}-influx results in contraction (Hermesmeyer, 1976; Hermsmeyer et al, 1981).

However, the Ca^{2+} channel facilitators potentiate the contractions induced by elevated concentrations of K^+, usually 10-15mM (Schramm et al, 1983a, b; Su et al, 1984; Loutzenhiser et al, 1984; Truog et al, 1984; Kanmura et al, 1984; Yamamoto et al, 1984; Cheung, 1985; Dube et al, 1985). Similar potentiation of the K^+-induced contraction has been reported for intestinal smooth muscle (Spedding & Berg, 1984). Furthermore, potentiation of K^+ - induced contractions by Ca^{2+} channel facilitators is followed by an additive increase

in $^{45}Ca^{2+}$ influx (Loutzenhiser et al, 1984; Yamamoto et al, 1984; Schramm et al, 1985). These results suggest that Ca^{2+} channel facilitators increase Ca^{2+} entry through voltage-dependent Ca^{2+} channels in the smooth muscle. In contrast to the above observation, effects of the Ca^{2+} channel facilitators on contractions-induced by receptor agonists are inconsistent. The Ca^{2+} channel facilitators have little effect on the NA-induced contraction in rabbit aorta (Schramm et al, 1983a, b; Loutzenhiser et al, 1984), a slight potentiating effect on the phenylephrine- induced contraction in the rat tail artery (Su et al, 1984), the NA-induced contraction in mesenteric artery (Kanmura et al, 1984) and the histamine-induced contraction in porcine coronary artery (Dube et al, 1985) and a pronounced potentiating effect on the BHT-920-induced contraction in rat tail artery (Su et al, 1984) and NA and methoxamine-induced contractions in the saphenous vein (Cheung, 1985). Such experiments are normally carried out at normal or high $[Ca^{2+}]$ (1 - 2.5mM) and hyperoxia (95%O_2). In our studies Bay K was not able to potentiate NA or KCl-induced contractions in 2.5mM Ca^{2+}, but it did at lower Ca^{2+} concentrations whether in normoxia or hyperoxia. In the portal vein NA-induced as well as KCl-induced contractions have been shown to be entirely dependent on extracellular Ca^{2+} (Sigurdsson et al, 1975). If Bay K and nifedipine are assumed to affect selectively the voltage dependent Ca^{2+} channel, the contractions induced by NA as well as high K^+ can be attributed to activation

of this Ca^{2+} channel. In this particular case, the receptor activation may induce membrane depolarisation resulting in opening of the voltage dependent Ca^{2+} channel (Bolton, 1979).

The possibility of an additional effect through receptor dependent but voltage-independent channels cannot be ruled out. In other vessels, there is evidence of a receptor linked Ca^{2+} channel that is distinct from voltage dependent Ca^{2+} channel. In rabbit aorta (Mekata 1974, 1976) and main pulmonary artery (Haeusler & Thorens, 1980), high K^+ induces contractions which correlate with membrane depolarisation whereas NA induces similar contractions without, or with only a small, depolarisation. Addition of high K^+ and NA together elicits additive contraction (Karaki et al, 1985) as well as additive increase in $^{45}Ca^{2+}$ uptake (Karaki & Weiss, 1980; Meisheri et al, 1981). These additional changes cannot depend only upon membrane depolarisation. Furthermore, in a Ca^{2+}-free and Sr^{2+} substituted solution, high K^+ induces both contractions and subsequent increases in $^{89}Sr^{2+}$ uptake whereas NA is almost ineffective on both muscle tension and $^{89}Sr^{2+}$ uptake (Karaki et al, 1986b). Ba^{2+} also enters the high K^+-depolarised but not the NA-treated rabbit aorta (Karaki et al, 1986c). Thus Sr^{2+} and Ba^{2+} can penetrate the high K^+-activated channel more than the NA-activated channel. These results support the idea that voltage dependent and receptor- linked Ca^{2+} channels exist in

this aortic smooth muscle. In the rat portal vein Sr^{2+} can substitute for Ca^{2+} to a large extent in contraction induced by depolarising, high K^{2+} solution, but not for NA-induced contraction (Uvelius et al, 1974; Arner et al, 1981, 1983). This supports the idea that voltage dependent and receptor-linked Ca^{2+} channels might exist in the rat portal vein.

Our results show that Bay K 8644 facilitates both KCl- and NA- induced contractions at low $[Ca^{2+}]_o$ to a similar extent and that this effect is not significantly different between hyperoxic (>590mm Hg) and physiological (110 mmHg) O_2 levels. This similarity between Bay K 's facilitation of KCl and NA might suggest that NA-induced contraction through receptor activation may induce membrane depolarisation resulting in opening of the voltage-dependent Ca^{2+} channel (Bolton, 1979). Fiol de Cuneo (1983) also showed that NA and KCl-induced contractions in the rat portal vein were equally susceptible to Ca^{2+} antagonists.
Alternatively, receptor-operated channels in this tissue might be susceptible to dihydropyridines.

Bay K 's inability to facilitate NA induced contraction in 2.5mM Ca^{2+} in the portal vein might be shared by other vascular smooth muscle such as the aorta (Schramm et al, 1983) since such experiments are usually carried out in physiological saline containing 2.5mM Ca^{2+}. Recently, Karaki et al (1986) showed that CGP 28392 a

dihydropyridine analogue of Bay K 8644 has a relatively selective activating effect on voltage dependent Ca^{2+} channels in rabbit aorta. However, it also activates receptor-linked Ca^{2+} channels in rabbit aorta when Ca^{2+} concentrations are low. In rat aorta and guinea-pig taenia CGP activates both types of Ca^{2+} channels (Karaki et al, 1986).

Bay K was, however, able to facilitate the spontaneous phasic activity of the non-stimulated tissues at 95% O_2 and 2.5mM Ca^{2+}. This may suggest some difference in excitation-contraction coupling between the spontaneous activity and drug induced effects. Relative resistance to Ca^{2+} antagonists, including nifedipine, of the spontaneous phasic activity of the rat portal vein compared with the tonic contraction induced by either NA or KCl has been reported by several workers (Fiol de Cuneo et al, 1983; Hicks, 1983 a & b; Campbell et al, 1986; see Chapter 5). It therefore appears a paradox that Bay K 8644 should have a reverse effect, i.e relatively more efficacy on the spontaneous activity.

Results obtained to Ca^{2+} cumulative re-addition in KCl 50mM depolarised tissues were interesting. The spontaneous activity was abolished and the response to Ca^{2+} re-addition was qualitatively similar to that in quiescent vascular tissues such as the aorta. Although Bay K facilitated the Ca^{2+} response at 16% O_2, it could not at 95%. On the other hand, with the use of a milder

depolarising solution of KCl 20mM, which did not abolish the spontaneous activity, Bay K caused a similar facilitation at both O_2 tensions. The implication of this might be that the altered state of contractility of the tissue (50mM KCl in zero Ca) might alter the excitation-contraction coupling. This result argues against the use of supra physiological levels O_2 which might lead to oxygen toxicity (Bowman & McGrath, 1982). The argument for the use of supraphysiological pO_2 is to avoid the development of an anoxic core (Pittman & Duling, 1973), but Hellstrand et al, 1977 showed that an anoxic core does not develop in the portal vein.

CHAPTER 5

ASSAY OF BAY K 8644 AT DIFFERENT OXYGEN TENSIONS.

SUMMARY

1. Bay K (10nM - 0.3μM) did not significantly affect the equilibrium response to KCl (50mM) at 0.31mM Ca^{2+}, i.e. 5min after addition of KCl. [Bay K] $\geq$ 1uM attenuated these responses. This effect was similar in 16% and 95%O_2.

2. [Bay K] $\geq$ 1nM enhanced the non-equilibrium response to KCl 50mM, i.e. the peak response from 0 to 2min after KCl 50mM in 0.31mM Ca^{2+}.

3. Bay K facilitated responses to KCl and NA to a similar extent at oxygen tensions from 95% down to 1%. The effects obtained with CGP 28392, though of lesser potency and more variability, gave similar results.

4. At 0% O_2 (hypoxia), responses to KCl were more resistant to hypoxia than were NA-induced responses. In fact, the responses induced by KCl in hypoxia were greater than the equivalent responses at 16% O_2. Subsequent assay of Bay K at hypoxia showed that Bay K 's facilitatory effect was much reduced during hypoxia than during oxygenation.

5. It was concluded that responses at 0% could already have been facilitated.

INTRODUCTION

In the previous chapter it was shown that Bay K 8644 could facilitate clearly at low Ca^{2+} concentrations, consequently Bay K as well as CGP 28392 has been assayed at 0.31mM Ca^{2+} in this chapter.
The assay at different O_2 tensions has been carried out with a view to determining the mechanism of excitation contraction coupling of NA and KCl responses at different O_2 tensions.

METHODS

Bay K concentration-response versus NA and KCl-induced contraction in Krebs containing 0.31mM Ca^{2+}.

Since it was impossible to facilitate responses to NA or KCl in 'normal' Ca^{2+} containing Krebs, but potentiation was clear at lower [Ca^{2+}], the potency of Bay K was assayed in Krebs containing 0.31mM Ca^{2+} at various pO_2 levels.

Cumulative concentration/response experiments.

This series of experiments was carried out for KCl only. After 5min response to KCl had been taken in 0.31mM Ca^{2+}, increasing concentrations of Bay K were then added cumulatively into the tissue bath in the continued presence of KCl.

Non-cumulative concentration/response experiments.

In this series of experiments the non-cumulative concentration/ response relationships for increasing concentrations of Bay K 8644 or CGP 28392 were determined against responses to either NA or KCl. After the initial equilibration with 16%O_2, the tissues were challenged with either NA 10μM or KCl 50mM until reproducible contractile responses were obtained. The tissues were then washed twice with 2.5mM Ca^{2+} and allowed to rest for 10min, after which they were washed 2-3 times with zero Ca^{2+} Krebs and left in this for 5min. The zero Ca^{2+} Krebs was replaced by 0.31mM Ca^{2+} Krebs for 5min , after which control responses to either NA 0.3μM or KCl 50mM were taken, allowing 5min drug contact time. The tissues were washed with 0.31mM Ca^{2+} Krebs allowing 10min rest before a second response was repeated. After washout, the tissues were incubated with Bay K or CGP for 15 - 30min, before repeating the test concentration of the activator. Thereafter, tissues were kept in 0.31mM Ca^{2+} and the procedure was repeated in the presence of increasing concentrations of either Bay K or CGP 28392.

Experiments in different O_2 tensions were carried out in different tissues, but in each experiment the preparation was first equilibrated with 16%O_2 and NA or KCl tested, before switching the gas to the required O_2

tension. These could thus serve as standards with which to compare tissues if necessary.The tissues were equilibrated with the test O_2 tension for 30min in 2.5mM Ca^{2+}, before washing with zero Ca^{2+} 2-3 times. The above procedure for assaying Bay K or CGP in 0.31mM Ca^{2+} was repeated.

The pD_2 values were determined by expressing the change in tension in the presence of the Ca^{2+} facilitator as percentage of the maximum change in tension also in the presence of the facilitator.

Interaction of nifedipine with Bay K 8644.

These experiments were carried out using the same protocol as for 0.31mM Ca^{2+} experiments above. Only one concentration of nifedipine was tested per tissue in the presence of increasing concentrations of Bay K 8644 in tissues contracted with either NA 0.3μM or KCl 50mM.

The concentrations of nifedipine tested were 1nM, 10nM and 0.1μM.

The experiments were carried out at only 16% O_2.

RESULTS

1.Cumulative Bay K concentration versus response to KCl 50mM

The tissues were exposed to 50mM KCl for 5min in 0.31mM Ca^{2+}. On addition of KCl a contraction occurred which, as in Ca^{2+} 2.5mM, waned to about 50% of its initial

height by 5min. Bay K was then added cumulatively from 1nM to 0.3μM . It sometimes produced a small further increase in tone but the change in the mean was not significant (P>0.05). From 1μM Bay K , the tone started to drop. The time control tissue showed a small further decline, after the initial 50% wane, during the period of observation. This result was similar in the two O_2 tensions tested (16% & 95%). Upon washing out with zero Ca^{2+} and subsequent exposure to 2.5mM Ca^{2+} physiological saline, the Bay K treated tissues showed enhanced phasic responses compared with controls.

Thus Bay K could not enhance the equilibrium response to KCl 50mM. We therefore decided to look in detail at the Bay K versus concentration/response relationship in a non-cumulative manner, administering each concentration individually before addition of KCl.

2. **Non cumulative Bay K concentration versus response to KCl- 50mM or NA 0.3μM in Krebs containing 0.31mM Ca^{2+}:**

a. At 16%O_2 and 95%O_2

(i) KCl 50mM

The tissue was sensitive to Bay K using the peak response during the 5min exposure to KCl as an index. The KCl-induced contraction was potentiated by Bay K

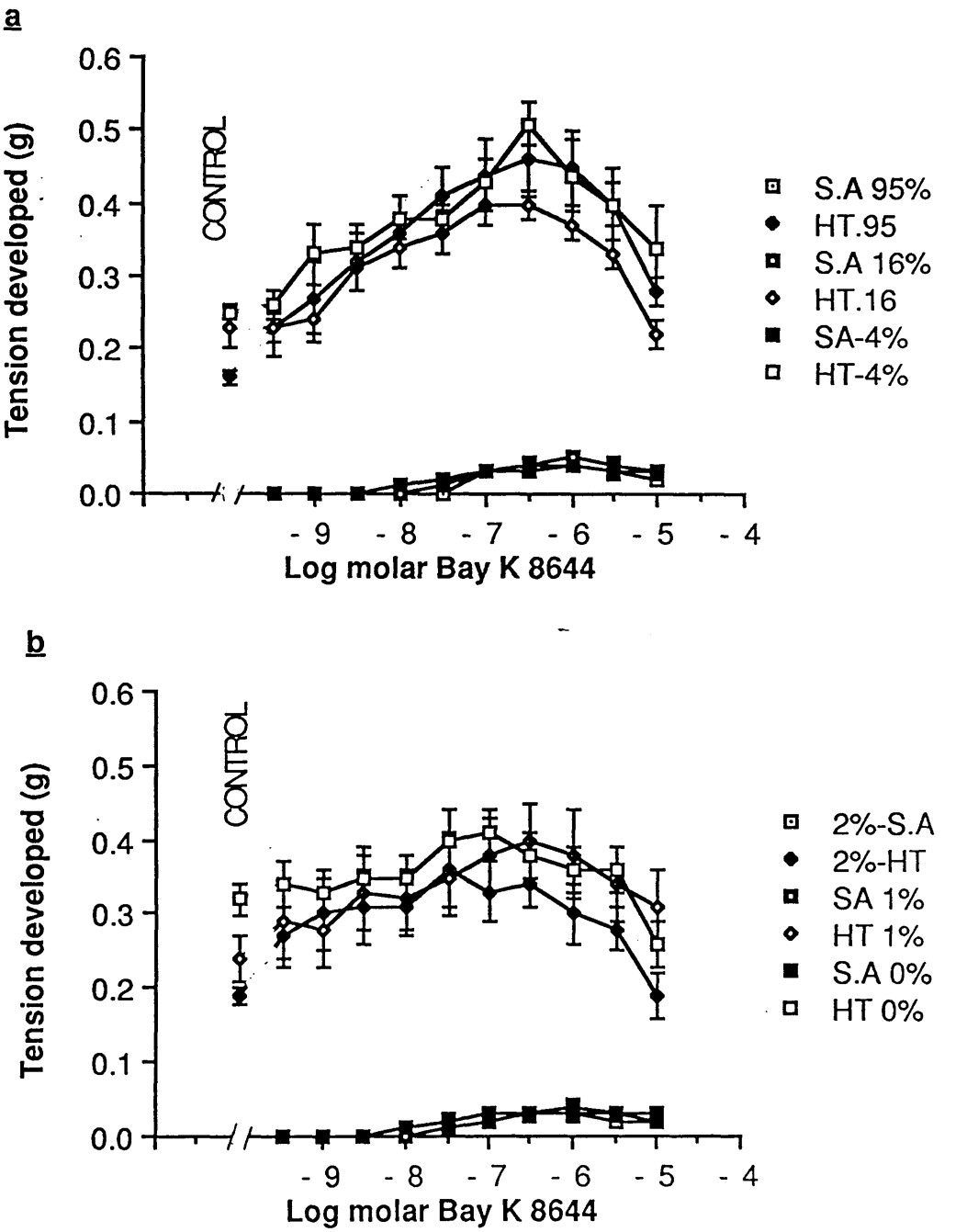


Fig 5.1

Bay K 8644 concentration/ response relationship at different O_2 tensions in (a) 95%O_2 - 4%O_2 and (b) 2%O_2 - 0%O_2.

Bay K's -induced facilitation was assessed by the increase in response to KCl 50mM.

The experiments in different O_2 tensions were unpaired. The points represent means ± s.e.m. of n = 6 - 9 experiments.

from 1nM, in a concentration-dependent manner. However from 1μM the potentiation started to drop.

In 0.31mM Ca^{2+} the response to KCl was monophasic. However, Bay K 8644 induced a biphasic contraction to KCl. The threshold was at 30nM Bay K and by 0.3μM Bay K the biphasic response was clearly discernible in each O_2 tension examined.

When the results were expressed as a percentage of the maximum change in tension at each O_2 level the pD_2 values for Bay K obtained were as shown on Table 5.1. In 16% O_2 pD_2 was 8.2±0.3 n=6, while the corresponding values in 95% O_2 and in 4% (see section 2.b) were 8.8±0.2 and 8.18 ± 0.2 respectively. These pD_2 values in the different O_2 tensions were not statistically significantly different from each other. The absolute sizes of the mean responses are shown in fig 5.1a.

When the results were expressed as a percentage of the control maximum response to 50mM KCl in 2.5mM Ca^{2+}; the results in the two O_2 tensions were similar and again there was no statistically significant difference in the facilitatory effect of Bay K in the two O_2 tensions. The maximum facilitation was achieved at 0.1μM and, by 3μM, Bay K caused attenuation of the contractions Fig 5.1a.

Although Bay K enhanced the peak of the KCl 50mM

induced contractions in 0.31mM Ca^{2+}, the decline of the contraction with time still occurred. This is consistent with the failure of Bay K to enhance the equilibrium response at 5min and beyond and thus the failure to see potentiation with cumulative addition of Bay K .

(ii). NA (0.3μM).

From Bay K 3nM there was a concentration dependent increase in NA-induced contraction.
Bay K did not alter the qualitative response of the tissue to NA; a contraction characterised by contractile spikes superimposed on the tonic contraction was usually obtained.

The pattern of response was the same in the two O_2 tensions. The biphasic response to NA was absent in control responses to NA in 0.31mM Ca^{2+}, but in the presence of Bay K a few of the tissues showed biphasic responses to NA and this seemed linked with the sensitivity of the tissue. Only the most sensitive tissues showed the biphasic response at Bay K concentrations of 0.1μM - 1μM. This phenomenom was rare in the higher O_2 tension.

The pD_2 value for potentiation by Bay K in 16%O_2 was 8.08±0.1, n=6; and in 95% was 8.04±0.15, n=6. As in KCl - depolarised tissues the values in the two different O_2 tensions were not significantly different from each

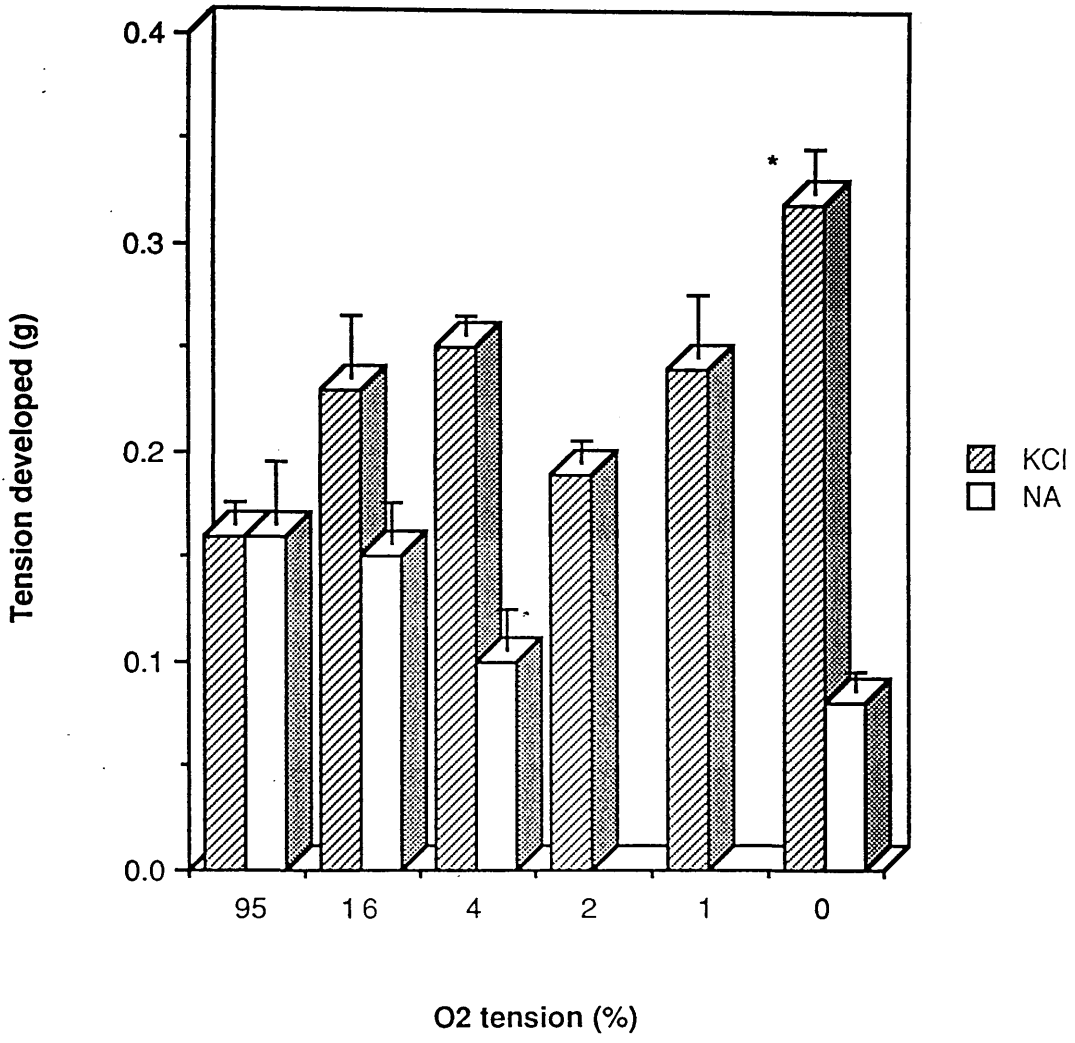


Fig 5.2
The effect of decreasing O_2 tension on the control responses induced by KCl 50mM and NA 0.3μM in Krebs containing 0.31mM Ca^{2+}.
The histograms show the mean of at least 6 experiments while the vertical bars represent ± s.e.m.
* indicate that KCl response in hypoxia (0%O_2) was statistically significantly ($P < 0.05$) higher than the response at normoxia (16%O_2).

other. This was similar when the results were expressed as a percentage of control NA 10μM maximum in 2.5mM Ca^{2+}. See Fig 5.3. The pD_2 values for Bay K in NA contracted were not statistically significantly different ($P > 0.05$) from the equivalent values obtained for Bay K in KCl contracted tissues at 16%O_2 and 95%O_2.

b. <u>At 4% to 1%O_2</u>

(i) <u>KCl</u>

At lower oxygen tensions i.e 4%, 2% and 1%O_2 respectively, control responses to KCl were not significantly different from those at 16%O_2. The control values in g in the different O_2 tensions (%) examined were 0.16±0.01 (95), 0.23±0.03(16), 0.25±0.01(4), 0.19±0.01(2), 0.24±0.03(1), and 0.32±0.02(0), n = 6-9. (see fig 5.2). The only group which was significantly different from 16% was that at 0% (discussed below). Under these lower oxygen tensions i.e 4%-1%, Bay K was able to facilitate the KCl 50mM induced contractions in 0.31mM Ca^{2+}. The pD_2 values were not statistically significantly different from that at 16%. Except for the pD_2 at 2% in which there was a slight increase (though not significant) when compared with at 16%. See fig 5.1b and table 5.1.

	O2 TENSION	pD2 values	P values
KCl (50mM)	95%	8.8 ± 0.2	NS
	16%	8.2 ± 0.3	
	4%	8.18 ± 0.2	NS
	2%		
	1%	8.44 ± 0.13	NS
	0%	7.78 ± 0.11	NS
NA (0.3μM)	95%	8.04 ± 0.15	NS
	16%	8.08 ± 0.1	
	4%	7.89 ± 0.27	NS
	0%	-	

Table 5.1 shows the pD2 values obtained for non-cumulative concentration /response for Bay K 8644. The portal veins were contracted with either KCl 50mM or NA 0.3μM in Krebs containing 0.31mM Ca2+. The pD2 values were calculated from the % increase in response to either KCl or NA in the presence of Bay K 8644.
Values are means ± s.e.m of 6 - 9 experiments.
pD2 value for KCl or NA at 16%O2 has been compared with those at other O2 tensions. NS indicates not statistically significant from 16%O2.

(ii) NA

In the lower O_2 range, NA was examined only at 4%. The control response to NA was depressed (fig 5.3) compared to that at 16% or 95%O_2. However the facilitation was as good as at higher O_2 as a proportion of the control. The pD_2 was 7.89 $\pm$0.27 n=4, which was not statistically significantly different from that at 16%O_2.

c. Hypoxia (0%O_2)

(i) KCl

Gassing the tissues with 5%CO_2 in 95%N_2 paradoxically gave a control response to KCl 50mM, which was higher ($P < 0.05$) than in any of the higher O_2 tensions. Although Bay K was able to potentiate this response to some extent, the absolute and proportionate increases were significantly reduced ($P<0.05$) when compared with the response in 16%O_2. The facilitatory effect of Bay K in hypoxia was statistically significant ($P < 0.05$) from 30nM to 0.3µM. (Fig 5.1b).

Qualitatively the control response was similar to that obtained in the presence of O_2.

(ii) NA

NA-induced responses were more susceptible to hypoxia

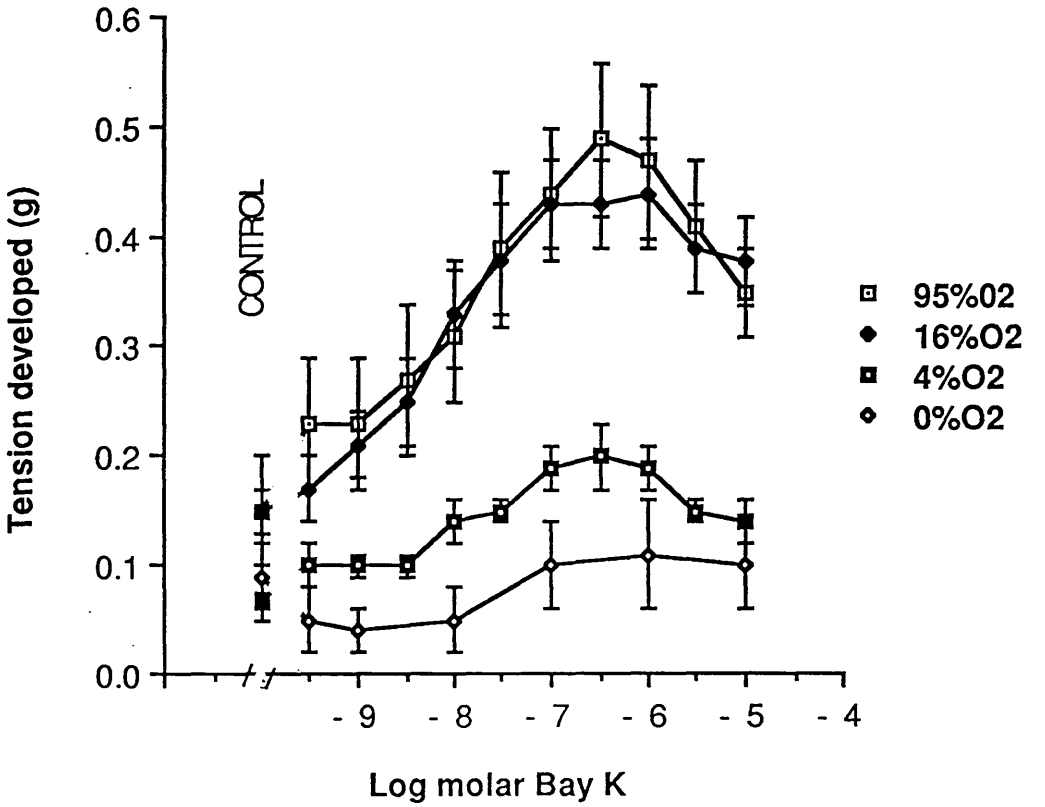


Fig 5.3

Bay K 8644 concentration/ response relationship at different O_2 tensions (95%O_2 - 0%O_2).

Bay K's induced facilitation was assessed by the increase in response to NA 0.3μM (95%O_2 - 4%O_2) by Bay K in Krebs containing 0.31mM Ca^{2+}. The experiments in the different O_2 tensions were unpaired. The points represent means ± s.e.m of n =6 - 9.

than were those to KCl. NA 0.3μM induced contractions in hypoxia were reduced to about 50% of the response in either 16% or 95%O_2. The control values at the different O_2 tensions were 0.16g±0.03(95), n=9; 0.15g±0.02(16), n=9; 0.10g±0.02(4), n=4; 0.08g±0.01(0), n=4. See fig 5.3.

Since the responses to NA 0.3μM were depressed at 0%, making it difficult to assess facilitation, an attempt was made to assay Bay K against a higher concentration of NA (10μM) to give a response approximately the size of that at higher O_2 tensions. The control response to the latter concentration of NA was 0.14g±0.05, n=6. There was a time-dependent decrease in response to NA at this concentration in those conditions: only from Bay K 8644 0.1μM to 10μM was there ever an increase in response and this was not statistically significant. No attenuation by Bay K 10μM was observed. See fig 5.3.

d. Time matched control experiments at 95%O_2

Control tissues which were not treated with Bay K showed a slight increase between the first and the second response and thereafter there was a drop in the response, which subsequently remained steady for the next 2hrs. Thereafter there was a gradual but slight increase in contraction to KCl 50mM. See Fig 5.4a & b.

In the next stage of the experiment we determined the

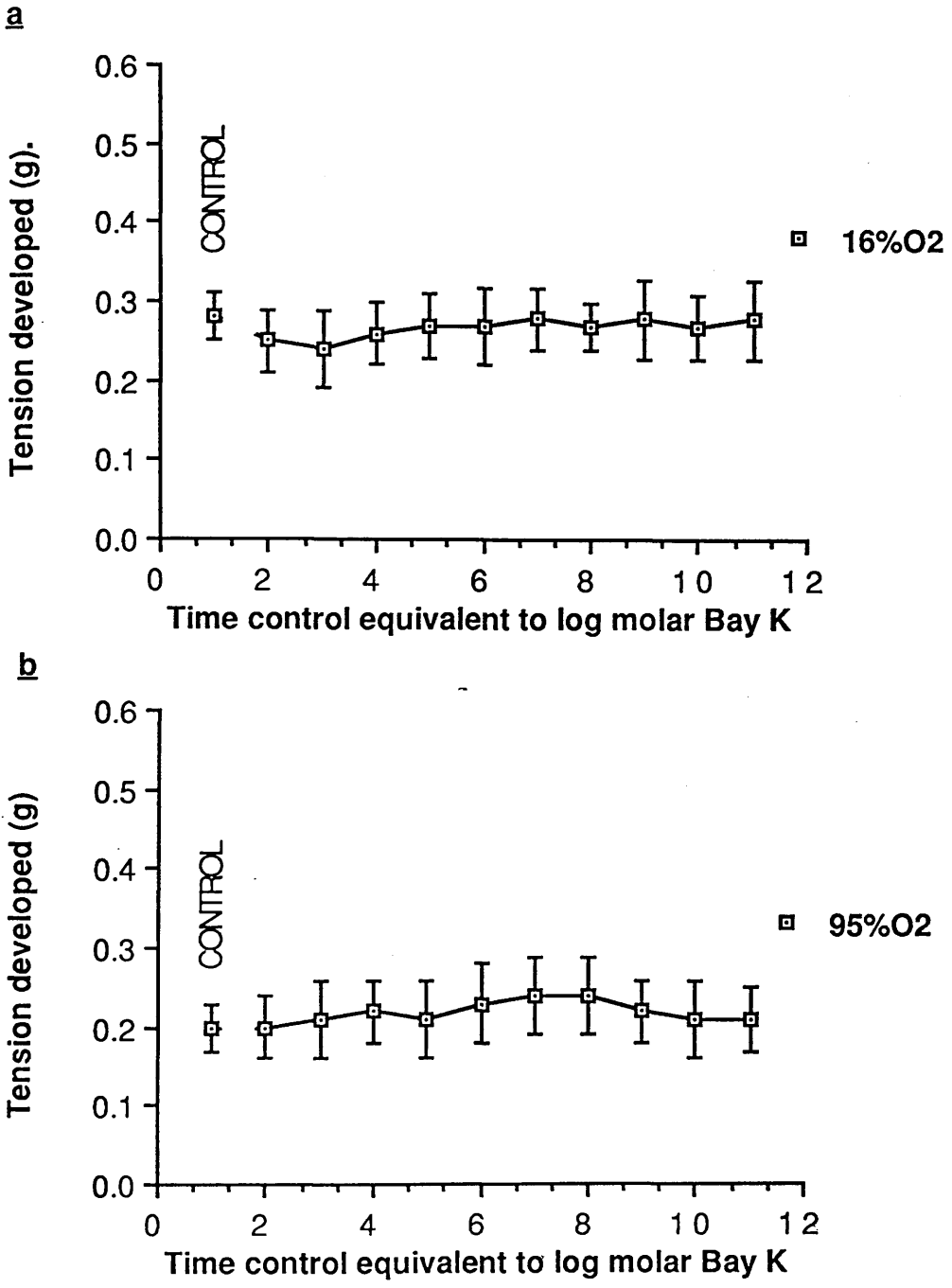


Fig 5.4
The time-matched control response to KCl 50mM in Krebs containing 0.31mM Ca^{2+} at (a) 16%O_2 and (b) 95%O_2.

Bay K concentration/response relationship in different levels of buffered Ca^{2+}.

3. Effect of Ca^{2+} lack on KCl induced response at 16% and at 0%O_2

A comparison between lack of oxygen and of Ca^{2+} was made. As expected KCl could not cause contraction in nominally "Ca^{2+}-free Krebs" in either 16% or 0%$_2$. Bay K was ineffective in recovering the response to KCl in Ca^{2+} -free Krebs. The relationship to [Ca^{2+}] was then investigated further (see section 4, below).

4. Bay K concentration - response in different buffered [Ca^{2+}]$_o$

The concentrations of free Ca^{2+} examined were 10uM, 30μM , 0.10mM, 1.25mM and 2.5mM each buffered with EGTA + NTA. The Bay K concentration-response relationships in these various levels of Ca^{2+} were determined versus KCl 50mM using a non cumulative protocol. The experiment was carried out in 16% only since Bay K caused a similar facilitation in 95% O_2 and 16% O_2 (fig 5.1a).

The Bay K concentration - effect relationship was illustrated in two ways. First, the response was represented as % of an initial control response to KCl 50mM in 2.5mM Ca^{2+} (fig 5.5a). This shows that the slope was steeper at Bay K concentrations from 1nM to 1μM, at

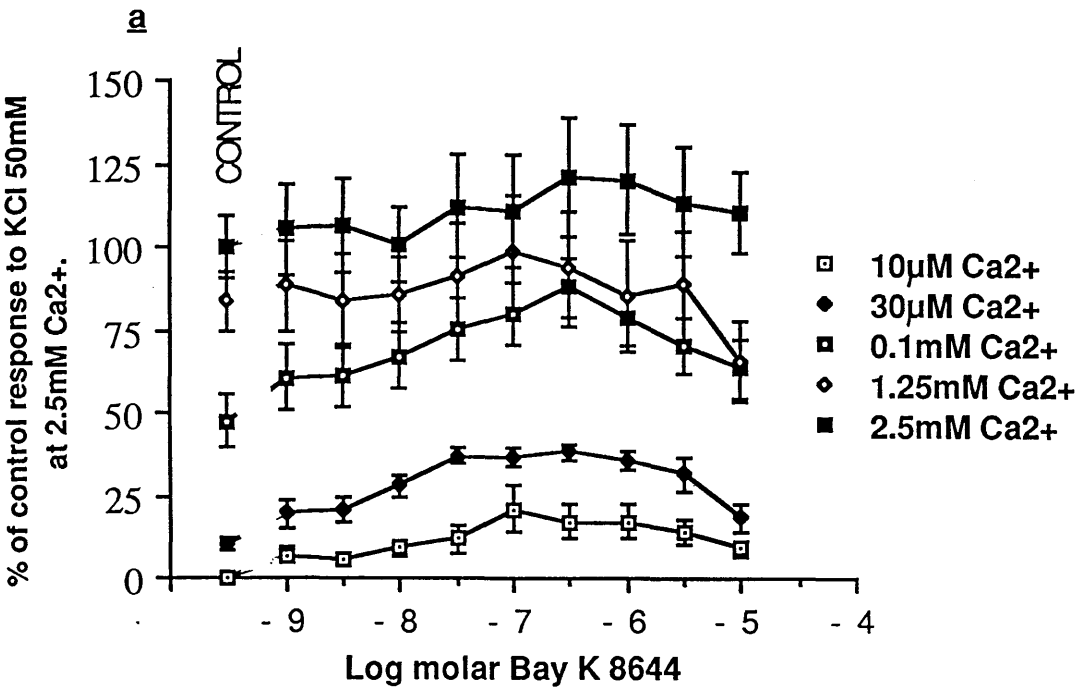
Fig 5.5

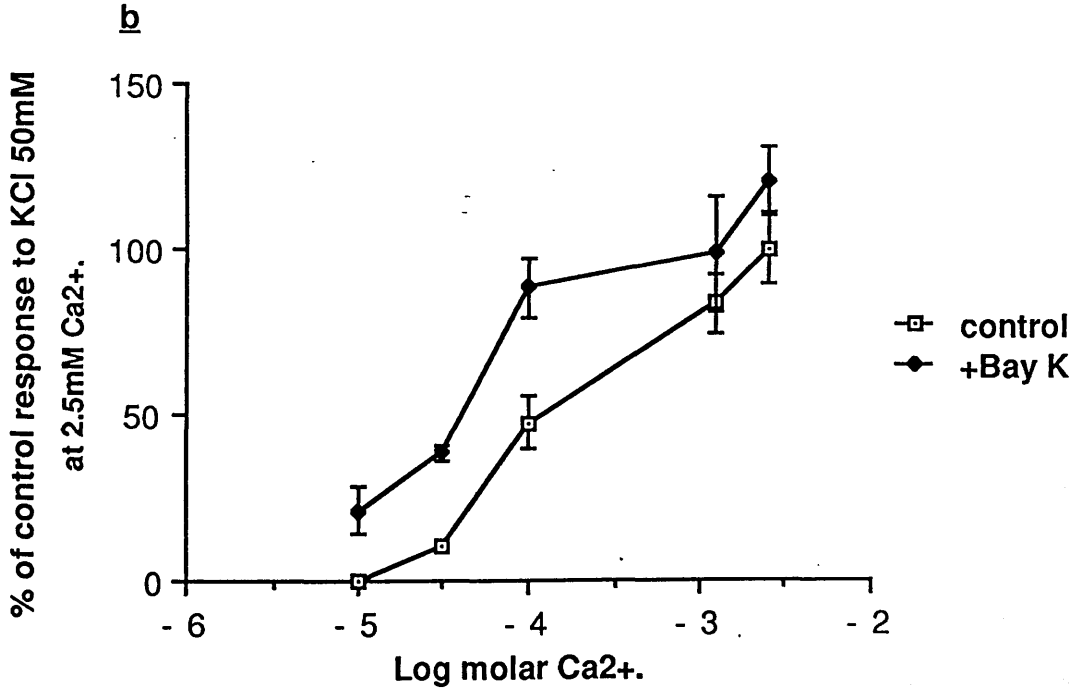
Bay K 8644 concentration/ response relationship at different concentrations of buffered Ca^{2+} at 16%O_2.

The experiments were unpaired and the results are expressed as (a) response plotted as percentage of control response to KCl at 2.5mM Ca^{2+} versus [Bay K 8644] and (b) the same data plotting the responses as percentage of control response to KCl in 2.5mM Ca^{2+}) versus [Ca^{2+}] for controls and for the maximally facilitated response at each Ca^{2+} concentration. Points represent means + s.e.m, n = 4.

The responses in the control at each Ca2+ level were compared with those in the presence of Bay K 8644.

*, ** represent $P < 0.05$ and 0.01 respectively.


a
150
125
100
75
50
25
0
CONTROL
% of control response to KCl 50mM at 2.5mM Ca2+.
- 9
- 8
- 7
- 6
- 5
- 4
Log molar Bay K 8644
10µM Ca2+
30µM Ca2+
0.1mM Ca2+
1.25mM Ca2+
2.5mM Ca2+


b
150
100
50
0
% of control response to KCl 50mM at 2.5mM Ca2+.
- 6
- 5
- 4
- 3
- 2
Log molar Ca2+.
control
+Bay K

low Ca^{2+} levels 10μM, 30μM and 0.1mM than at the higher levels of Ca^{2+} i.e 1.25mM and 2.5mM Ca^{2+}, see fig 5.5a. Secondly, responses were represented as % of the control response in each Ca^{2+} level. This compensated more for individual variation and showed that responses at 30uM Ca^{2+} were most potentiated, followed by those in 0.1mM Ca^{2+}. In 2.5mM Ca^{2+} the potentiation to Bay K varied greatly between tissues and the overall picture was an increase in the mean values which was not statistically significant at any concentration of Bay K .

The peak responses within 5min exposure to KCl were used in these experiments. See fig 5.5a. The detailed form of responses to KCl 50mM in these experiments was as follows : control responses to KCl in 10uM to 0.1mM Ca^{2+} were monophasic and without spontaneous activity in the tissue prior to or after KCl addition. However in the presence of Bay K , biphasic contractions appeared to KCl in 0.1mM Ca^{2+} from 30nM Bay K in 1 out of 3 tissues, and from 0.1μM Bay K in the remaining tissues. In the lower Ca^{2+} buffers (10μM and 30μM) biphasic contraction occurred in the presence of higher concentrations of Bay K (from 0.3μM). At the higher concentrations of Ca^{2+} (1.25mM and 2.5mM), KCl 50mM-induced contractions were clearly biphasic in the controls. In the presence of Bay K the phasic response was a little enhanced in these few tissues sensitive to Bay K in 2.5mM Ca^{2+}, while the tonic response was hardly affected. See fig 5.5b.

Comparing responses in the buffered Ca^{2+} at 0.1mM Ca^{2+} with those in unbuffered Ca^{2+} at 0.31mM Ca^{2+}, control responses without Bay K were higher in "buffered" than in "unbuffered", even though the Ca^{2+} level in the unbuffered was higher. The Bay K concentration response curve was steeper in the unbuffered saline than in the buffered Ca^{2+}. A possible explanation of this is that Bay K Ca^{2+} facilitating effect decreases as the response increases.

5. CGP 28392

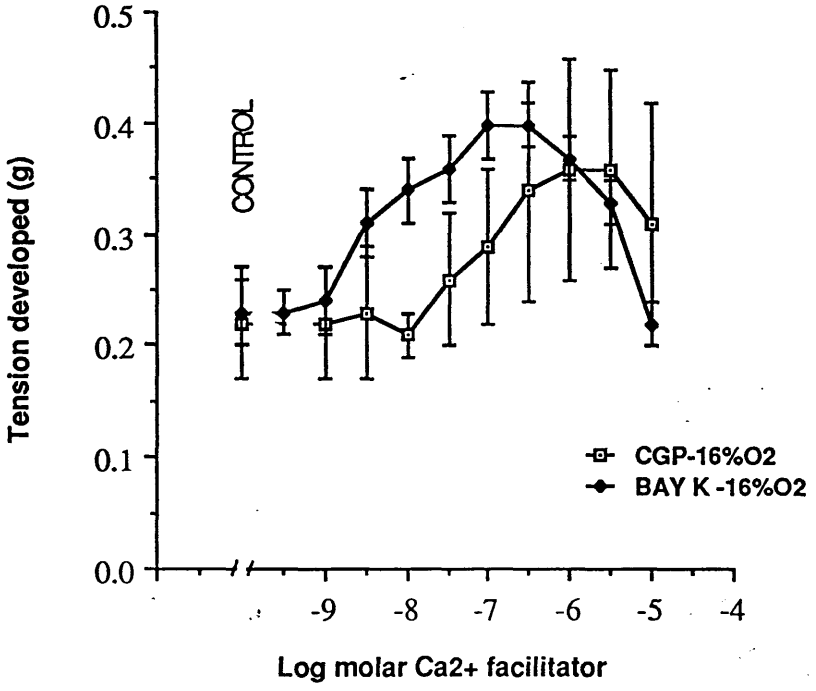
CGP 28392 experiments were carried out in unbuffered and in buffered Krebs at 0.31mM.

a. Concentration-effect in 0.31mM Ca^{2+} (unbuffered)

(i) Effects on KCl 50mM induced contraction

KCl at 95% and at 16%O_2.

CGP (10nM - 1µM) was less potent than Bay K in potentiating KCl 50mM- induced contraction . From 3nM, CGP caused a concentration-related increase in contraction which peaked at 1µM. The pD_2 value at 95%O_2 was 7.16±0.02 (n =4). CGP was about an order of magnitude less potent than Bay K . The inhibitory



Flg 5.6

Concentration response/ relationship for Bay K 8644 and CGP 28392 at 16%O_2.

Facilitation was assessed by the increase in response to KCl 50mM in Krebs containing 0.31mM Ca^{2+}.

The experiments with Bay K and CGP were unpaired. Points represent means ± s.e.m of n = 4-9 experiments.

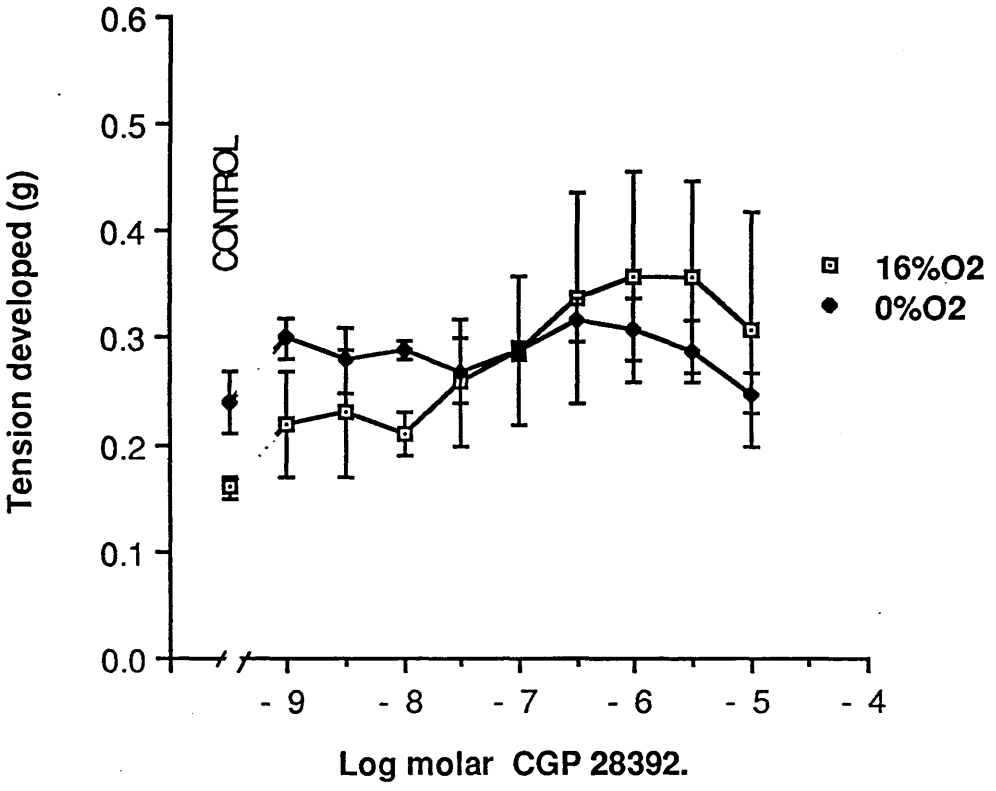


Fig 5.7

CGP 28392 concentration/ response relationship at 16%O_2 and 0%O_2.

CGP's induced facilitation was assessed by the increase in response to KCl 50mM.

The experiments in the different O_2 tensions were unpaired. The points represent means ± s.e.m of n = 4-6 experiments.

potency of CGP at 95% is similar to that of Bay K . See fig 5.6. The trend was the same in the 16%O_2 tension. Table 5.2 shows the pD_2 values from CGP in KCl 50mM contracted tissues at different O_2 tensions.

KCl at 0%$_2$

Even though control responses to KCl 50mM without CGP 28392 at 16%O_2 and at 0% were lower than those obtained for the Bay K series of experiments at equivalent 0%O_2 tensions, control responses to KCl at 0% were higher they than were at 16%O_2. CGP-induced facilitation was similar to Bay K 's, see fig 5.7. The maximum facilitation (calculated from the maximum change in tension expressed as a percentage of the control response) was compared with the value for Bay K .
At 0%, as in presence O_2, CGP was less potent than Bay K as its concentration response lay to the right of that of Bay K . See fig 5.8.

Concentration-effect in 0.31mM Ca^{2+} (buffered)

A similar experiment was carried out in buffered Ca^{2+} at the same $[Ca^{2+}]_{free}$ as in unbuffered. In this case it was difficult to demonstrate the Ca^{2+} facilitating effect on KCl-induced contraction. Both CGP-treated and control tissues showed a time dependent decrease in response when CGP concentrations from 3nM to 10μM were tested. See fig 5.9. The apparent lack of effect of CGP

	O2 TENSION	pD2 values	P values
KCl (50mM)	95%	7.16 ±0.02	NS
	16%	7.17 ±0.14	
	0%	6.92 ±0.33	NS
NA (0.3μM)	95%	6.38 ±0.07	
	16%	6.82 ±0.14	*

Table 5.2 shows the pD2 values obtained for non-cumulative concentration/response of CGP 28392. The portal veins were contracted with either KCl 50mM or NA 0.3μM in Krebs containing 0.31mM Ca2+. The pD2 values were calculated from the % increase in response to either KCl or NA in the presence of CGP 28392.
Values are means + s.e.m n=4 -6.
pD2 value for KCl or NA has been compared with those at other O2 tensions.
*, is $P < 0.05$, while NS is not statistically significant from 16%O2.

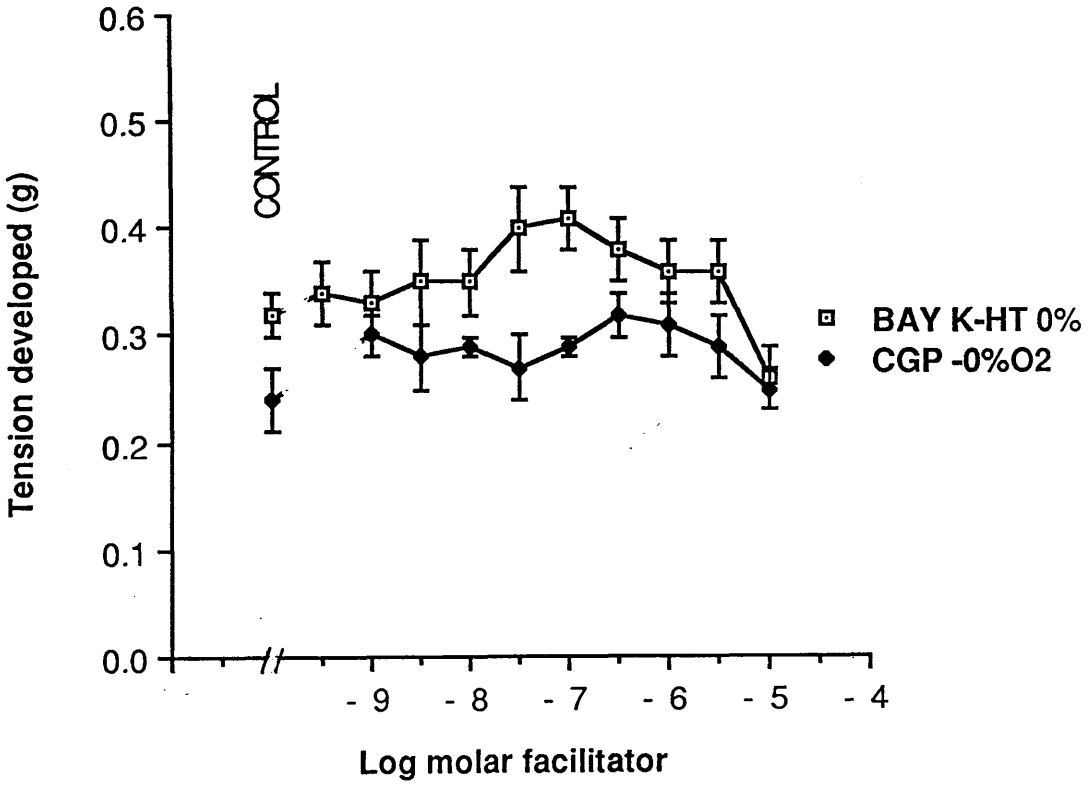


Fig 5.8

Concentration response/ relationship for Bay K 8644 and CGP 28392 at $0\%O_2$.

Facilitation was assessed by the increase in response to KCl 50mM in Krebs containing 0.31mM Ca^{2+}.

The experiments with Bay K and CGP were unpaired. Points represent means ± s.e.m of n = 4-6 experiments.

was due to the higher Ca^{2+} level, 0.31mM in these experiments than the 0.1mM in Bay K experiments.

(ii). Effects on NA 0.3uM-induced contraction

Concentration-effect in 0.31mM Ca^{2+} (unbuffered)

In the same concentration range (10nM - 1μM) that potentiated responses to KCl, CGP 28392 potentiated responses to NA 0.3μM at 16% and at 95% O_2. CGP was also less potent than Bay K in potentiating responses to NA (fig 5.10). The pD_2 value at 95% was statistiaclly signicantly lower ($P < 0.05$) than at 16%. The reason for this is unknown. However, the pD_2 value at 16% O_2 was not statistically significantly different from the value obtained for KCl at 16% O_2 (see table 5.2). In general, (as for Bay K 8644) the values obtained for NA were slightly lower than those for KCl.

6. Nifedipine's interaction with Bay K 8644.

a. In tissues contracted with KCl.

In the presence of low concentration of nifedipine, 1nM, there was a small leftward shift in Bay K CRC. From 10nM nifedipine, there was a rightward shift in Bay K CRC, with an enhancement of its maximum (Fig 5.11a).

b. In tissues contracted with NA.

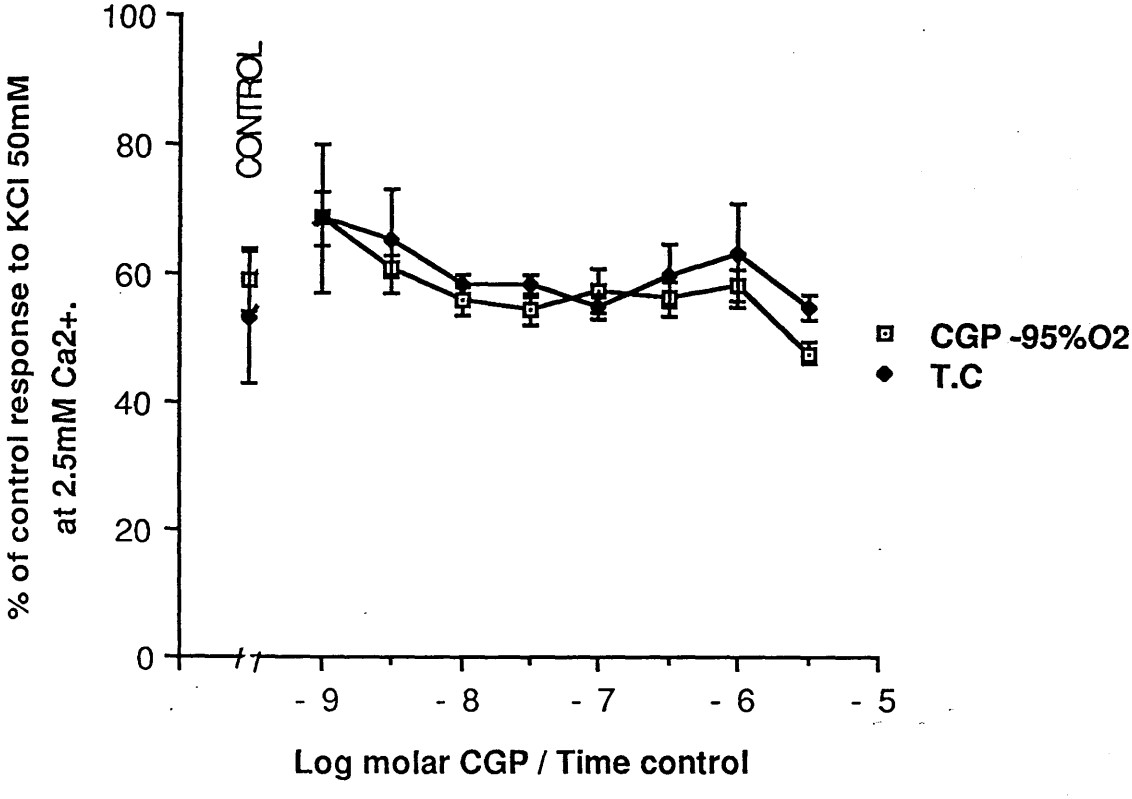


Fig 5.9

CGP 28392 concentration/ response relationship at buffered Ca^{2+} level 0.31mM at 95%O_2.

A time matched control is also shown. This response was not statistically significantly different from CPG treated ($P > 0.05$). Points represent means ± s.e.m, n = 4.

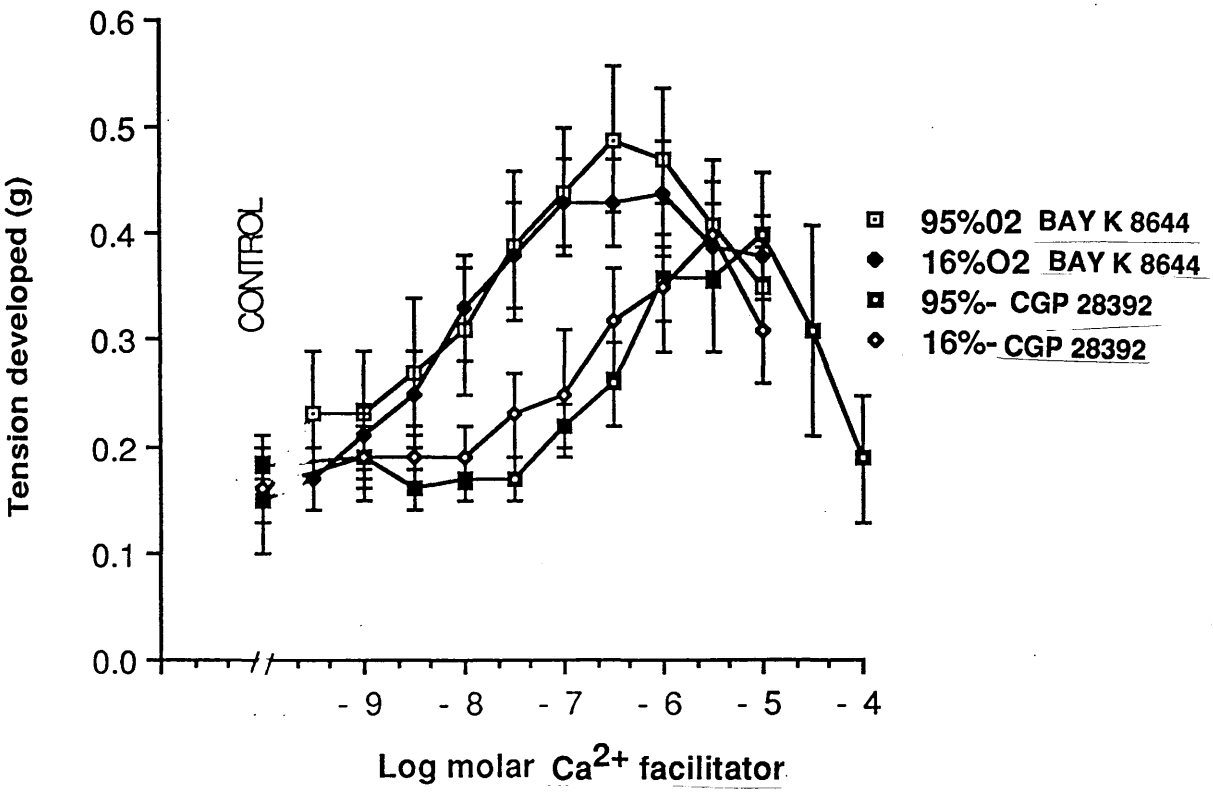


Fig 5.10

The concentration response/ relationship for Bay K 8644 and CGP 28392 at 16%O_2 and 95%O_2 .

Facilitation was assessed by the increase in response to NA 0.3μM by either Ca^{2+} facilitator in Krebs containing 0.31mM Ca^{2+}.

The experiments with Bay K and CGP were unpaired. Points represent means ± s.e.m, n = 4-6.

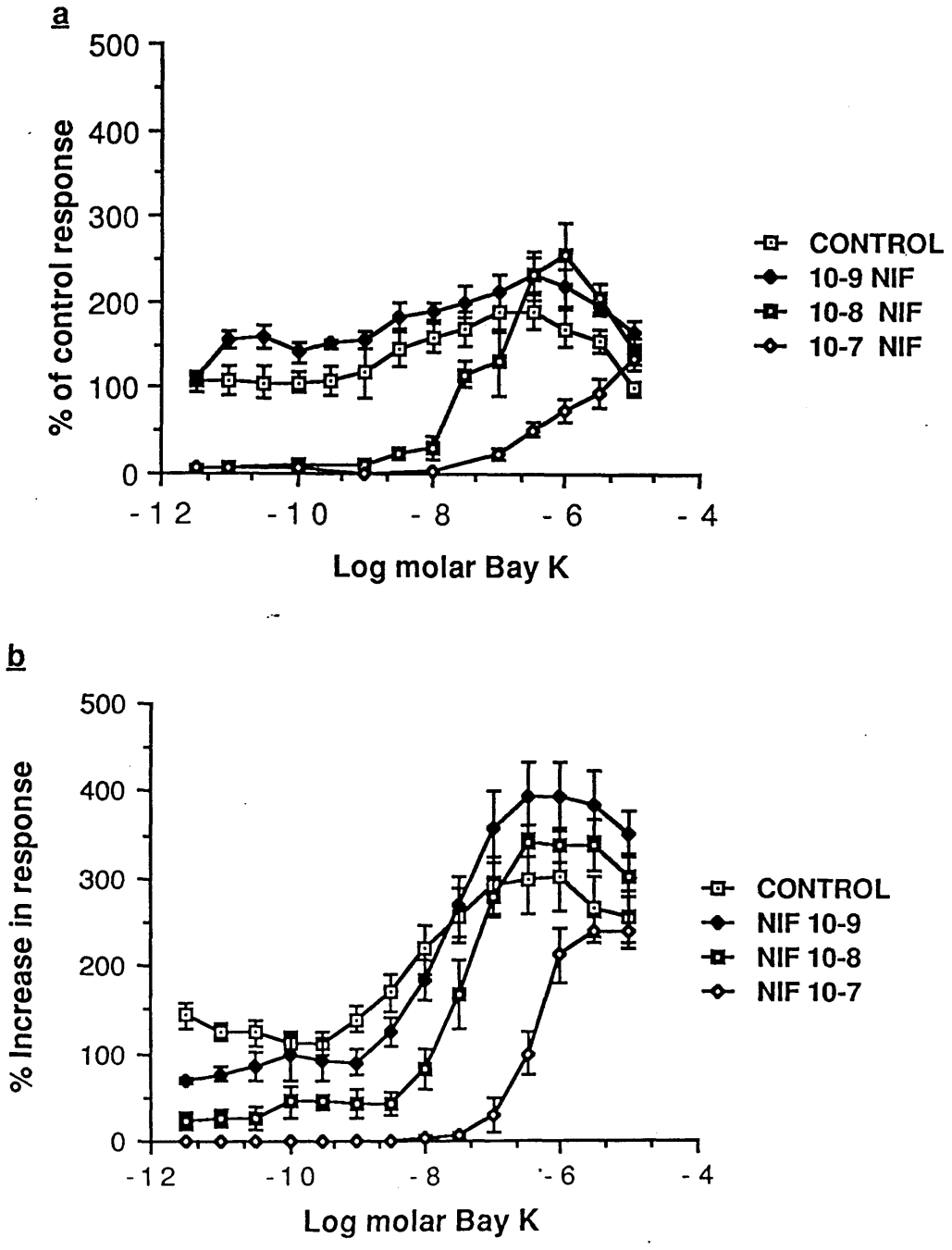


Fig 5.11.
Nifedipine's interaction with Bay K 8644 -induced effects in (a) KCl 50mM contracted tissue and (b) NA 0.3μM contracted tissues at 16%O_2.

Tissues were pretreated with nifedipine 15 -30min, before producing Bay K CRC in the presence of nifedipine.
One concentration of nifedipine was used per tissue. Each concentration of nifedipine was tested on at least 4 tissues.

Nifedipine 1nM did not potentiate Bay K 's in NA contracted tissues, however, it caused a small but statistically insignificant shift of Bay K 's CRC. As for KCl, nifedipine from 10nM caused a parallel shift of Bay K 's CRC with an enhancement of its maximum (Fig 5.11b)

DISCUSSION

Validity of method

In this series of experiments, the optimal assay of Bay K could be carried out at 0.31mM Ca^{2+}. Clearer facilitation of responses to either NA or KCl was observed at this concentration over a wider range of O_2 levels. The potency of Bay K was similar to that described in other vascular tissues (Schramm et al, 1983; Dube et al, 1985; Mikkensen et al, 1985). Also the dual action or the "partial agonist" effect is clearly seen in this assay method; the facilitatory agonist effect from 1nM to 0.3μM, and the inhibitory effect at 1uM and above. Since the concentration/response relationship for Bay K 8644 is bell-shaped, it is possible that the inhibitory effect has started before the maximum facilitatory effect has been reached. Consequently there is no true "maximum" and differences in the maxima, e.g. between Bay K 8644 and CGP 28392 or with O_2 or Ca^{2+} levels, are of no quantitative significance. Similarly, pD_2 values may be overestimates

of potency since they are based on the concentration to achieve 50% of a response less than maximum. Nevertheless our estimated pD_2 values for Bay K are comparable with those in the literature, (e.g) (i) 7.94 (calculated from the EC_{50} of 11.4 ±1.0nM) in porcine coronary artery (Dube et al, 1985); (ii) about 8 in both the isolated rabbit aorta and the isolated perfused guinea pig heart (Schramm et al, 1983); (iii) 7.65 in dog heart sarcolemma (Vaghy et al, 1984). Erne et al (1984) obtained a pD_2 value of 6.65 for CGP in human platelet. This value is slightly lower than those obtained in this study. However, it was noted that most previous workers used single concentrations of either Bay K 8644 or CGP 28392 in their studies. This present "assay" for Ca^{2+} channel facilitation was quite sensitive to Bay K , particularly at the lower $[Ca^{2+}]$, where spontaneous activity is virtually abolished in the controls and small changes were therefore easily detectable. High phasic activity limits the subtlety of detection at high $[Ca^{2+}]$. Comparing Bay K 8644 with CGP 28392, the latter was about one order of magnitude less potent than Bay K , in its agonistic potency, while the inhibitory effects were more similar. The potencies of Bay K and CGP 28392 are similar to those described by Mikkelsen (1985) assessed by the increase in the spontaneous activity of non-stimulated rat portal vein. Bay K potencies determined in different O_2 tensions were not significantly different from each other. The only deviation from this was when oxygen gassing was

eliminated altogether (0%: Po_2 5±2mmHg). At slighlty higher but still low Po_2 (from 4% to 1%), the potency of Bay K remained the same as at 16% O_2 in KCl stimulated tissues. Only at 0% O_2, did a radical change occur: control responses to KCl were significantly higher than at 16%, and Bay K was capable of little facilitation. Facilitation was still significant at 30nM and 0.1μM but inhibition was observed from 0.3μM rather than from the higher concentration of 1μM found at higher O_2 tensions. This result might suggest that in hypoxia, KCl-induced control responses were already facilitated and as a result there was little scope for Bay K to facilitate them. Vanhoutte et al (1980a, b) showed that larger responses were obtained to KCl in hypoxia than in normoxia in canine coronary artery. In addition larger responses were also obtained to NA in hypoxia (contrary to our observations in the RPV- see section below) and 5-HT.

In the present study, NA-induced responses in low Po_2 i.e 4% and 0% showed a considerable decrease from control responses at higher Po_2. However, as for KCl. at 0% O_2 the degree of potentiation relative to the control (at 0% O_2 tension) was reduced. Again, as for KCl, in 4% O_2, potentiation was straightforward though starting from a lower control size. Again it is only at 0% O_2 that Bay K 's effectiveness is lost.

Hellstrand (1979) showed that about 25% of the KCl-induced response was resistant to hypoxia, while only

10-15% of the NA -induced response remained in hypoxia (Hellstrand, 1977 & Ebeigbe, 1979). These experiments were carried out in 2.5mM Ca^{2+}. We too, have confirmed this relative resistance of response to KCl in a series of separate experiments, though a higher percentage of the response to KCl (45%) remained in hypoxia. The relative resistance of KCl to oxygen compared with that to NA has also been reported in another blood vessel, namely the rabbit aorta (Shibata & Briggs, 1967; Coburn, 1977). Several hypotheses have been put forward to explain O_2-resistant, KCl-induced contractions, e.g. lower O_2 consumption, smaller anoxic core (Shibata & Briggs, 1967; Ruiz et al, 1981), O_2-sensitive mechanisms other than supply of ATP to contractile machinery (Needleman & Blehm, 1970; Namm & Zucker, 1973) or anaerobic metabolism to supply ATP (Peterson &.Paul, 1974).

Experimental conditions of high KCl , 0%O_2 mimic those of ischaemia, which are characterised by acidosis, hyperkalemia and metabolite accumulation. Hellstrand et al (1977) showed that in the rat portal vein metabolic activity was higher in hypoxia than in normoxia, thus leading to accumulation of metabolites and acidosis (Sigurdsson & Grammp, 1981). Recently, endogenous Ca^{2+} channel activators have been identified, including substance P (Govoni,1987; Perney & Miller, 1987) and palmitoyl carnitine (Bigaud & Spedding, 1986; Mir & Spedding, 1986). Palmitoyl carnitine is a lipid metabolite which accumulates in the sarcolemma during

myocardial ischaemia and might therefore be a candidate for an endogenous ligand produced during ischaemia, exacerbating calcium overload. Therefore the presence of palmitoyl carnitine or similar compounds during hypoxia facilitation would already have occurred, which could make further facilitation by Bay K 8644 impossible.

However, to test the hypothesis that responses were already facilitated at 0% O_2, we considered the following in the next section:

1. If the responses to KCl were already facilitated and Bay K could not cause much facilitation, then the tissue response at 0% should be less susceptible to nifedipine in 0% than in 16% O_2.
2. The CGP 28392 compound, an analogue of the Bay K compound should mimic Bay K 's effect.
3. The effect of the endogenous ischaemic metabolite , palmitoyl carnitine should be similar to that of Bay K , and the responses at 0% should be abolished by an antagonist of palmitoyl carnitine synthesis.
4. The sensitivity to extracellular [Ca^{2+}] of the rat portal vein at 0% should be low.

Comparison of the response in hypoxia to that in Ca^{2+}-free

Ebeigbe et al (1980) showed that in the rat portal vein, the effect of Ca^{2+} withdrawal was similar to that of hypoxia, an observation based on the use of NA only.

This is not consistent with the observations made in this study since neither NA or KCl-induced responses were abolished in hypoxia vis-a-vis abolition of such responses in Ca^{2+}-free Krebs. Indeed Sigurddson & Grammp (1981) showed that, at 2.5mM Ca^{2+}, responses to various acitvators,e.g. NA, ACh, KCl, 4-aminopyridine and Ba^{2+} are depressed but not completely abolished by hypoxia. However the situation at low Ca^{2+} is different for KCl-induced contraction where the responses to became larger.

Interaction of nifedipine with Bay K 8644

Nifedipine no doubt antagonises the Ca^{2+} facilitatory effect of Bay K , at concentrations between 1nM and 0.1μM depending on the agonist the tissue is stimulated with. It is well documented that the Bay K and nifedipine interaction is on the dihydropyridine (DHP) receptor sites (Schramm et al, 1984; 1985; Spedding, 1985, Thomas et al, 1985).

The potentiation of Bay K induced effect by nifedipine at 1nM is interesting. Evidence for such paradoxical nifedipine potentiation of Bay K has been provided in other tissues. Calcium antagonists have been reported to have positive inotropic effects on canine papillary muscle, at low concentrations (Himori et al, 1976); Schramm et al, 1982 showed nifedipine at 2.9nM caused potentiation of Bay K induced contractions of KCl 15mM partially depolarised rabbit aorta. The absence of

statistical information in the report of Schramm et al precludes a determination of the significance of this observation. Recently, Dube et al 1985 made a similar observation in porcine coronary artery. They showed that pretreatment for 25 - 90min with 80% inhibitory concentrations of either nimodipine or diltiazem potentiated Bay K - induced contractions. Higher concentrations of diltiazem caused an inhibition of Bay K induced contraction, while pretreatment with higher concentrations of nimodipine caused further potentiation of potentiation elicited by Bay K . Dube et al proposed that Bay K functioned as a partial calcium antagonist at two functionally distinct 1,4-dihydropyridine 'receptor sites'. Schwart et al, 1985 showed that nitrendipine also potentiated Bay K -induced contraction of porcine coronary artery.

CHAPTER 6

MODULATORY EFFECTS OF HYPOXIA ON Ca^{2+} CHANNEL FUNCTION

SUMMARY

1. Ca^{2+} concentration/response at 0.31mM Ca^{2+} level used to assess facilitation by Ca^{2+} facilitators, at normoxia was not statistically different from that at hypoxia.
2. Assessment of nifedipine's inhibition of contraction to KCl showed that there was a progressive resistance to nifedipine as O_2 was lowered, particularly at hypoxia.
3. The phasic component of KCl-induced contraction was more resistant to nifedipine than was the tonic component.
4. As for Bay K and CGP, the maximum facilitation induced by palmitoyl carnitine was depressed in hypoxia compared to normoxia.
5. Control responses to KCl were more susceptible to POCA in hypoxia than they were in normoxia.
6. Results are consistent with the hypothesis that the Ca^{2+} channels may have already been facilitated in hypoxia.

INTRODUCTION

"When attempting to relate the therapeutic effects of drugs to their pharmacological properties, it is important to remember that the tissue conditions prevailing in pathological states may profoundly affect

the drug activities. In ischaemic or anoxic conditions, a drop in the activity of the sodium-potassium pump and an activation of K^+ efflux may be responsible for an increase of extracellular K^+, which would in turn modify the membrane potential and the function of calcium channels" (Godfraind, Miller & Wibo,1986).

This above quotation seems to be highly pertinent to the differential results obtained at normoxia and hypoxia with respect to KCl induced responses. The inability of Bay K to facilitate well in hypoxia may mean that contractions induced in hypoxia may already have been facilitated, (though not necessarily due to a change in $[K^+]_o$). Hence the inability of Bay K to cause further facilitation. This hypothesis is further supported since control responses to KCl in hypoxia are in fact higher than in 16%O_2 .

In this study this hypothesis of the responses being facilitated in hypoxia has been tested in three ways.
(1) If the responses in hypoxia are already facilitated, by an action on the Ca^{2+} channels, the Ca^{2+} sensitivity should be increased in hypoxia and therefore the Ca^{2+} concentration/ response curve in hypoxia should lie to the left of that in normoxia.
(2) If the responses in hypoxia are less sensitive to Bay K than they were in normoxia, because the channels are already facilitated, then it would be expected that the responses in hypoxia might be less sensitive to nifedipine as well, either by pharmacolgical antagonism

at the dihydropyridine site or physiological antagonism within the Ca^{2+} channel..

(3) If the response is already "fully" facilitated (as indicated by the ineffectiveness of Bay K in hypoxia) by accumulation of acyl carnitines in hypoxia, then exogenous palmitoyl carnitine should have a similar profile Bay K . Palmitoyl carnitine (PC) is an endogenous Ca^{2+} facilitator that accumulates during ischaemic conditions (Spedding, 1987; Spedding & Mir, 1987). PC has been shown to activate Ca^{2+} channnels directly (Spedding & Mir, 1987).

In addition, the effects of POCA, an inhibitor of the synthesis of palmitoyl carnitine, were examined on the response to KCl in hypoxia and on palmitoyl carnitine's effect on KCl-induced responses: this should block the effects of endogenous PC and provide corroboration or otherwise for the above hypothesis.

METHODS:

First series:

Ca^{2+} dependency of the RPV at hypoxia:
non-cumulative Ca^{2+} re-addition.

After an equilibration at 16% O_2 in Krebs containing 2.5mM Ca^{2+} the tissues were contracted with KCl 50mM until contractile responses were reproducible. The

tissues were then washed with "normal" Krebs and allowed 15min rest. They were then washed 3 times with zero Ca^{2+} saline and, after 5min in the latter, KCl 50mM was added. A response was taken followed by wash out. Thereafter the tissue were exposed to increasing Ca^{2+} concentrations, allowing 10min in each before KCl 50mM was tested for 5min.

For the experiment in hypoxia, after the inital equilibration with 16% O_2, the gas was switched to 95% N_2:5% CO_2 for 30min. Subsequently the tissues were washed with zero Ca^{2+} saline as in normoxia, and the rest of the procedure above was repeated.

Second series

a. Concentration /effect relationship for nifedipine versus KCl 50mM contractions at 0.31mM Ca^{2+}.

The concentration/response relationship for nifedipine was examined on KCl 50mM induced responses at 0.31mM Ca^{2+}, using the previously described protocol for Bay K . Tissues were examined at both 0% and 16% in unpaired experiments. The tissues were incubated for 15-30min with the first concentration of nifedipine, before a response to KCl 50mM was taken for 5min. The tissues were washed with 0.31mM Ca^{2+} Krebs, and the next concentration of nifedipine was added to the bath for 10min before taking another response to KCl 50mM.

In a separate series of experiments, effects of addition of nifedipine on the spontaneous activity of the RPV were examined.

b. Nifedipine's effect at 2.5mM Ca^{2+}

At low Ca i.e. 0.31mM Ca, the characteristic biphasic response to KCl normally obtained at high Ca was absent. We therefore decided to look at response to KCl at high Ca in order to study the effects of nifedipine on the different components of KCl-induced response at three levels of oxygen tension, hyperoxia 95%O_2, normoxia 16%O_2 and hypoxia 0%O_2. The response to KCl 50mM at high Ca^{2+} usually consisted of two components, phasic and tonic. The phasic response was transient usually lasting 15-30s, while the tonic response was slower to develop and of longer duration..
The protocol used was the same as for 0.31mM Ca^{2+} except that 2.5mM Ca^{2+} was used throughout the experiments.

3. Third series:

Palmitoyl carnitine and POCA exeriments

a. Palmitoyl carnitine

The protocol was the same as for Bay K assay (see method Chapter 4). The tissues were incubated with each concentration of palmitoyl carnitine for 15 - 30min

before in 0.31mM Ca^{2+} testing it against KCl 50mM-induced contraction.

b. POCA

POCA was studied in experiments where 2 tissues were run in parallel with KCl 50mM as activator (1) control without POCA (10µM and 100µM), (2) POCA alone and (3) POCA in combination with palmitoyl carnitine (1µM - 100µM). The tissues were incubated with POCA for 15min to 20min. The same time was used for palmitoyl carnitine.

ANALYSIS OF RESULTS

Results from hyperoxia or hypoxia were normally compared with the control response in normoxia, using either unpaired or unpaired t- tests, as appropriate. $P < 0.05$ was considered to be statistically significant.

In nifedipine experiments at 2.5mM Ca^{2+}, the phasic and the tonic responses to KCl 50mM were analysed separately, each as a percentage of its own maximum. pIC_{50} values were obtained by linear regression analysis of the log concentration/ response relationship.

Drugs:

Nifedipine (Bayer), palmitoyl carnitine (Sigma), POCA (phenyl alkyl oxirane carboxylic acid), chemical name: sodium 2-[5-(4-chlorophenyl)-pentyl]-oxirane-2-

carboxylate -obtained from Dr. Mike Spedding, Syntex Research Centre, Research Park, Edinburgh.

RESULTS

1. First series:

Ca^{2+} DEPENDENCY OF THE RPV AT 16%O_2 AND 0%O_2.

In this series of experiments, the peak responses by 1min to KCl at each concentration of Ca^{2+} during non-cumulative Ca^{2+} re-addition at 0% were not statistically significantly different ($P > 0.05$) from the equivalent responses at 16%O_2 for the peak response by 1min. However as the Ca^{2+} concentration increased there was a qualitative difference between the response at low Ca^{2+} from 0.16mM - 0.63mM and at high Ca^{2+} of 1.25mM and above. The time courses of the responses are shown as insets in fig 6.1a . Note the fast phasic contraction at the high Ca^{2+} which contrasts with the slow contraction at low Ca^{2+}.

For the 5min response, howvever, the Ca^{2+} concentration/ response curve at 0% lay to the right of that at 16%O_2 and the responses at each concentration were significantly different ($0.001 < P < 0.05$) as shown in fig 6.1b

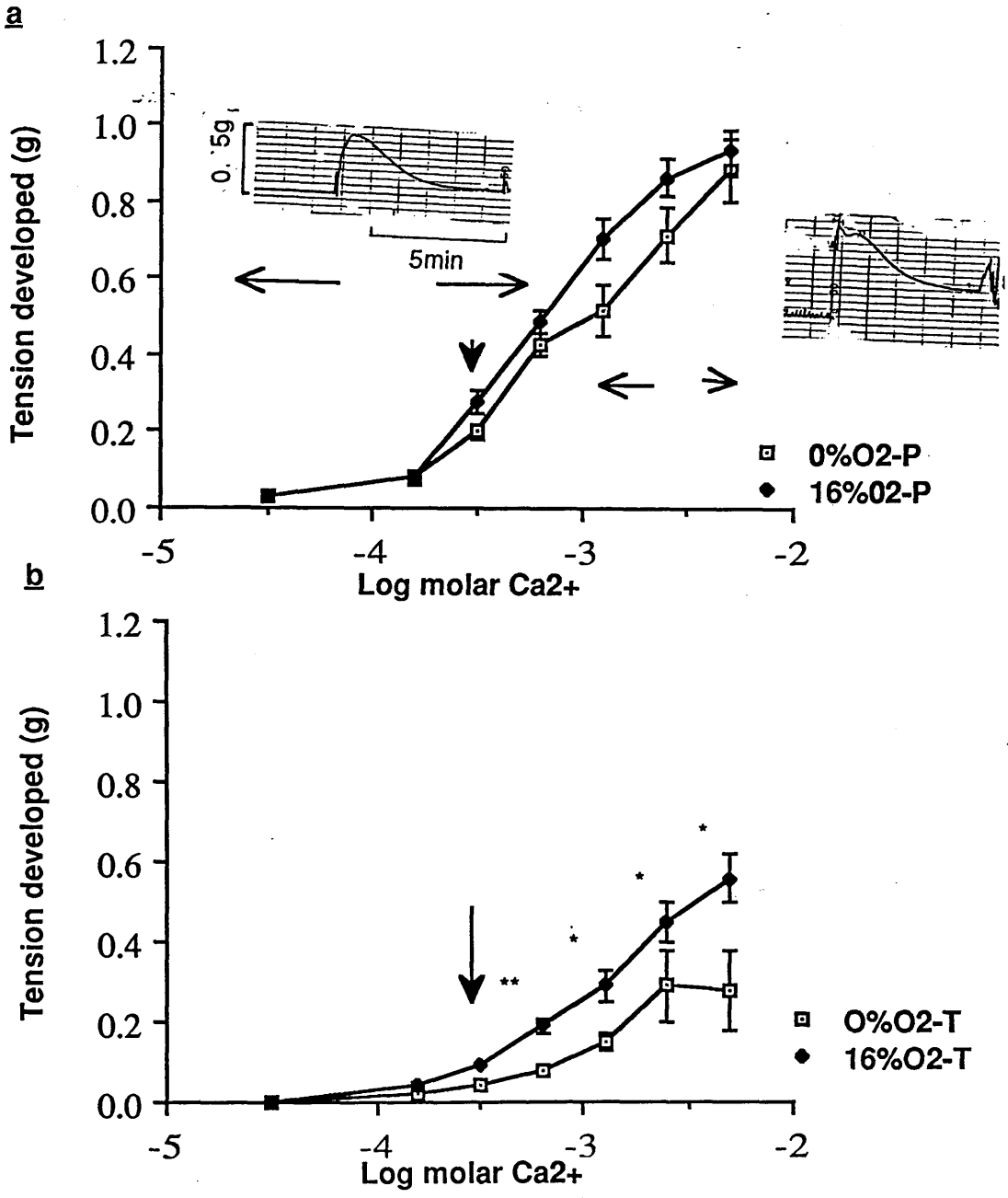


Fig 6.1.
The $[Ca^{2+}]$/ response relationship in RPV contracted with KCl 50mM during non-cumulative Ca^{2+} re-addition at $0\%O_2$ (hypoxia) and $16\%O_2$ (normoxia) in unpaired experiments.
(a) shows the 1min response (phasic) and (b) 5min response (tonic). However, qualitatively different responses are obtained by 1min as indicated in the insets. Horizontal arrows indicate the $[Ca^{2+}]o$ range the responses occur, while the vertical arrow indicates the $[Ca^{2+}]o$ used in the assay of Ca^{2+} facilitators.
The responses at each $[Ca^{2+}]$ were not statistically significantly different from each other at $0\%O_2$ and $16\%O_2$ ($P< 0.05$). On the other hand, the responses at 5min at $16\%O_2$ (b) were significantly higher (*, ** shown are for $P < 0.05$ and 0.01 respectively). The points show means $\pm$ s.e.m., n = 5 - 6. Vertical arrow as for (a).

2.Second series:

NIFEDIPINE

Spontaneous activity

During the equilibration period in Krebs containing 2.5mM Ca^{2+},
switching from 16% to 0% reduced the height of the spontaneous activity to 36.4.0±3.0%, n=14 (pooled from various data). Replacement of this Krebs with one containing 0.31mM Ca^{2+} led to the abolition of the spontaneous activity.

a. Concentration /effect relationship for nifedipine versus KCl 50mM contractions at 0.31mM Ca^{2+}.

At hypoxia

(i) Nifedipine on KCl

Nifedipine 1nM potentiated contractions to KCl 50mM by 30.7±3.3%, n=6, and induced spontaneous activity. At the higher concentration of nifedipine 10nM, the KCl-induced contraction was smaller than with 1nM nifedipine but was still higher (9.6±5.0% potentiation) than the

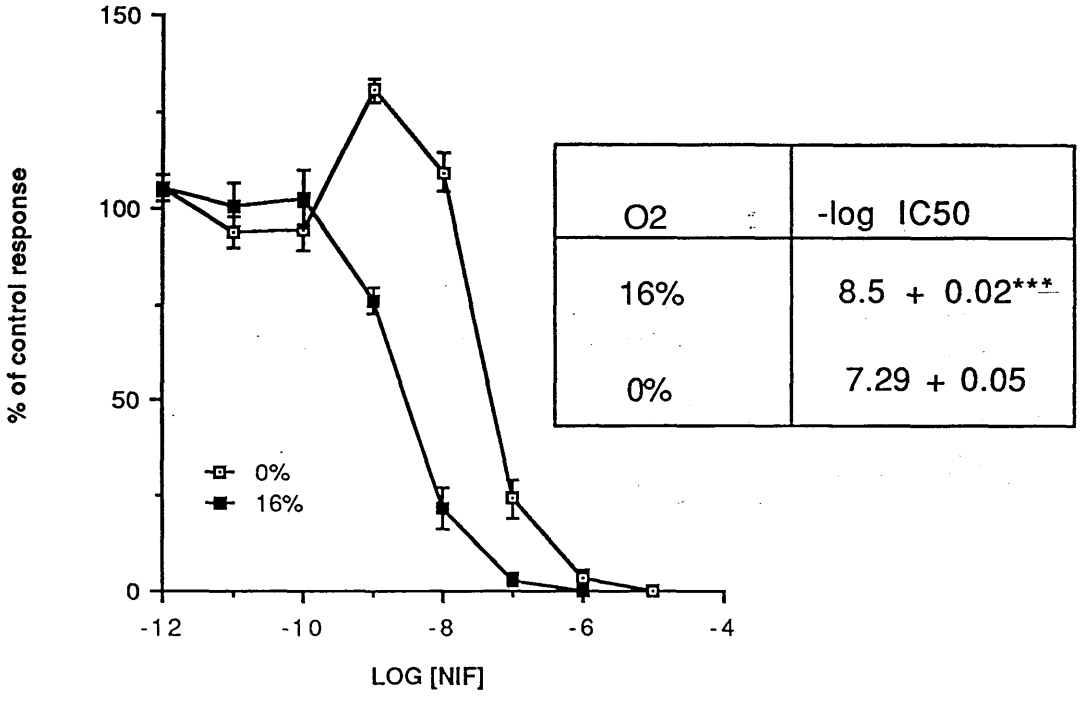
control and spontaneous activity was still evident. Higher concentrations of nifedipine caused inhibition and 100% inhibition was obtained by 1μM (Fig 6.2).

The hypoxia -resistant, KCl-induced responses were compared with responses obtained at 16% O_2. At 16% O_2, nifedipine inhibited responses from 1nM in 2 out of the 6 tissues, in the remaining tissues the threshold was at 0.1nM. In all six tissues 100% inhibition was obtained at 1μM.It therefore appears that the hypoxia -resistant, KCl-induced responses were less susceptible than those at 16% O_2. Fig 6.2 shows that from nifedipine 0.1nM, the mean concentration response curve for nifedipine at 0% O_2 lies to the right of that at 16% O_2. The mean -log IC50 value for nifedipine against KCl at 16% O_2 was significantly higher ($P < 0.001$) than at 0% O_2.

(ii) Concentration / effect relationship for nifedipine versus NA 1uM contraction at 0.31mM Ca^{2+} in 0% O_2

The nifedipine concentration/response curve at 0% in the tissues contracted by NA 1μM, like that for KCl 50mM lay to the right of that at 16% from 1pM to 1nM. At 10nM, % inhibition was shifted the right of 16%. However the mean -log IC50 for nifedipine at 16% O_2 was not statistically different ($P > 0.05$) from that at 0% O_2. See fig 6.3a. Fig 6.3b compares the mean -logIC50 for nifedipine against NA with nifedipine against KCl.

Nifedipine concentration response at 16% and at 0%O2.



O2	-log IC50
16%	8.5 + 0.02***
0%	7.29 + 0.05

Fig 6.2

The concentration /response relationship for nifedipine obtained from the inhibition of KCl 50mM-induced contraction of RPV at 16%O_2 and 0%O_2 at 0.31mM Ca^{2+}. The nifedipine concentration/response curve at 0%O2 lies to the right of that at 16%O_2.

The inset shows the -logIC50 values for nifedipine at the two O_2 tensions. The value at 16%0_2 was significantly higher ($P< 0.001$) than that at 0%O_2. The points and values represent means $\pm$ s.e.m, n = 6.

Fig 6.3.

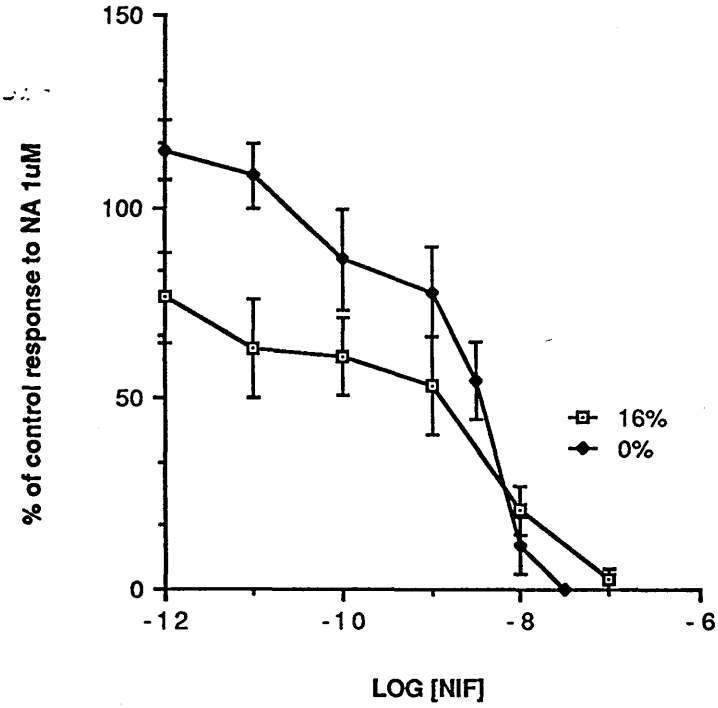
(a) The log concentration/response relationship for nifedipine obtained from the inhibition of NA 1μM -induced contraction of RPV at 16%O_2 and O%$_2$ at 0.31mM Ca^{2+}.

The nifedipine concentration response at 0%O_2 lies to the right of that at 16%O_2 up to 10nM.

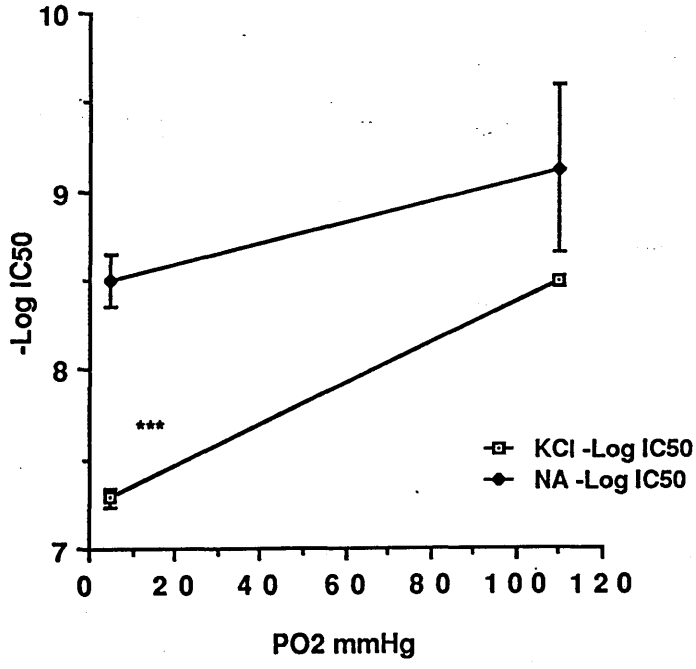
(b). Compares the -log IC50 for nifedipine against KCl 50mM and NA 1μM at 16%O_2 and 0%O_2. The -logIC50 for nifedipine against KCl was significantly higher ($P < 0.001$) at 16%0_2 than at 0%O_2. On the other hand, the log IC50 for nifedipine against NA was not statistically significantly higher at 16%O_2 than at 0%O_2.

The points are means ± s.e.m., n =4- 6.

a


150
100
50
0
% of control response to NA 1uM
-12
-10
-8
-6
LOG [NIF]
16%
0%

b


10
9
8
7
-Log IC50
***
0
20
40
60
80
100
120
PO2 mmHg
KCl -Log IC50
NA -Log IC50

b. At 2.5mM Ca^{2+}

(i) At 16% O_2

a. Nifedipine on non-stimulated RPV

The spontaneous activity (S.A) of the RPV was inhibited by nifedipine from 30nM and by 0.3μM there was 100% inhibition . The -log IC50 value for nifedipine against S.A was 7.67±0.1, n=4. This value is lower than those against different components of KCl's induced contraction at same O_2 tension (see below).

Nifedipine on KCl

Control responses to KCl 50mM

Contraction to KCl 50mM in Na-substituted Krebs was usually a phasic contraction followed by a tonic contraction which waned. The ratio of the tonic (5min measurement) to phasic components (1min measurement) was 0.56±0.02 n=4.

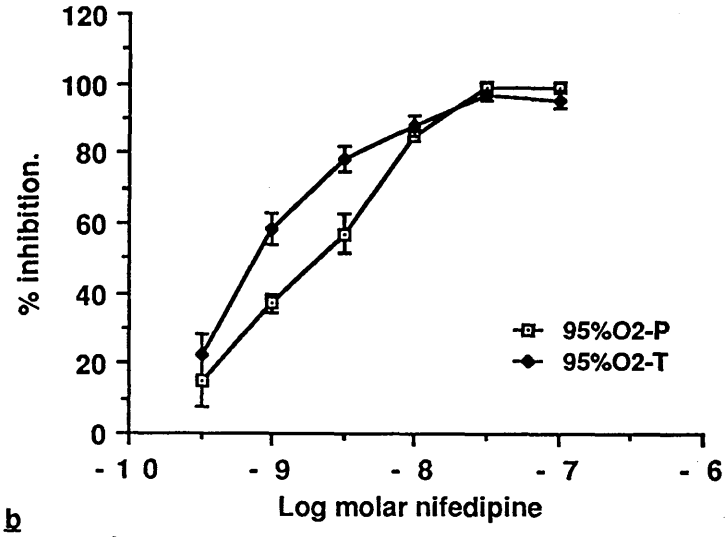
In the presence of nifedipine

Nifedipine inhibited the response from 0.3nM - 30nM. The phasic component of KCl 50mM -induced contraction was more resistant to nifedipine than was the tonic from

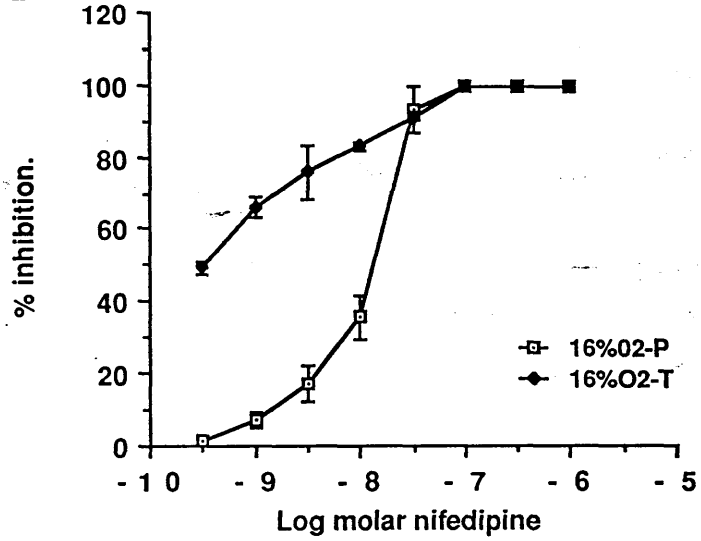
Fig 6.4.

The % inhibition of KCl 50mM -induced contraction against log molar nifedipine at (a) 95%O_2, (b) 16%O_2 and (c) 0%O_2 respectively at 2.5mM Ca^{2+}. The concentration response curve of the phasic component generally lies to the right of that of the tonic component, suggesting that it is more resistant to inhibition by nifedipine.
The points represent means ± s.e.m., n=4.

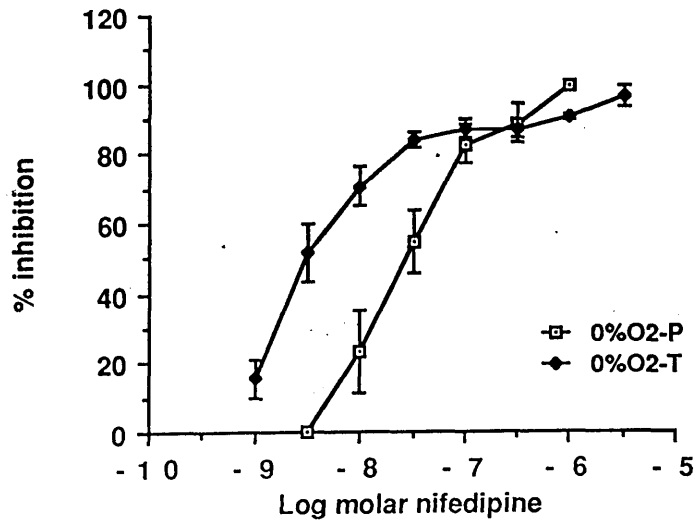
a


120
100
80
60
40
20
0
% inhibition.
95%O2-P
95%O2-T
- 10
- 9
- 8
- 7
- 6
Log molar nifedipine

b


120
100
80
60
40
20
0
% inhibition.
16%02-P
16%O2-T
- 10
- 9
- 8
- 7
- 6
- 5
Log molar nifedipine

c


120
100
80
60
40
20
0
% inhibition
0%O2-P
0%O2-T
- 10
- 9
- 8
- 7
- 6
- 5
Log molar nifedipine

0.3nM -10nM (fig 6.4b). The -log IC_{50} for nifedipine versus the phasic and tonic components were 7.88±0.05 and 9.37±0.02 respectively (Fig 6.5). At 30nM, the phasic contraction was 100% inhibited, but there remained approximately 10% of the tonic contraction that was resistant to nifedipine up to 1μM.

(ii). 95% O_2

Control response to KCl.

The tonic response was better maintained than in 16% and the ratio of the tonic to phasic components was 0.79±0.07, n=4.

In the presence of nifedipine

The KCl 50mM-induced responses were more susceptible to nifedipine than were those at 16 O_2% (see fig 6.5 which shows the mean -log IC50 values for nifedipine against KCl at different O_2 tensions). Responses were inhibited from 0.3μM to 30nM (fig 6.4a). As in 16% O_2, the responses were 100% inhibited at 30nM. At higher concentrations, 1uM and above, "the resistant response" was only about 3% of the control tonic response, i.e smaller than at 16% O_2.

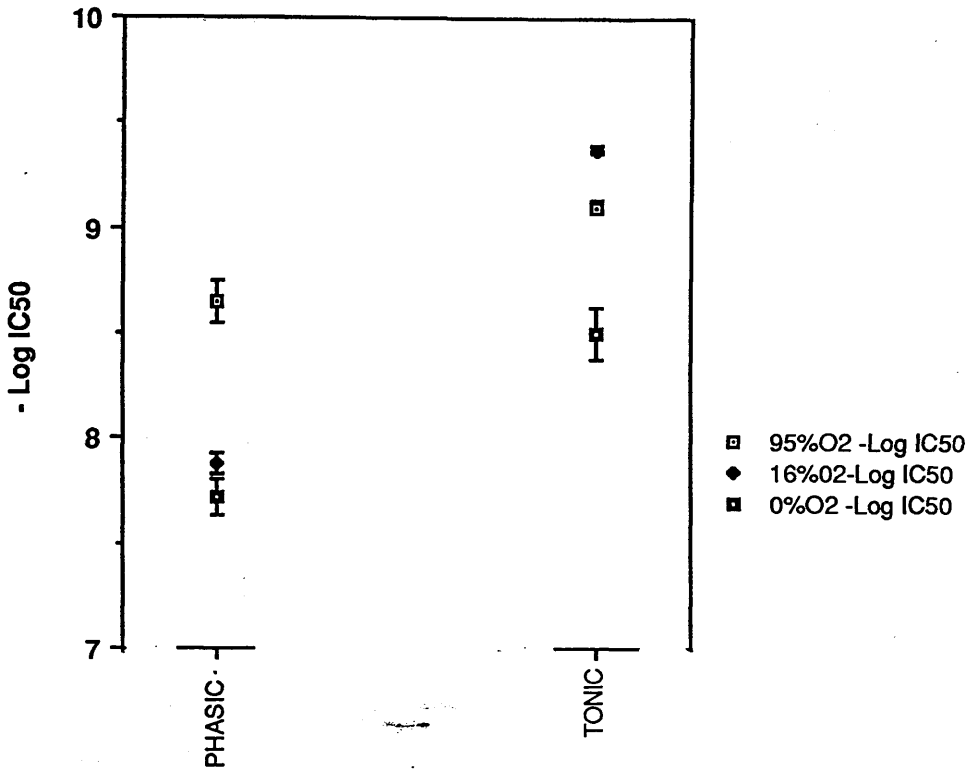


Fig 6.5

The -log IC50 values for nifedipine against different components of KCl 50mM -induced response at different at different O_2 tension at 2.5mM Ca^{2+}. The fig shows that the phasic component was more resistant than was the the tonic component irrespective of the O_2 tension. In addition, responses at 0%O_2 were more resistant than at 95%O_2 or 16%O_2. Data was derived for fig 6.4.

The points represent means $\pm$ s.e.m., n=4. *, indicate $P < 0.05$ when values at 16%O_2 were compared with those at 95%O_2 and 0%O_2.

(iii). 0% O_2.

Control response to KCl

The tonic response was much reduced relative to the phasic response.

Responses were of two types, varying between tissues. One type showed a prominent phasic component but the response then waned i.e. the subsequent tonic contraction was reduced more than 70% compared with the phasic contraction. The other type was like the typical contraction in higher O_2 tension consisting of phasic and tonic and further "phasic-like" component intermediate between the other two. The tonic component in this type of contraction to KCl was usually about 50 -60% of the size of the phasic component. The mean tonic to phasic component ratio was 0.40 ± 0.06, n=4. See inset for the traces of typical responses obtained to KCl (fig 6.1a).

The phasic component was more resistant than the tonic to decreasing O_2 tension (fig 6.4c). The responses at 0% were in general more resistant than normoxia and hyperoxia (see fig 6.5). The phasic contraction was 100% inhibited at nifedipine 0.1μM. As at normoxia, there was a nifedipine -resistant tonic contraction remaining after the phasic contraction has been inhibited. This was 10% of the control tonic response and was evident up to nifedipine 10μM.

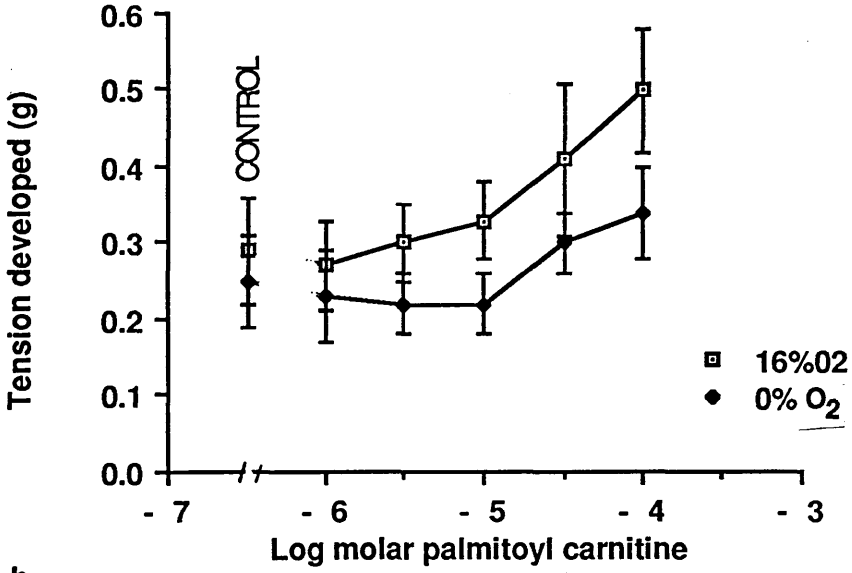
Fig 6.6.

The log concentration response relationship for palmitoyl carnitine (PC) at 0.31mM Ca^{2+} at 16%O_2 and 0%O_2.

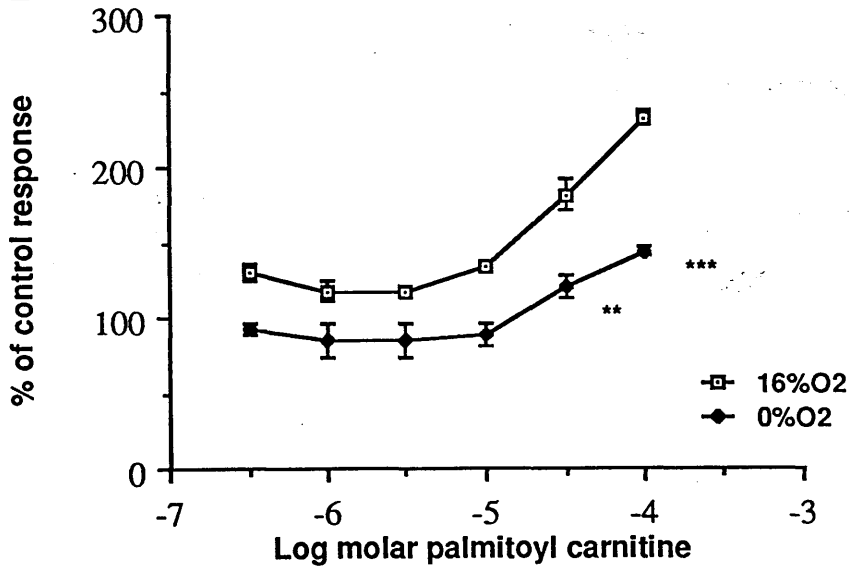
(a). A plot of tension developed (g) versus the log molar PC.
(b). A plot of percentage of control response to KCl 50mM versus log molar PC.
PC's concentration response curve at 0%0_2 lies to the right of that at 16%O_2.

Facilitation was assessed by the increase in response to KCl 50mM in the presence of PC.
A maximum for PC was not reached because of the high concentrations necessary.
The experiments were unpaired. Points represent means ± s.e.m., n = 4.
** and *** represent P values < 0.01 and 0.001. The points P values are shown indicate statistically significant depression of PC's facilitation in hypoxia.

a


Tension developed (g)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CONTROL
16%02
0% O2
- 7
- 6
- 5
- 4
- 3
Log molar palmitoyl carnitine

b


% of control response
300
200
100
0
***
**
16%O2
0%O2
-7
-6
-5
-4
-3
Log molar palmitoyl carnitine

3.Third series

a PALMITOYL CARNITINE

(i). At 16% O_2.

Concentrations of PC lower than 10uM did not facilitate KCl-induced contractions but at this concentration and above there was facilitation. In these experiments, the maximum facilitation by palmitoyl carnitine was not attained because of the small quantity of the drug available and the high concentrations required. Spontaneous activity of the tissue that was suppressed in the control response in 0.31mM Ca^{2+} re-emerged from 10uM palmitoyl carnitine; quantification of it became difficult as there was frothing which caused irregular mechanical artefacts on the recording of tension. The frothing could be reduced by decreasing the rate of gassing, but was not done because it would have affected the bath O_2 tension. Fig. 6.6 shows a comparison of the log concentration/ response relationship for PC at normoxia and hypoxia. The CRC at normoxia lies to the left of that at hypoxia. Fig 6.7a shows a comparison of the log concentration/ response relationship for PC, Bay K 8644 and CGP 28392 at normoxia. PC's CRC lies farthest to the right, suggesting that it is much less potent than Bay K 8644.

Fig 6.7

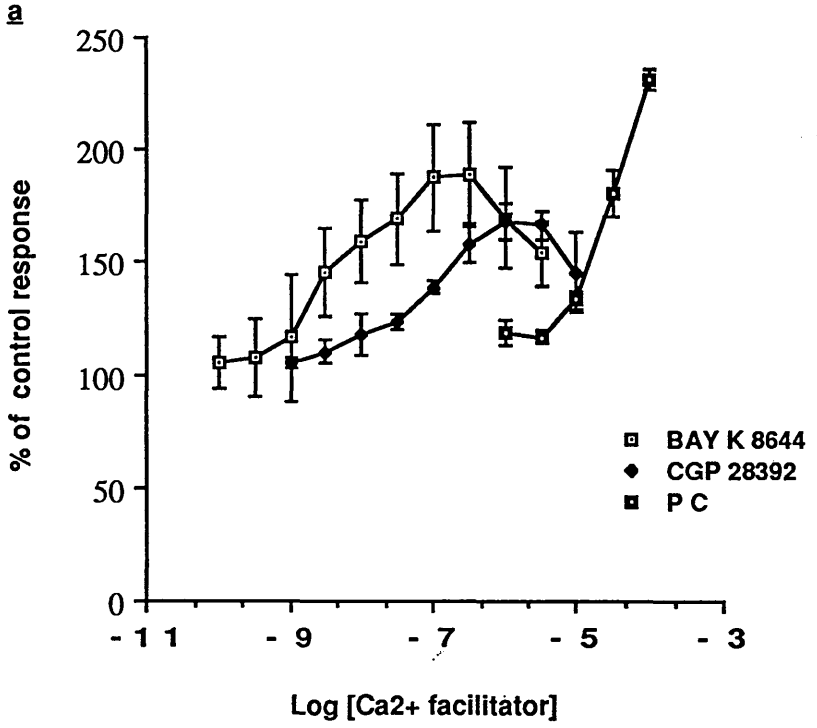
(a) The log concentration /response relationship for Bay K 8644, CGP 28392 and palmitoyl carnitine as percentage of control response to KCl 50mM against the log molar concentration of each of the different Ca^{2+} facilitators at 0.31mM Ca^{2+} at 16%O_2.

The points show the means ± s.e.m. of 4 -6 experiments.

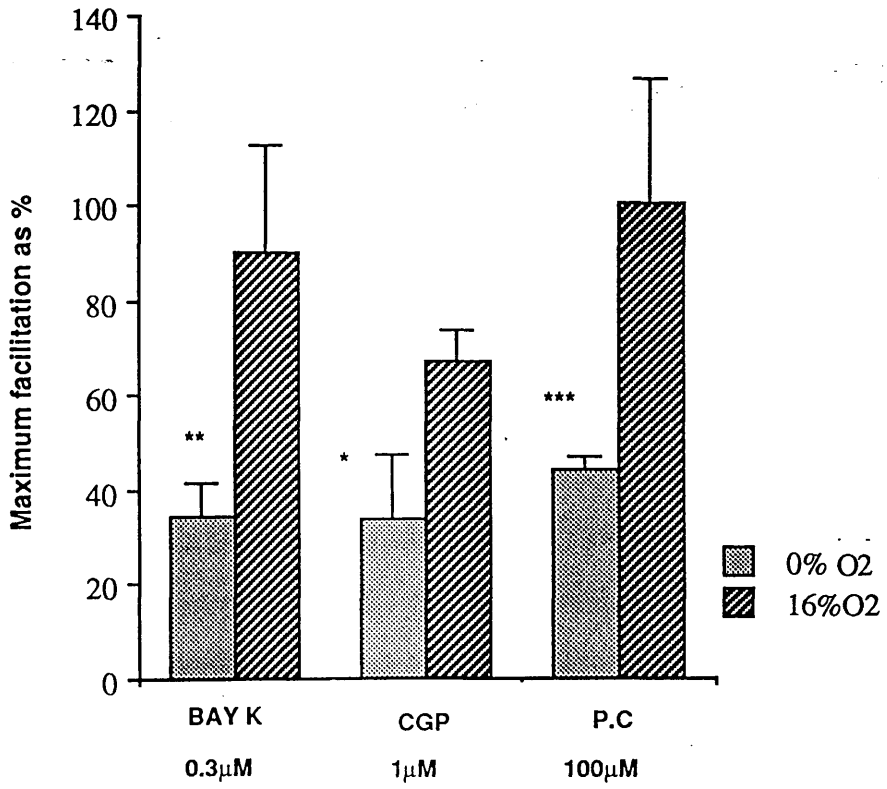
(b). The maximum facilitation of KCl 50mM -induced contraction by Bay K 8644, CGP 28392 and palmitoyl carnitine at 0.31mM Ca^{2+} at 16%O_2 and 0%O_2.

The points show means ± s.e.m, n = 4 - 9. Statistically significant values represent comparison between maximum facilitation in 0%O_2 and in 16%O_2. *, ** and *** show $P < 0.05 < 0.01 < 0.001$.

a


250
200
150
100
50
0
% of control response
- 1 1
- 9
- 7
- 5
- 3
Log [Ca2+ facilitator]
BAY K 8644
CGP 28392
P C

b


140
120
100
80
60
40
20
0
Maximum facilitation as %
**
*
***
BAY K
0.3μM
CGP
1μM
P.C
100μM
0% O2
16%O2

(ii) At 0%.

In the presence of palmitoyl carnitine

In this series of experiments, the control responses to KCl 50mM at 0.31mM Ca^{2+} were quite similar to those at 16%O_2. Palmitoyl carnitine caused a potentiation from a higher concentration threshold of (30μM) than found at 16%O_2. The potentiation at 100uM was 44.3±2.9%, n=4 compared to 100.9±28.2%, n=4 potentiation at 16%.The maximum concentration examined was 100μM. Fig 6.6 shows the log concentration/ response relationship at hypoxia. Fig 6.7b shows a comparison of the maximum facilitation obtained at normoxia with that at hypoxia for Bay K , CGP 28392 and PC. For all three Ca^{2+} facilitators this maximum was significantly smaller ($0.001 < P < 0.05$) at hypoxia. Data for Bay K 8644 and CGP were obtained from Chapter 5.

b. POCA

(i). At 16%O_2.

On KCl 50mM induced-response

POCA 10μM had no effect on the response to KCl 50mM, but at 100μM there was a small decrease (fig 6.8a). The interesting thing, however, was that upon washout,

tissue previously exposed to POCA 100μM showed a 75.6% potentiation (n = 2) to subsequent contraction to KCl 50mM. Recovery was incomplete (31.3% potentiation, n = 2) even after 1hr (fig 6.8b). The time-matched control on the other hand showed reproducible responses until the last 45min of the experiment during which there was a 21% reduction in response.

On palmitoyl carnitine induced-effects.

Tissues (n = 2) treated with both POCA 10μM and palmitoyl carnitine 3uM showed an inital 10% decrease in response to KCl 50mM, which remained unchanged in the presence of increasing concentrations of palmitoyl carnitine (1μM - 100μM). As for POCA on control response to KCl, there was a post washout potentiation of 50% compared to the control.

(ii). At $0\%_2$

On KCl 50mM induced contraction

15-20 min incubation with POCA 10μM caused 20% (n = 2) reduction of the control response to KCl 50mM. The higher concentration of 100μM caused 42% (n=2) inhibition of KCl 50mM induced contractions (fig 6.8a). The recovery rate was fast and was complete by 15min (fig 6.8b). Increasing the incubation period to 50min did not enhance the inhibition.

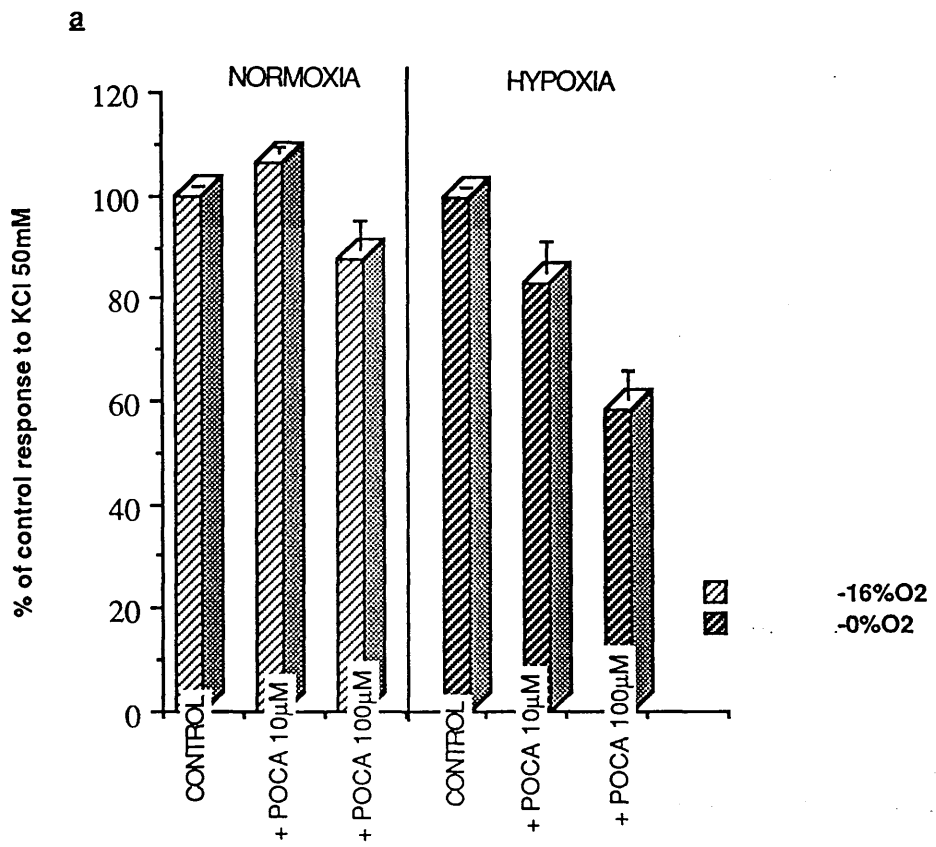


Fig 6.8.

The effect of POCA10µM and 100µM on the response to KCl 50mM at 0.31mM Ca^{2+} at 16%O2 (normoxia) and 0%O_2(hypoxia).

At hypoxia, POCA 100µM reduced the response to 60% of control. Recovery after POCA washout was slow and it took more than hour for complete recovery (not shown).

At normoxia, POCA 100µM reduced the response to 80% of control. Therefore the KCl-induced response was less susceptible to POCA than was that at 0%O_2. Recovery was not delayed, in fact there was a post-washout potentiation. This potentiation was still present even 1 hour after the POCA washout (not shown).

Columns show means of n=2.

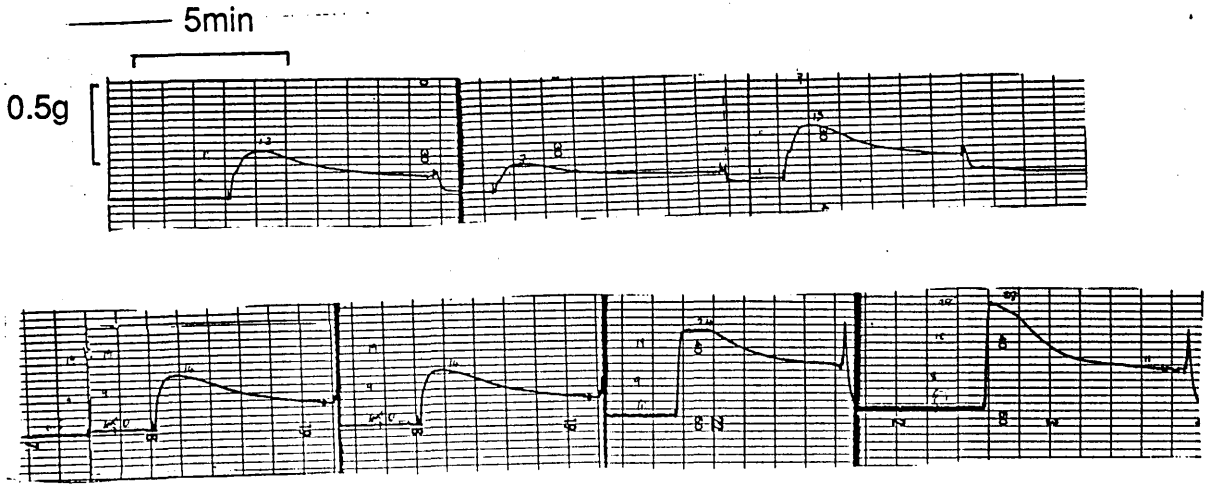


Fig 6.8b

Shows the trace of contraction of KCl 50mM in hypoxia (upper panel) and normoxia (lower panel).

In hypoxia, the 1st response was the control, while the 2nd response was in the presence of POCA 100μM (note that POCA reduced KCl response). The 3rd response was recvery response 10min after POCA wash out (recovery was fast).

In normoxia, the 1st response was control, while the second was in the presence of POCA 100μM (note that this response is less susceptible to POCA). The 3rd and 4th responses were taken 10min and 30min after POCA wash out (note that this response was potentiated even up till 30min after POCA). Recovery was more than 60min (not shown).

On palmitoyl carnitine-induced effects

POCA 10μM, had no effect on the small potentiation induced by palmitoyl carnitine (30μM and 100μM). A higher concentration of POCA was not examined because of the small quantity available.

DISCUSSION .

Ca2+ dependency at hypoxia

Irrespective of the oxygen tension employed, there was a qualitative difference between the peak response at low Ca^{2+} and at Ca^{2+} 0.63mM and above at 1min. However there was no statistically significant difference between the $[Ca^{2+}]$/ response relationship at $0\%O_2$ (hypoxia) and $16\%O_2$ (normoxia). The Ca^{2+} concentration of 0.31mM, in which the sensitivity of the RPV to Ca^{2+} facilitators has been assessed, usually allowed a response to KCl that reached a peak by 1min. It was this peak response that was measured and is referred to in documenting the $[Ca^{2+}]$/ response relationship at 1min. It was expected that if responses were already facilitated at hypoxia then the Ca^{2+} response curve should lie to the left of that at normoxia. However, this was not the case in these experiments. While the Ca^{2+} response/relationship

at 1min at hypoxia did not differ from that at normoxia, it did differ if responses at 5min (which are normally wane from earlier values) were taken. The $[Ca^{2+}]$/ response curve at hypoxia based on the 5min response usually lay to the right of that at normoxia. At the Ca^{2+} level of 0.31mM, in which the Ca^{2+} facilitators were assessed, the absolute difference between responses at hypoxia and normoxia was small compared with the higher Ca^{2+} levels, although the proportionate difference remained high (see fig 6.1b).

Nifedipine on KCl-induced responses.

At 0.31mM Ca^{2+}

The concentration/response curves for inhibition by nifedipine in the presence of either KCl or NA at 0% lay to the right of those at 16%.

The greater inhibition by nifedipine (1pM to 1nM)of responses to NA compared with those to KCl in 16%O_2 was not expected. This might be partly explained by the lower control response to NA 1µM at 16%O_2. However at nifedipine 10nM and above the potency was similar with both KCl and NA. NA-induced contractions at 0% were more resistant to very low concentrations of nifedipine than were those at 16%O_2, but from 1nM the inhibition was not significantly different at 0% and 16%O_2.

Nifedipine, at the low concentrations of 1pM to 1nM,

potentiated KCl-induced responses at 0.1nM and 1nM, while inhibition was obtained from 10nM. This increase in response was also observed during the study on the interaction of Bay K with nifedipine (1nM) and is discussed in Chapter 5. In the presence series of experiments nifedipine concentrations lower than 1nM were used to find out whether the threshold concentration for nifedipine's agonism was lower than 1nM. However 1pM up to 0.1nM produced no effects. In this series of experiments the agonism by nifedipine was not observed in normoxia. However, no explanation is apparent for this observation of inconsistency in nifedipine's agonist effect.

At 2.5mM Ca^{2+}

The tonic response to KCl irrespective of the O_2 tension was consistently more susceptible to nifedipine than was the phasic contraction. This is consistent with the observations made by Fiol de Cuneo et al (1983) and Campbell et al (1986). However the IC50 values obtained in the present experiment at 16%O_2 (shown in brackets) were higher than those obtained by Campbell et al, which were 7.69 (8.65) and 8.5 (9.1). The "phasic response" in this case is equivalent to what Campbell et al (1986) referred to as "spike" in their paper. They identified three components to KCl -induced contraction, namely spike, phasic and tonic. They argued that it is usually 2 components that are described by most authors even

though there is evidence for 3. Evidence from the present study indicates 3 but for consistency with NA effects it was decided that two phases be described. The difference in the log IC50 values from this study compared with theirs might be explained by differences in methodology. They employed the cumulative concentration/ response method while the non-cumulative was employed in this study; in addition, they did not substitute NaCl for KCl.

There was a progressive resistance of nifedipine's inhibition of the different components of KCl-induced contraction going from 95%O_2 to 0%O_2. This is consistent with the observation made at the lower Ca^{2+} level, 0.31mM, in which the -log IC50 value for nifedipine at 16%O_2 was significantly higher than it was at 0%O_2. The resistance obtained as O_2 tension is lowered, particularly at hypoxia, has been predicted from the results obtained in the previous Chapter where Bay K 8644's facilitatory effect diminished in hypoxia and indeed this held true for CGP as well. This effect was not exclusive to the synthetic Ca^{2+} facilitators alone since the endogenous Ca^{2+} facilitator palmitoyl carnitine (Mir & Spedding 1986; 1987; Spedding, 1987; Bigaud & Spedding, 1986; Duncan et al 1986) showed the same trend. Therefore if the hypothesis that the responses in hypoxia were already facilitated probably by the accumulation of acyl carnitines, of which PC is one, then PC should mimic Bay K 8644's effect. The results in this series of experiments corroborate that.

The resistance to nifedipine is likely if the endogenous substance interacts with the dihydropyridine sites. Such interaction of PC with the dihydropyridine site has been shown recently by Spedding & Mir (1987). These authors showed that it was possible to antagonise the Ca^{2+} facilitating effect of PC with the dihydropyridine Ca^{2+} antagonists, e.g. nifedipine (class 1 - Ca^{2+} antagonist) or verapamil or diltiazem (class 11 - Ca^{2+} antagonists) but not with flunarizine or cinnarizine (class 111 - Ca^{2+} antagonists). They then predicted that, in ischaemia, voltage operated channels (VOCs) activated by acyl carnitines would be relatively resistant to compounds like nifedipine, verapamil and diltiazem, but not to the class 111 Ca^{2+} antagonists- cinnarizine and flunarizine. Though only nifedipine was used in my experiments it would have been interesting to see what effects flunarizine would have in ischaemic conditions since the rat portal vein has been shown to be quite resistant to this compound in hyperoxia (van Neuten et al 1978; Marriot, 1985).

An alternative hypothesis to explain the increasing resistance to nifedipine as O_2 tension is lowered is that there is more dependence on intracellular Ca^{2+} at lower O_2 tension as opposed to extracellular Ca^{2+}. The relative resistance of the phasic component (at 2.5mM Ca^{2+}) to nifedipine compared to the tonic could also suggest an intracellular Ca^{2+} contribution to this response. The phasic component of the KCl response is

thought to be due to Ca^{2+} released from a superficially bound pool triggered by an early influx of extracellular Ca^{2+} and is associated with electrical spike activity (Bennett, 1972). Both the electrical spike activity and the phasic contraction have been shown to be maximal within the first minute of exposure to KCl (Gollenhofen et al, 1973; Sigurddson et al, 1975; Taranenko et al, 1978; Weston & Jetley, 1980). The tonic component of KCl's response, which is of a slower time course than the phasic one , is related to a continuing influx of calcium through voltage sensitive channels (Gollenhofen & Hermstein, 1975; Gabella, 1978). In my experiments the tonic component of KCl-induced response and indeed that of NA was more susceptible to changes in O_2 tension than was the phasic one. It therefore seems there is a parallel between O_2 tension lowering and inhibition by Ca^{2+} antagonists of extracellular Ca^{2+}. This does not, however imply a common mechanism of action (see discussion - Chapter 7).

On the other hand one could argue that the hypothesis of energy limitation (Fay, 1971) could have accounted for the relative ineffectiveness of either nifedipine or Bay K 8644 in hypoxia. According to Paul (1980) there are small phosphate stores vascular smooth muscle, which are depletable within mins by basal energy requirements of the tissue. In the present study control responses to KCl were reproducible in hypoxia even up to 3.5 hours (data not shown). If formation of

energy was limited during hypoxia such responses would be reduced progressively. Many workers have shown evidence against energy limitation during hypoxia. Chang & Detar (1980) showed that in the aorta, responses to low concentrations of NA were more susceptible to hypoxia than were those to high concentrations of NA. Since contractility is directly dependent upon energy utilisation (Paul et al, 1983) one would not have expected responses to high concentration of NA to be resistant to hypoxia. Other evidence against energy limitation comes from Needleman & Blehm (1970) and Namm & Zucker (1983), who, independently, showed that in hypoxia the presence of agonists, such as adrenaline, KCl or phenylephrine, did not lead to the depletion of intracellular ATP. In fact, phenylephrine 1μM -induced responses were reduced by hypoxia before any significant reduction in the total ATP content was detected (Namm & Zucker, 1973).

At nifedipine 0.1μM and above a resistant tonic component was observed at all O_2 tensions. This is characterised by a slow rise which peaked by 1-2min. There is really no explanation for this response if it is not an artefact. It usually appeared after the phasic contraction (which is usually more resistant to nifedipine than the previous tonic response) had been completely abolished. While this later emerging tonic response is resistant to nifedipine the earlier tonic response is readily suceptible to nifedipine.

Palmitoyl carnitine

Palmitoyl carnitine, a compound that accumulates during ischaemic conditions, behaved like Bay K 8644 in these experiments. It facilitated KCl induced contraction at 10uM -100uM. This is within the concentration range shown to cause Ca^{2+} facilitation in the guinea pig taenia coli i.e. 1μM-1000μM (Spedding & Mir, 1986; Spedding & Bigaud, 1986). The facilitation in normoxia was 100%, which was reduced to 40% in hypoxia even though the absolute size of the control response to KCl was similar or higher in hypoxia in the present series of experiments. It has been shown by Spedding & Bigaud (1986) that palmitoyl carnitine does not behave like Bay K in all its effects, e.g. while basal release of EDRF does not affect the contractile response to palmitoyl carnitine it does reduce that due to Bay K . On the other hand while EDRF -induced release by Ach was blocked by PC, Bay K had no effect. Such an influence on EDRF is not expected in the portal vein (data not shown) as the influence of EDRF in this vessel as well as other venous preparations except the rabbit saphenous (McGrath and Wilson's unpublished observation) is minimal (Furchgott, 1983). The other effects of PC not shared by Bay K 8644 are antihistaminic and anticholinergic effects (Spedding & Bigaud, 1986). In the present experiment it seems that palmitoyl carnitine's effect is on Ca^{2+} channel, and not its other

effects.

Fig 6.9. shows a schematic diagram of events that take place in ischaemia and how these modulate Ca^{2+} channel function.

POCA

The study on POCA was preliminary involving small numbers (n = 2) but certain key observations gave support to the hypothesis which derives from the data.

In hypoxia, the acyl inhibitor, POCA (100μM) caused an inhibition of about 40% of KCl 50mM induced contraction which took over 45min to recover. On the other hand the effect of POCA during oxygenation was less susceptible to POCA. The inhibition of KCl -induced contraction by POCA in hypoxia is consistent with the hypothesis that acyl carnitines could accumulate during ischaemia and that this accumulation could be blocked by POCA (Spedding, 1987). A similar result has been observed in neonatal myocytes, in which, POCA was shown to prevent accumulation of long chain acyl carnitines during hypoxia (Knabb et al, 1986). In the same study, these workers showed that in normoxic-perfused cells acyl carnitine was concentrated in mitochondria and cytoplasmic membranous components. Only small amounts were present in the sarcolemma. Hypoxia increased mitochondrial long chain acyl carinitines by 10-fold and sarcolemmal long chain carnitine by 70-fold These authors concluded that endogenous long-chain acyl

carnitines must be mediators of electropysiological alterations induced by hypoxia.

Although POCA had little effect on the control response to KCl as well as the facilitation induced by PC in normoxia, there was a post-washout potentiation. Such post washout potentiation could be due to an enhanced effectiveness of endogenous PC, possibly arising from a build-up, during the presence of POCA, of a precursor. However such a build up of PC might have been expected in hypoxia rather than in normoxia. It is interesting however that there was no such post-washout potentiation to POCA in hypoxia, possibly because PC cannot facilitate under this condition.

POCA was not explored further because of the high threshold inhibitory concentration of POCA 100μM, as a limited quantity of the drug was available. However, it would be interesting to see whether POCA at 100μM or a higher concentration would by blocking accumulation of acyl carnitines restore facilitation in hypoxia.

Fig 6.9

A schematic representation of events that take place in ischaemia and how these modulate Ca^{2+} channel function.

In ischaemia there is loss of ionic homeostasis as a consequence of ATP depletion . The mitochondria require ATP for active extrusion of Ca^{2+}. Loss of homeostasis leads to increased cytosolic Ca^{2+} via increased 2Na+/ Ca^{2+} exchange, increased K+ loss and increased Ca^{2+} influx.

Consequently, the normal ratio of extracellular Ca^{2+} : intracellular Ca^{2+} of 10,000 : 1. is grossly altered in favour of intracellular Ca^{2+}.

This increased cytosolic Ca2+ results in mitochondrial overload. There is increased synthesis of acyl carnitines e.g. palmitoyl carnitine, via acyl CoA and carnitines.

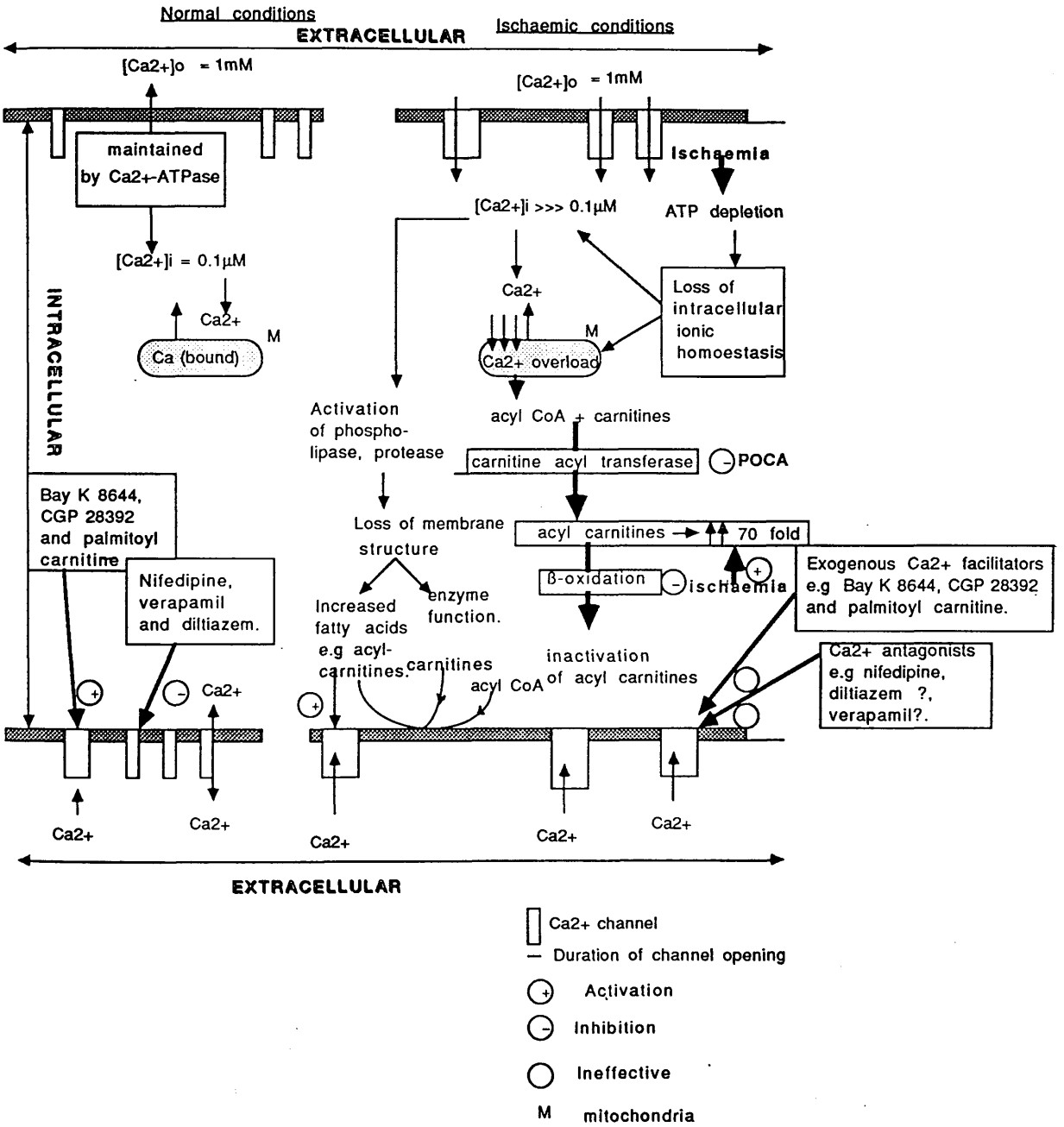
The increased cytosolic Ca^{2+} leads to activation of phosholipase which leads to the loss of membrane structure. The disruption of membrane structure causes the level of fatty acids to increase. These fatty acids include acyl carnitines.

Acyl carnitines levels are also increased in ischaemia because the ß-oxidation which leads to their inactivation is blocked.

Acyl carnitines activate Ca^{2+} channels directly. Consequently their accumulation causes full activation of the Ca^{2+} channels, therefore rendering further administration of Ca^{2+} facilitator ineffective, which explains why Bay K 8644, CGP 28392 and palmitoyl carnitine, itself, are ineffective in ischaemic conditions.

Since acyl carnitines act at the dihyropyridine site to block the effect of nifedipine, it would be expected that niifedipine would be ineffective in ischaemia.

POCA, carnitine acyl transferase inhibitor, would prevent the synthesis of acyl carnitines and therefore block their accumulation in ischaemia. It is therefore only in the presence of POCA that a facilitation of the Ca2+ channel can be observed.


Normal conditions
Ischaemic conditions
EXTRACELLULAR
[Ca2+]o = 1mM
[Ca2+]o = 1mM
maintained by Ca2+-ATPase
Ischaemia
[Ca2+]i >>> 0.1µM
ATP depletion
[Ca2+]i = 0.1µM
Ca2+
Ca (bound)
M
Ca2+
Ca2+ overload
M
Loss of intracellular ionic homoestasis
INTRACELLULAR
Activation of phospho-lipase, protease
acyl CoA + carnitines
carnitine acyl transferase
POCA
Bay K 8644, CGP 28392 and palmitoyl carnitine
Loss of membrane structure
acyl carnitines
70 fold
Nifedipine, verapamil and diltiazem.
β-oxidation
ischaemia
Exogenous Ca2+ facilitators e.g Bay K 8644, CGP 28392 and palmitoyl carnitine.
enzyme function.
Increased fatty acids e.g acyl-carnitines.
carnitines
acyl CoA
inactivation of acyl carnitines
Ca2+ antagonists e.g nifedipine, diltiazem ?, verapamil?.
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+
EXTRACELLULAR
Ca2+ channel
Duration of channel opening
Activation
Inhibition
Ineffective
M mitochondria

CHAPTER 7

Ca^{2+} MODULATORS AND HYPOXIC VASODILATATION.

SUMMARY

1. In rat portal vein, hypoxic relaxation against NA-induced tone was observed between 1 and 2 min after switching to N_2 in 5%CO_2. The greater the initial contraction by NA the greater proportionately, was the hypoxic relaxation, which was complete by 5min, and upon reoxygenation there was a contraction within 1min to 5min.

2. Pretreatment with Bay K 0.3μM or its addition during an estabilished hypoxic relaxation did not modify the response.

3. Bay K 0.3μM could reverse the relaxation to nifedipine 10nM or 30nM of either NA 1μM or KCl 50mM-induced contraction .

4. Increased $[Ca^{2+}]_o$= 12.5mM reversed nifedipine induced relaxation more than it did reverse hypoxic relaxation.

5. Addition of either nifedipine 10nM or verapamil 0.1μM, 2min after hypoxic relaxation had been induced caused depression of the contracile spikes superimposed on the relaxation, and blocked the subsequent reoxygenation contraction.

INTRODUCTION

Hypoxia is known to cause vasodilatation and the mechanism of this hypoxic vasodilatation is not fully elucidated. Several hypotheses have been put forward, namely : limitation of energy production (Altura & Altura, 1976; Detar & Bohr, 1968; Fay, 1973; Hellstrand, et al 1977; Namm & Zucker, 1973; Pittman & Duling, 1971; Smith & Vane, 1966); formation of endogenous vasoactive substances (Borda et al, 1980; Roberts et al, 1979; Smith & Vane, 1960); altered memrane permeability to ions (Vanhoutte, 1976); and a specialised oxygen sensor associated with smooth muscle (Coburn et al, 1979; Smith & Vane, 1966). Another attractive hypothesis to explain the phenomenon of hypoxic vasodilatation is Ca^{2+} withdrawal via Ca^{2+} sequestration or inhibition of Ca^{2+} influx as proposed by Ebeigbe et al (1980); these authors and Marriot (1985) observed that there was a link between hypoxic vasodilatation and Ca dependence of various vascular smooth muscle preparations. Tissues most susceptible to hypoxia were those highly dependent on extracellular Ca^{2+} while resistance to hypoxia was observed in tissues that utilised intracellular Ca^{2+} (Ebeigbe et al, 1980; Marriot, 1985).

The hypothesis that hypoxic vasodilatation is due to inhibition of Ca^{2+} influx was tested in the present study by using Bay K 8644, a Ca^{2+} facilitator that acts by increasing transmembrane influx of extracellular

calcium through specific calcium channels (Schramm et al 1983). If hypoxic vasodilatation acts by preventing Ca^{2+} influx, Ca^{2+} facilitation might reverse or prevent relaxation.

METHODS

Longitudinal strips of the portal vein were obtained as described previously (see methods Chapter 2). The tissues were set up in Krebs of the following composition (mmol): NaCl, 119; KCl 4.7; $NaHCO_3$, 24.8; KH_2PO_4, 1.2; $MgSO_4$,1.2; Glucose 11.5; $CaCl_2$, 2.5 or 0.31mM in some experiments. EDTA 23μM and cocaine 1μM were added to the Krebs.

The tissues were gassed with 16%O_2:79%N_2 :5%CO_2(Po_2=112±10mmHg). Attempts were made to maintain equilibrium between gas and saline by rapid gassing and by covering the bath with parafilm to minimise gain of O_2 by diffusion from the atmosphere. The tissues were allowed 1.5h for equilibration, after which responses were obtained to NA 10μM. The tissues were washed, allowed to recover, contracted with NA 1μM for 5min and washed again. A further response to the same concentration was produced but this time a hypoxic relaxation was obtained: after 2min of the response to NA the gas was switched to 95%N_2: 5%CO_2 (Po_2 = 5±2mmHg) for 5 - 10min and then the gas was switched back to 16%O_2 (reoxygenation) for a further 5min; the tissues

were then washed and allowed 10min recovery. The tissues were then subjected to the protocols listed below. The whole procedure was then repeated starting in 95%O_2 with 5%CO_2 rather than in 16%O_2.

To test the effect of Bay K 8644 on hypoxic-relaxation of NA -induced contraction, 4 series of experimental protocols were adopted: in each case the test sequence NA-hypoxia-reoxygenation was as described above.

First series:

Bay K versus hypoxic relaxation.

(i). Pretreatment of the tissues with Bay K 0.3μM for 15min before NA was added and hypoxic relaxation induced.

(ii). Bay K effect was administered on established hypoxic relaxation of NA induced contraction. 5min after hypoxia had been induced, Bay K was added to the tissue for a further 5min, and then the tissues were reoxygenated for 5min.

These procedures were carried out first in 16%O_2 and then repeated in 95%O_2. Each tissue was used as its own time control by establishing a third control test sequence: after the treatment (in either O_2 tension), NA was added to the tissue for 15min to observe any decay

in tissue response with time.

(iii). The effect of Bay K was observed at a lower [Ca^{2+}] (0.31mM) on hypoxic relaxation of NA induced contractions, as in procedure 1. i.e (i) was repeated at a lower [Ca^{2+}].

Each procedure was carried in separate experiments on 4-6 tissues.

Second series:

Bay K versus nifedipine-induced relaxation.

2 min after inducing contractions to NA 1μM or KCl 50mM, nifedipine 10nM was added and, after observing any relaxation, the nifedipine concentration was increased to 30nM. After washout with 2.5mM Ca^{2+} Krebs, the tissues were washed 2-3 times with zero Ca^{2+} saline and then placed in 0.31mM Ca^{2+}. Control responses were taken to NA 0.3μM or KCl 50mM, after which the tissues were incubated with Bay K 0.3μM for 15min. Then the response to either NA or KCl was repeated. This latter sequence gave responses of equivalent size to the control contraction at higher Ca^{2+} without Bay K . Relaxation to nifedipine was re-tested under these conditions.

Third series:

Additional Ca^{2+} versus hypoxia or nifedipine-induced relaxation.

(i) The effect of prior increased [Ca^{2+}], i.e. 12.5mM was examined, on hypoxic relaxation and nifedipine relaxation.

When the effects of increasing the extracellular calcium concentration were examined, the experiments were carried out in Krebs solution of above composition except that the KH_2PO_4 was reduced to 0.1mM.

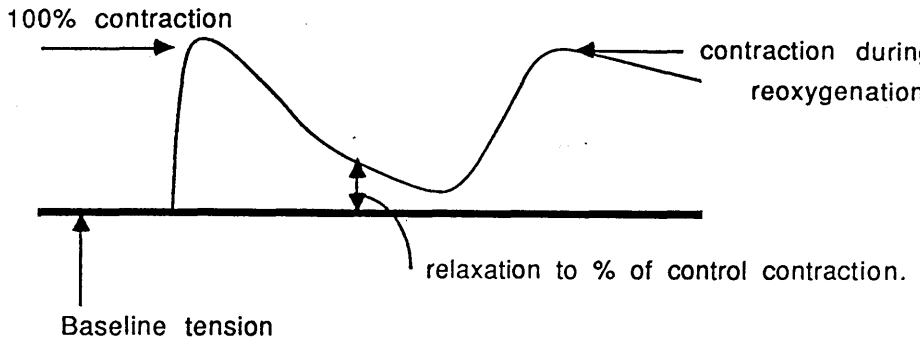
Fourth series:

The effects of the Ca^{2+} antagonists, nifedipine 10nM or verapamil 0.1μM, on the reoxygenation contraction were examined. 2min after inducing hypoxic relaxation of NA or KCl-induced tone; either Ca^{2+} antagonist was added for 10min, before reoxygenation for a further 5min.

ANALYSIS OF RESULTS

Hypoxic relaxation in this series of experiments has been quantified as relaxation to percentage of the control contraction to either NA or KCl at the maximum before hypoxia was induced. This has been illustrated in fig 7.1a. The control contraction was taken as 100%. The hypoxic responses in control and treated procedures have been compared using a paired student's t-test.

a



Schematic representation of the parameters measured during hypoxic relaxation of NA or KCl induced contraction

b

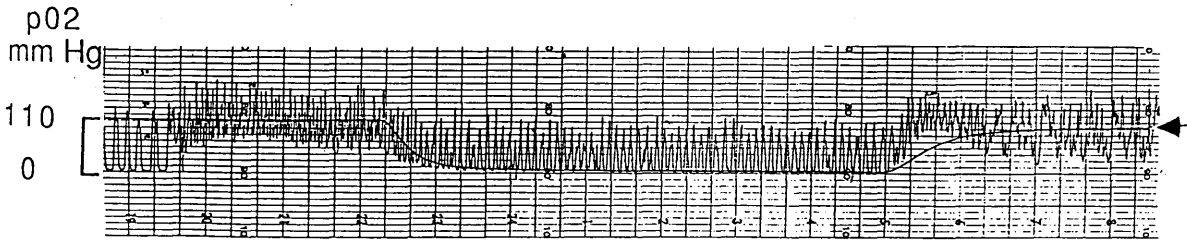


Fig 7.1

(a) A schematic representation of the parameters measured during hypoxic relaxation of NA 1μM or KCl 50mM -induced contraction.

(b) A recording of the tissue bath Po_2 simultaneously with the recording of the mechanical activity of the tissue. The onset of relaxation coincides with the onset of the drop in the bath Po_2. The mean bath Po_2 recorded was 5 ± 2mm Hg. The arrow shown in the fig. indicates the Po_2 trace. Calibration for Po_2 (mm Hg) is indicated far left.

RESULTS:

Basic responses

1. Effect of hypoxia on established contraction to NA (1uM)

:experiment in 2.5mMCa^{2+}

These experiments require a consistent, sustained contraction to NA as a basis for observing relaxation. Considerable quantitative variation in the time course of contraction necessitated drug-free, "normoxic" time controls in each experiment to indicate the extent of the progressive loss of tone which occurred. This makes quantitative comparison between groups of experiments difficult. We have expressed the responses as change in both baseline tone and in the peak of phasic activity.
The Po_2 drop in the bath was coincident with the onset of relaxation of the tissue to hypoxia as shown in fig 7.1b.
Since contraction in 95% was greater than in 16%O_2, a slightly greater relaxation by hypoxia was observed in 95%. The form of the relaxation to hypoxia was quite variable. Usually there were contractile spikes superimposed on such relaxations; the graphs in the figures quantify this.

The contraction upon reoxygenation with 16%O_2 or 95%O_2 was of similar magnitude to the contraction pre -

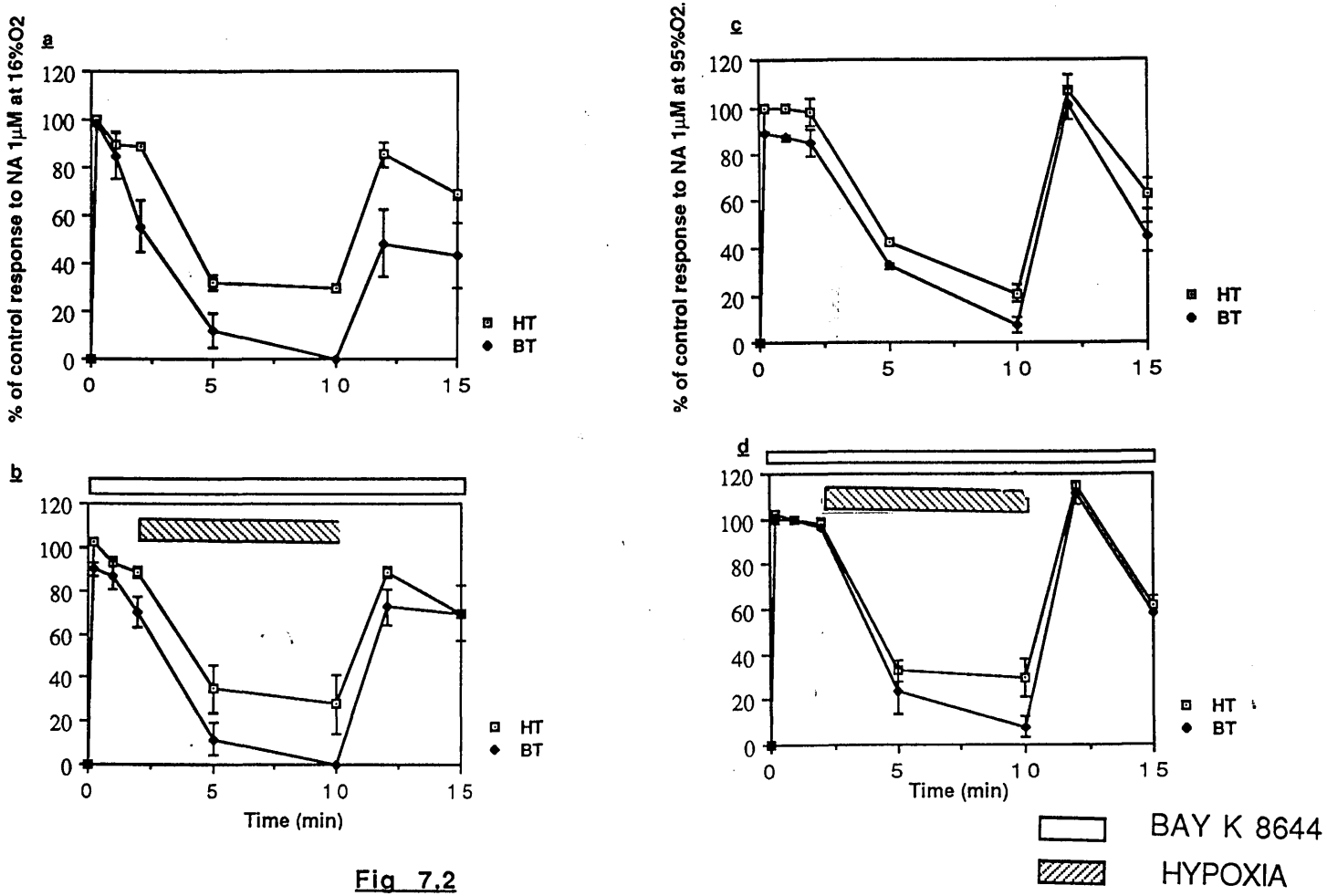


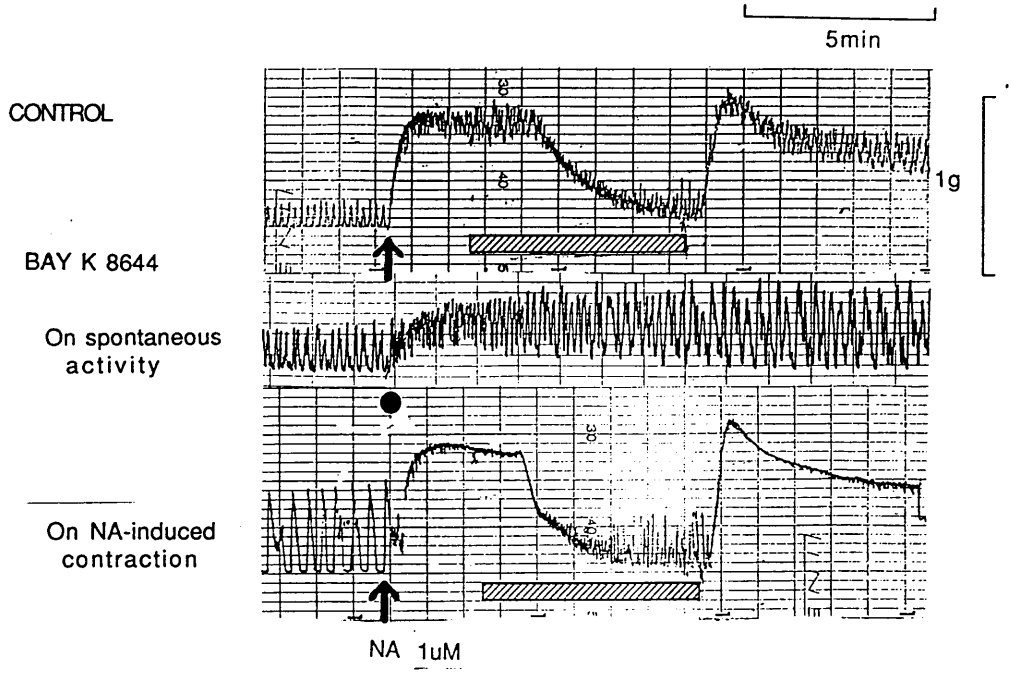
Fig 7.2

Effect of Bay K 0.3μM 15min pretreatment on hypoxic relaxation of NA 1μM -induced contraction of the isolated rat portal vein in either 16%O_2 (left) or 95%O_2 (right); (a) & (c) are controls before while (b) and (d) are after Bay K at 2.5mM Ca^{2+}.

HT= height of contraction.

BT= baseline tension.

At 95%O2



At 16%O2

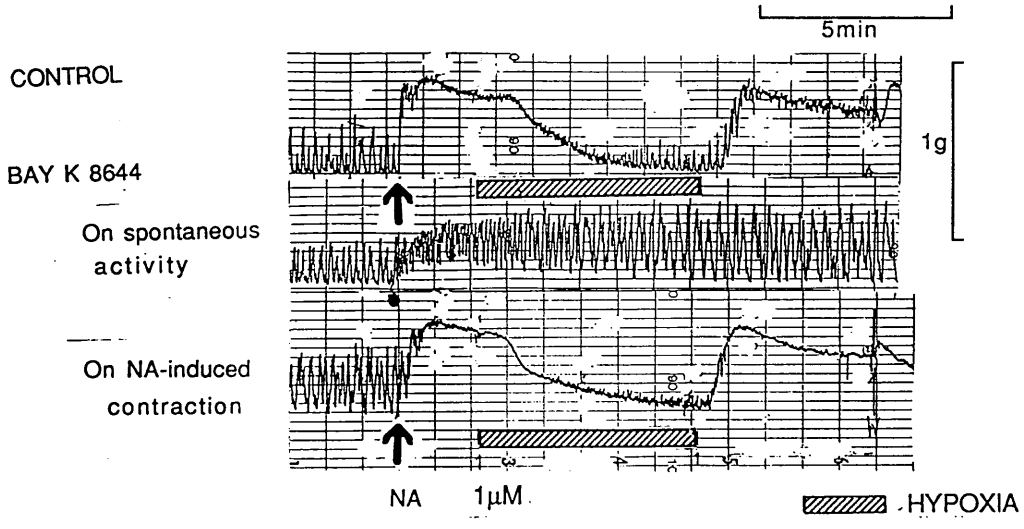


Fig 7.3

Example trace from which data was obtained at 95%O_2 (top) and 16%O_2 (below). Fig shows the control hypoxic relaxation of NA 1μM -induced contraction at 2.5mM Ca^{2+} (top panel) and in the presence of Bay K (bottom). The middle panel shows the effect on spontaneous activity of addition of Bay K 8644 prior to contraction to NA.

hypoxia. The contraction upon re-oxygenation was not, however, well maintained. (fig 7.2).

First series (a) NA- induced tone.

(i) Pretreatment with Bay K 0.3uM

The tissues were incubated in Bay K 0.3μM for 15min before NA- induced contraction with or without subsequent hypoxia was recorded.

Addition of Bay K 0.3uM caused a temporary increase in baseline tension for 2min and subsequently increased the phasic activity.
Responses to NA in either 16% or 95%O_2 were not potentiated by Bay K 0.3μM even though the spontaneous phasic activity was greatly increased (fig 7.2 a & b and Fig 7.3). Bay K did not significantly affect the hypoxic relaxation in 95%O_2, or in 16%O_2 (Table7.1).
The contraction upon reoxygenation was also unaffected. and Fig 7.2a & b.

(ii) Effect of Bay K 0.3μM on established hypoxic relaxation.

Bay K did not reverse the relaxation with this procedure. It did increase the frequency of the phasic activity superimposed on the relaxation, but not the amplitude of the phasic contractions. Neither was there an increased baseline tension. This happened

O2 tension	16%O2	95%O2
1. At 2.5mM Ca2+		
a. Bay K 15min pretreatment		
Control hypoxic relaxation	32.6 ± 2.7	25.9 ±1.0
+Bay K 0.3μM	30.0 ± 7.1 NS	30.7 ± 6.4 NS
b. Bay K on established hypoxia.		
Control hypoxic relaxation	44.1 ±5.6	37.0 ± 4.7
+Bay K 0.3μM	41.6 ± 6.3	44.2 ± 5.6
Recovery hypoxic relaxation	51.4 ± 6.8	42.8 ± 7.5
2. 0.31mM Ca2+-Krebs		
a. Hypoxic relaxation of Bay K-induced contraction.		60.9 + 5.4
b. Hypoxic relaxation of NA 0.3μM-induced contraction		
	35.5 ± 7.4	33.7 ± 6.3
+ Bay K 0.3μM	28.3 ±1.8 NS	29.1 ± 2.3 NS

Table 7.1 shows hypoxic relaxation of NA1μM -induced contraction to percentage of the initial control contraction before hypoxia without or with Bay K 0.3μM under different experimental conditions. The results are mean values of 4-6 observations. NS indicates that the values obtained for the control and Bay K treated under the various conditions were not statistically different from each other using the paired student's t-test.

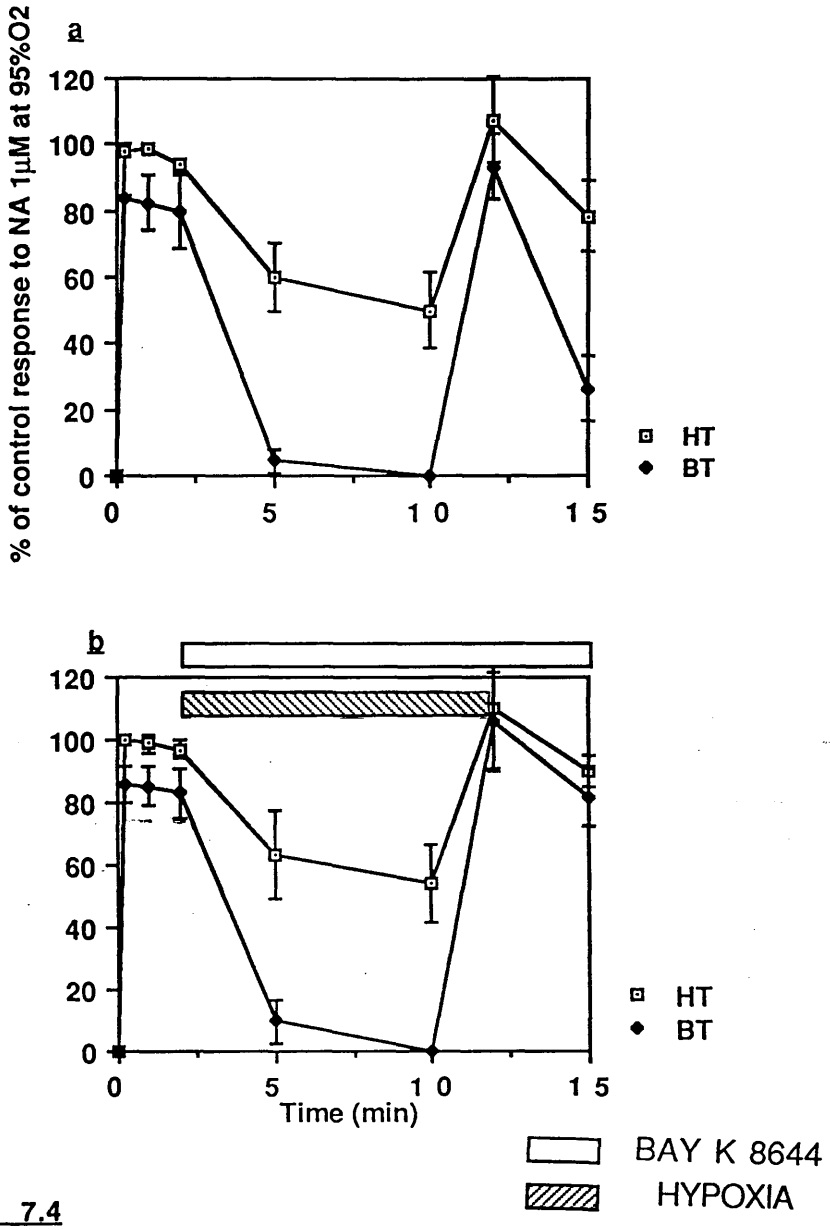


Fig 7.4

Effect of Bay K 0.3µM on established hypoxic relaxation of NA 1µM -induced contraction of the rat portal vein at 95%O_2. Figs (a) & (b) show relaxation before and during Bay K 0.3µM treatment respectively.

irrespective of the O_2 tension employed. See Figs 7.4a & b and Table 7.1. Bay K caused an inconsistent, slight increase in the height of the reoxygenation contraction, though there was an increase in the baseline.

(iii)Bay K on hypoxic relaxation in 0.31mM Ca^{2+}

In our previous experiments (Chapter 4) we have shown that Bay K facilitates both KCl and NA -induced contractions only at low $[Ca^{2+}]_o$ levels, see fig 7.5 a & b. The ineffectiveness of Bay K in reversing hypoxic vasodilatation in the experiment above in 2.5mM Ca^{2+} could be due to its inability to facilitate at this $[Ca^{2+}]_o$. This was tested by observing the influence of Bay K on the contraction to a submaximal concentration of NA (0.3µM) in 0.31mM Ca^{2+} and the associated hypoxic relaxation. Control responses to NA were taken and then the tissues were incubated in Bay K 0.3uM Krebs for 15mins and before repeating NA. Responses to NA were potentiated (fig 7.5) and were equivalent in size to responses induced by the higher concentration of NA 1uM in 2.5mM Ca^{2+} without Bay K . This gives a comparable basis of tone for examining hypoxic relaxation. In these circumstances there was no detectable difference in the hypoxic relaxation.

In fact in some individual experiments there was a greater relaxation in low Ca with Bay K , than in low Ca without it, though this was not significant comparing

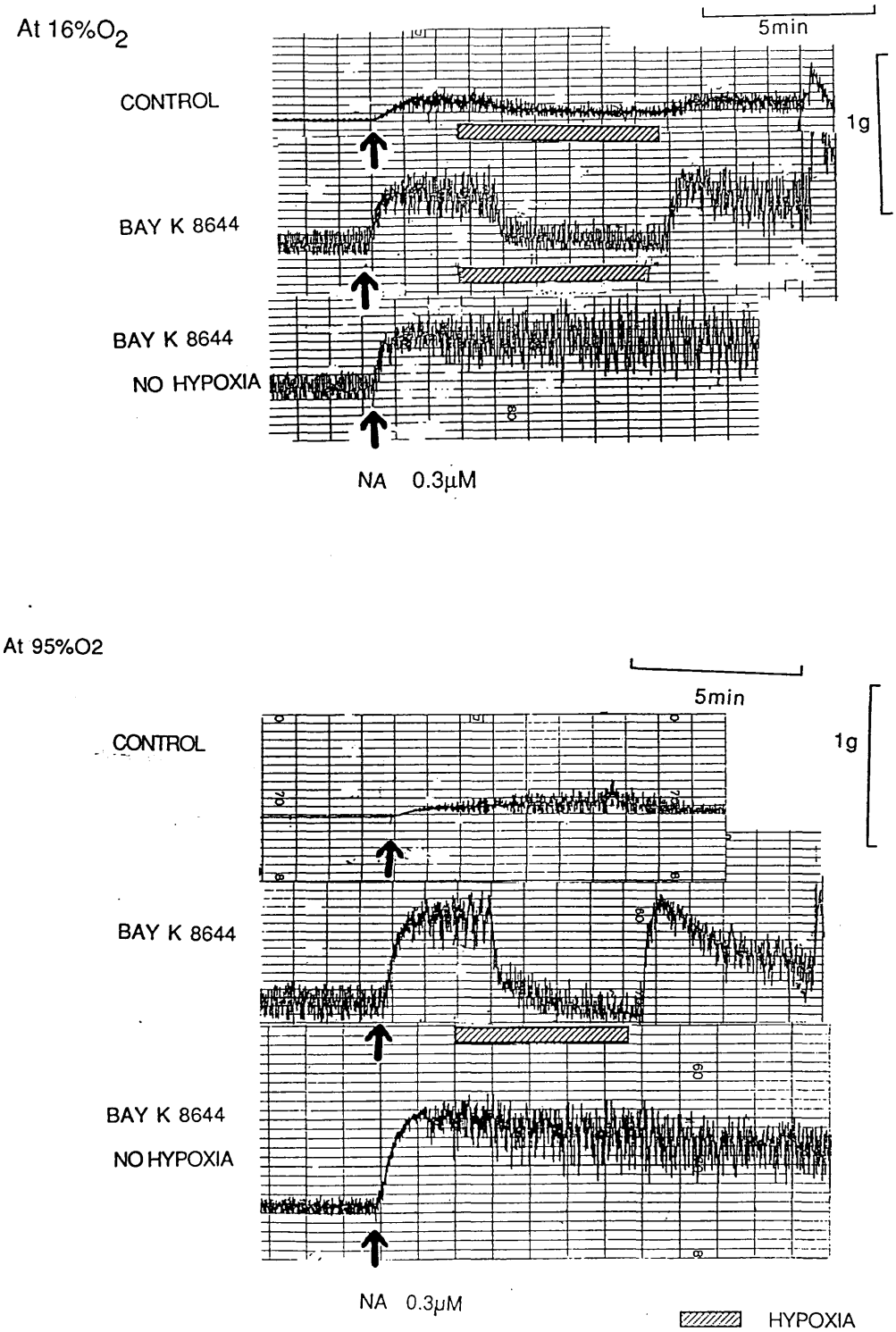


Fig 7.5

Responses to NA, hypoxia (hatched bar) and re-oxygenation in 16%O_2 (top) and 95%O_2 (bottom) at low (0.31mM) $[Ca^{2+}]_o$, without and with Bay K 0.3μM. Bottom: maintained response to NA in the same condition wihout hypoxia ('time control').

the means ($P > 0.05$) relaxations to % of control contraction to NA in 16% and 95% were 35.5 ± 7.4 and 33.7 ± 6.3 while the values were lower in the presence of Bay K 0.3μM, being 28.3±1.8 and 29.1± 2.3 respectively, See (table 7.1) .

Thus the trend for greater hypoxic relaxation at 95% than at 16%O_2 remained with lowered $[Ca^{2+}]_o$ in the presence of Bay K . As with other experimental protocols, Bay K did not potentiate the contraction upon reoxygenation, see fig 7.5. Bay K on its own contracted the tissue. 39% relaxation was obtained when hypoxia was induced. (table 7.1), see also fig 7.5a.

CGP 28392, another dihydropyridine Ca^{2+} agonist, at a concentration of 1uM did not modify hypoxic relaxation with any of the above protocols. Fig 7.6. shows CGP 23892 on hypoxic relaxation of NA 1μM induced contraction at 16% in Krebs containing 2.5mM Ca^{2+}. Like Bay K , CGP increased the spontaneous activity of the tissue without increasing the response to NA. Hypoxic relaxation was of similar magnitude in control and treated tissues.

First series (b): KCl-induced tone

Effects of hypoxia on K^+ induced contractions were more reproducible than on those to NA. Absence of spontaneous activity superimposed on K^+ induced contractions could

At 16%02

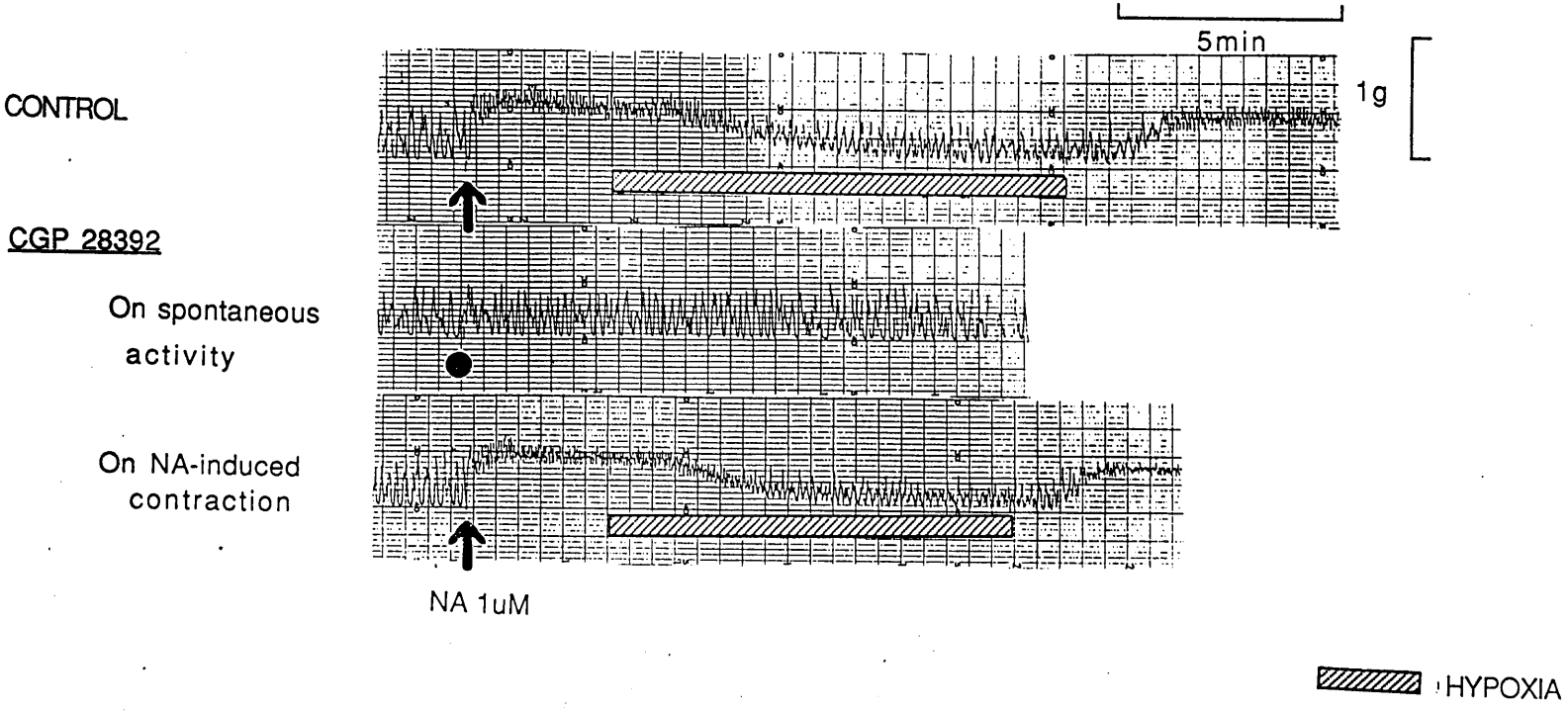


Fig 7.6
Example trace of the effect of CGP 28392 15min pretreatment on hypoxic relaxation of NA 1μM -induced contraction of the RPV at 2.5mM Ca^{2+} and 16%O_2.

account for this greater consistency. The typical response to KCl 50mM, was a phasic contraction which lasted 15s, followed by a fade and then a tonic contraction which peaked by 2min and subsequently waned. By 10min, contraction to KCl wanes to 52.4±5.3, n=12, of the initial contraction. Therefore there was a consistent time dependent decrease in contraction to KCl. When hypoxic relaxation was induced 2min into the KCl -induced contraction, the effect could be corrected for the time -dependent decrease in contraction, more easily than with the more variable NA-induced contraction.

(i)Pretreatment with Bay K .

Pretreatment with Bay K 0.3uM increased spontaneous activity but did not significantly alter the contraction to KCl 50mM. When hypoxia was induced the relaxation compared with the control was not significantly different ($P > 0.05$). The mean hypoxic relaxations to % of contraction to KCl 50mM before hypoxia were 17.3±3.0 (70.6%) and 23.2±2.8% (75.6%) n=6 for the control and Bay K treated in 16%O_2 respectively. The corresponding values at 95%O_2 were 14.3±5.7 (66.7%) and 17.2±5.9% (69.6%), n=6 for the control and Bay K treated respectively. Fig. 7.7. The values in brackets have been corrected for time dependent decrease in the tonic response to KCl.

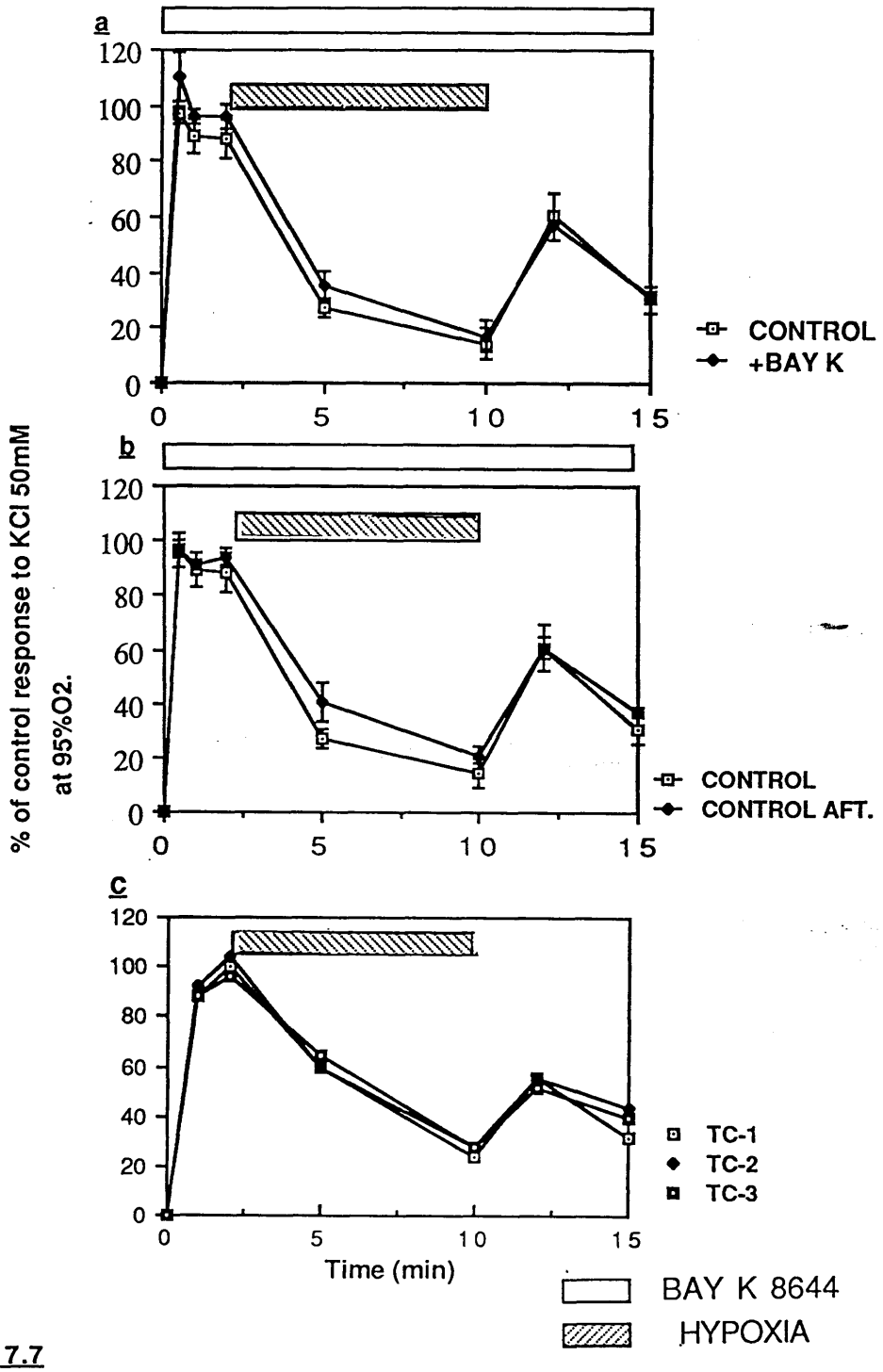


Fig 7.7

Effect of Bay K 0.3μM 15min pretreatment on hypoxic relaxation of KCl 50mM -induced contraction and subsequent re-oxygenation contraction. (a) before and after Bay K treatment. (b) control response before Bay K and recovery response after Bay K. (c) the time control response for 3 successive hypoxic relaxations of KCl-induced contraction.

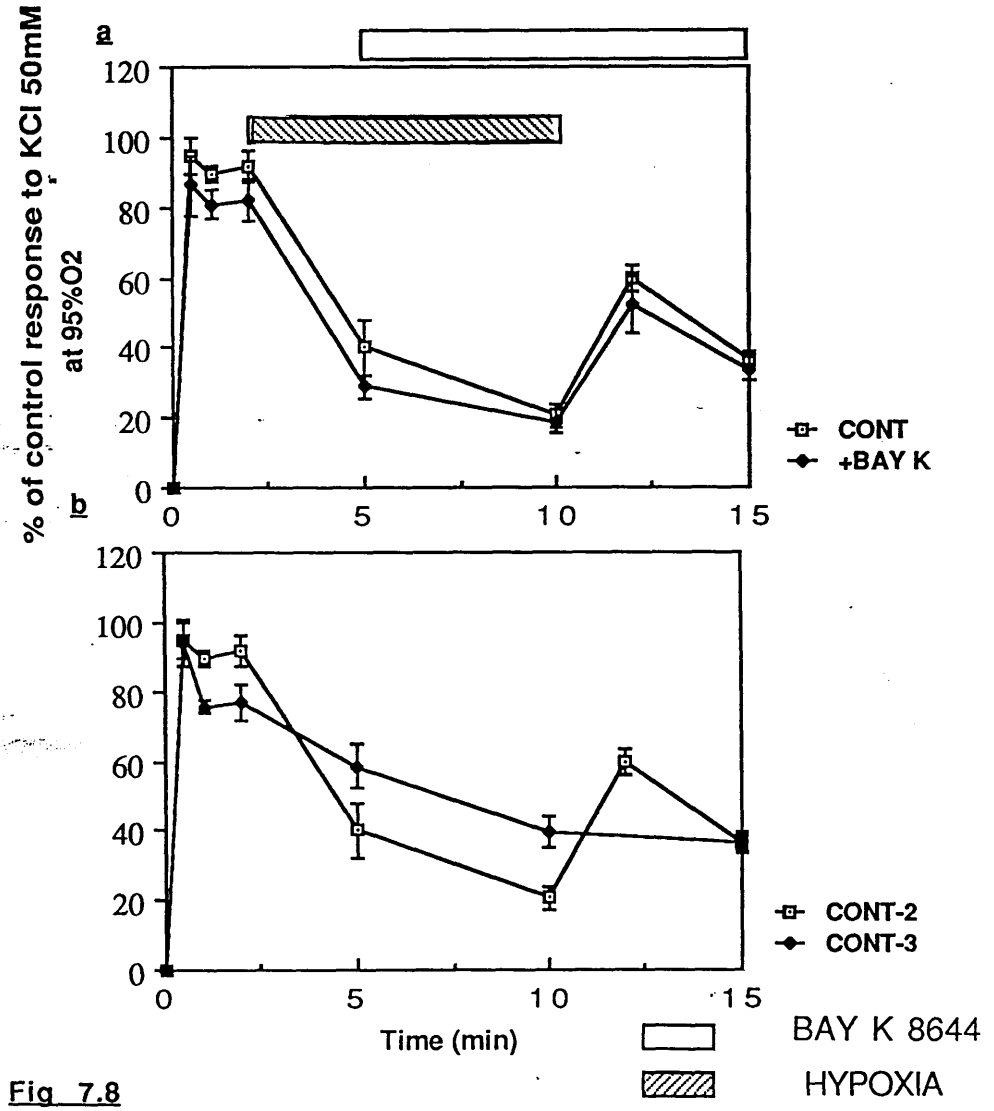


Fig 7.8

Effect of Bay K 0.3μM on established hypoxic relaxation and subsequent re-oxygenation in KCl 50mM contracted portal vein.

(a) hypoxic relaxation without and with Bay K treatment. (b) compares hypoxic relaxation and subsequent contraction (CONT-2) with time control response (CONT-3) to KCl over a 15min period.

(ii) Effect of Bay K on established hypoxic relaxation of KCl induced contraction.

The relaxation was slightly enhanced when Bay K 0.3uM was added during hypoxia. The maximum relaxations to % of the control contraction for Bay K treated in 16% were 26.8%±3.4 (78%) and 22.4±3.6% (74%) and in 95% were 20.7±3.2% (72%) and 19.0±3.6% (71%) See Fig 7.8. The values in brackets have been corrected for time depedent decrease in the tonic response to KCl.

Second series:

Bay K versus nifedipine-induced relaxation

In control experiments run in 2.5mM Ca^{2+}, nifedipine 10nM and 30nM relaxed the tissues to between 30% and 50% of the control contraction to NA 1μM, while the KCl 50mM induced contraction was relaxed to 20 to 30% of control contraction. When the experiment was carried out at a reduced Ca^{2+}, i.e. 0.31mM Ca^{2+}, responses to the same concentrations of NA and KCl were reduced. When Bay K was added for 15min, responses to NA 0.3μM and KCl were equivalent to Bay K -free controls in 2.5mM Ca^{2+} under these conditions. Nifedipine 10nM or 30nM now did not relax the NA induced tone while KCl induced contractions were only partially relaxed. See figs 7.9 & 7.10 and table 7.2.

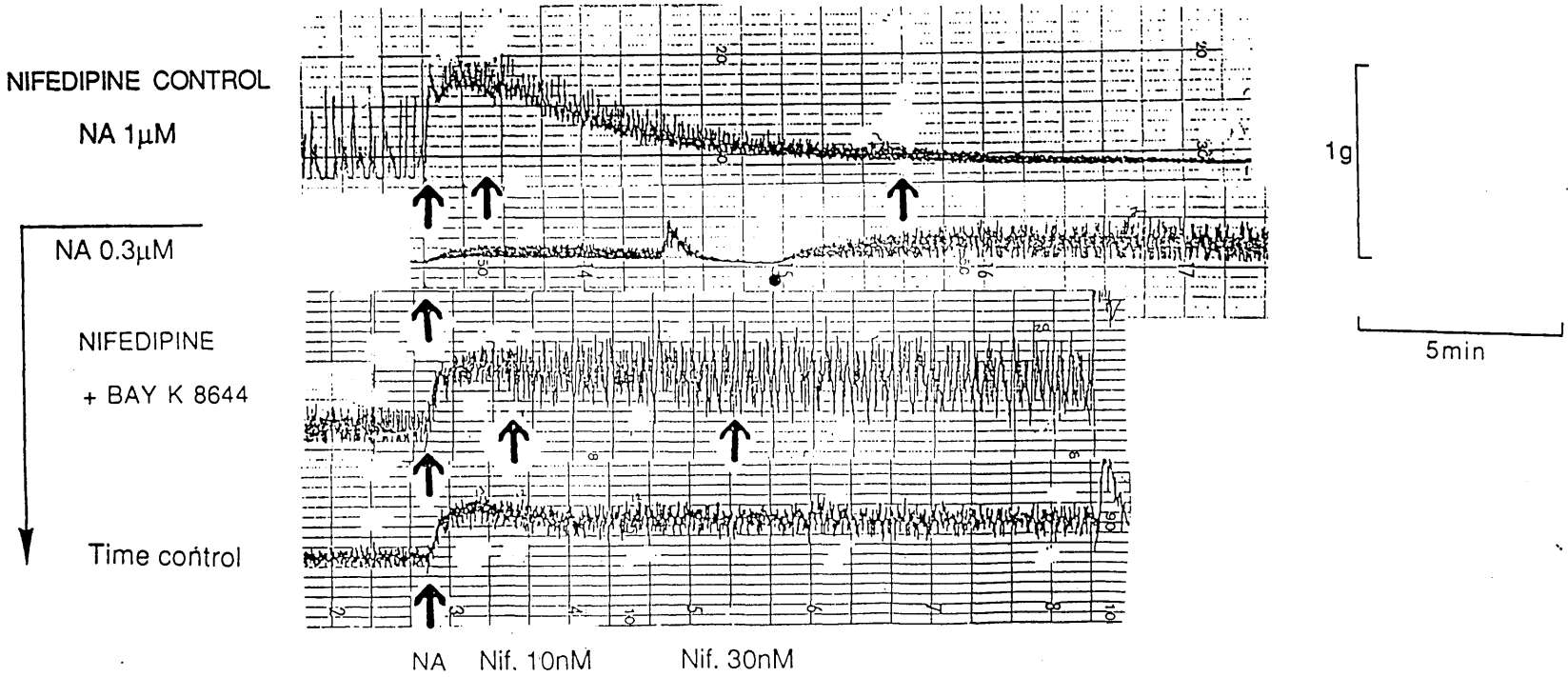


Fig 7.9

Nifedipine 10nM and 30nM -induced relaxation of NA 1μM -induced contractions and reversal of this relaxation by pretreatment with Bay K. Upper panel shows the control response to nifedipine 10nM and 30nM-induced relaxation. 2nd panel shows control response to NA 0.3uM at low Ca^{2+} 1.e. 0.31mM and addition of Bay K 8644 (at dot). 3rd panel shows response to NA in the presence of Bay K at 0.31mM Ca^{2+}: subsequent addition of nifedipine did not cause any relaxation. 4th panel shows the time control responses to NA in the presence of Bay K 0.3μM.

Third series:

(ii) Effect of prior additional Ca^{2+} i.e. 12.5mM on subsequent contraction, hypoxic relaxation and nifedipine induced relaxation.

Although responses induced by NA 1μM at 12.5mM Ca^{2+} were similar to those at 2.5mM Ca^{2+}, SA was depressed at 12.5mM Ca^{2+}. In the presence of additional Ca^{2+}, both nifedipine-induced relaxation and hypoxic relaxation were significantly reversed ($P < 0.05$), although a greater reversal of nifedipine relaxation by additional Ca^{2+} was observed (Table 7.2).

The protection against nifedipine-induced relaxation by high [Ca^{2+}] was reversible. Subsequently, in 2.5mM Ca^{2+}, nifedipine- induced relaxation was restored. Recovery to NA 1uM was about 50% of the control. See table 7.2.

The reoxygenation contraction was a little enhanced but not otherwise different from that in 2.5mM Ca^{2+}.

Fourth series:

Effect of Ca^{2+} antagonists on established hypoxic relaxation and subsequent reoxygenation contraction.

In the presence of nifedipine 10nM or verapamil 0.1μM, the contractile spikes superimposed on the hypoxic relaxation were depressed to 31.5±4.6% (n=6) and 6.0%± 0.4 (n=4) of the control contraction to NA 1uM,

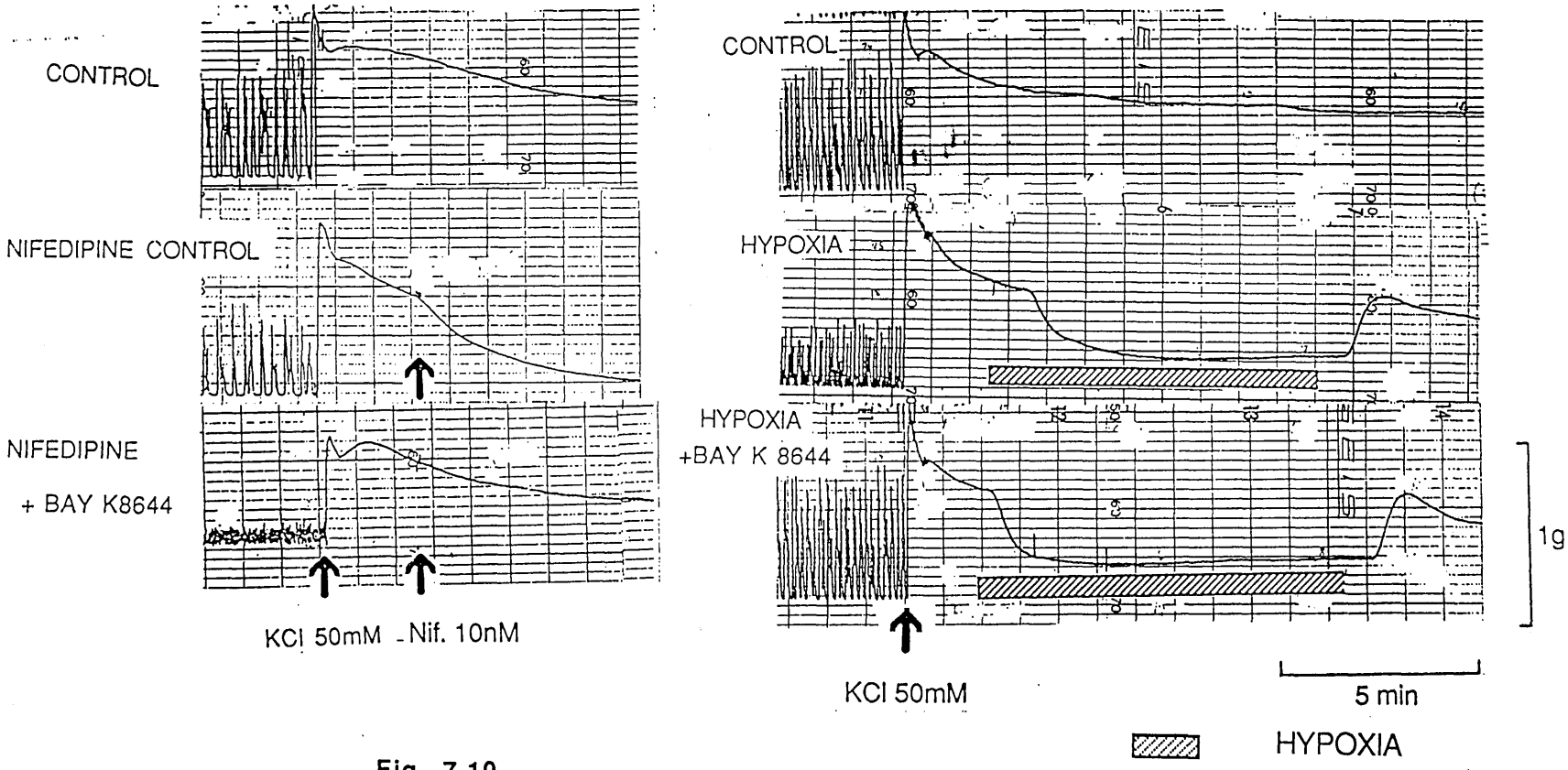


Fig 7.10

A representative trace comparing nifedipine and hypoxia-induced relaxation of KCl 50mM induced response without and with Bay K 8644. The panels on the left show the control response to KCl 50mM, in the presence nifedipine 10nM and nifedipine plus Bay K 0.3µM respectively. The panels on the right show the same sequence replacing nifedipine with hypoxia.

A.

	Control		Reponse during hypoxia		Re-oxygenation contraction	
Control (2.5mM Ca2+)	100	NS *	42.0 ±5.9(8)		100.8 ±8.9 (8)	
Excess Ca2+ i.e. 12.5mM	100		54.0 ± 5.8 (8		116.1± 8.4 (8)	NS
Recovery	100		48.3 ±8.4 (8)		99.3 ± 2.0 (8)	

B.

	Control	Response during nifedipine (10nM)	
Control (2.5mM Ca2+)	100	29.3 ±5.1 (4)	
Excess Ca2+ i.e. 12.5mM	100	74.4 ± 11.2 (4)	*
Recovery	100	28.9 ± 6.2 (4)	NS

C.

	Control	Response during hypoxia	Hypoxia/ Ca antagonist		Reoxygenation response	
	100	45.2 ±5.5 (6)	*			
Nifedipine (10nM)			31.5 ±4.6	(6)	29.0 ±4.9	(6) NS
Verapamil (0.1μM)	100		6.0 ± 0.4	(4)	8.1 ± 1.4	(4) **

Table 7.2 shows (A) the effects of excess Ca2+ on hypoxic-relaxation and on (B) nifedipine -induced relaxation.
(C). In a seperate series of experiments. effects of nifedipine and verapamil were examined on hypoxic relaxation and subsequent re-oxygenation contraction.
The values shown are relaxation to percentage of the control contraction to NA 1μM.
Values represent means + s.e.m. The number of observations are shown in brackets.
*, ** show P values < 0.05 and 0.01 respectively. The values compared are indicated.

respectively. The reoxygenation contraction was diminished by either antagonist. Nifedipine and verapamil inhibited the re-oxygenation contraction were to 29.0±4.9% (n=6) and 8.1± 1.4% (n = 4) of control contraction to NA respectively. See Table 7.2

DISCUSSION

Nature of the hypoxic relaxation of rat portal vein.

The degree of hypoxic relaxation against NA 1μM, even though variable, was still lower than that reported by Ebeigbe in his experiments. In our experiments the relaxation was to between 30 and 45% of the control contraction to NA 1μM in the separate experiments while Ebeigbe using a similar protocol obtained hypoxic relaxation of NA induced contraction was to between 0- and 5% of the control contraction. Such difference in our results could have arisen from the use of isometric transducers in the present experiments and isotonic in his.

It is worth noting that spontaneous contractile spikes were not completely suppressed in hypoxia. This appears at variance with previous reports by Hellstrand et al, (1977) and Sigurdsson et al,(1981a). But Sigurdsson & Grampp in another paper in (1981b), demonstrated that a total suppression of the spontaneous contractile spikes was not obtained in hypoxia probably

because the Po_2 in that particular experiment was about 10mmHg compared with < 5mmHg in their earlier experiments. In our experiment the same protocol (Sigurdsson & Grampp, 1981) of shifting between normoxia and hypoxia was adopted. The bath Po_2 during hypoxia as determined by a Po_2 electrode was 5 ± 2mmHg. The use of an isometric rather than isotonic transducer could have accounted for picking up the small spikes of the responses not recorded by other workers. This could also explain why earlier workers could not demonstrate responses of the portal vein to agonists in hypoxia (Sigurdsson & Grampp, 1981).

Bay K 8644 on hypoxic relaxation

Bay K treatment in a variety of protocols was unable to reverse hypoxic relaxation.

Hypoxia was proposed to inhibit Ca^{2+} influx by Ebeigbe (1979) because of the correlation which he showed between Ca^{2+}-free relaxation and hypoxic relaxation: those vessels which were susceptible to withdrawal of Ca^{2+} were also susceptible to hypoxia. Ebeigbe also showed in Ca^{2+} flux studies that hypoxia produced decreased Ca^{2+} uptake in $[Ca^{2+}]_o$ in NA or KCl stimulated preparations, but an increased Ca^{2+} uptake in the non- stimulated tissues. Marriot (1985), using Ca^{2+} antagonists, showed that there was a correlation between external Ca^{2+} dependency and susceptibility to hypoxia. In the same study Marriot showed that vascular smooth

muscles that are dependent on extracellular Ca^{2+}, such as the rat portal vein, are highly susceptible to changes in $[Ca^{2+}]$ as well as to hypoxia; confirming Ebeigbe et al's hypothesis.

In fact Marriot's study of the effects of Ca^{2+} antagonists on the portal vein in hypoxia was hampered since responses to NA or KCl were already depressed making it impossible to investigate the responses in the presence of Ca^{2+} antagonists. The susceptibilty of the rat portal vein to Ca^{2+} antagonists is well documented in hyperoxia -95% (Jetley & Weston, 1980; Fiol de Cuneo et al, 1985; Campbell et al, 1986) and in normoxia (16%), in our own studies e.g. a concentration of 0.1uM - 0.3uM of nifedipine would cause 100% inhibition of the spontaneous activity as well as of the agonist-induced response. By contrast, in the rat aorta, the NA -induced responses are more resistant to Ca^{2+} antagonists than are KCl induced responses, and the responses are also resistant to hypoxia (Marriot, 1985).

Bay K is said to promote transmembrane Ca^{2+} influx through voltage operated channels (Schramm et al, 1983). It acts in an opposite manner to its close dihydropyridine analogue nifedipine. It might have been possible that if hypoxia was to inhibit Ca^{2+} influx in these same channels, and consequently result in relaxation, Bay K might be able to reverse it. But in our experiments Bay K could not reverse the hypoxic relaxation despite various attempts at manipulating the

protocol to detect such an effect. In the absence of hypoxia the effects of nifedipine could be readily antagonised by Bay K . Does this mean that hypoxic relaxation is not wholly due to inhibition of $[Ca^{2+}]_o$ entry and that the correlation of sensitivity to hypoxia and to withdrawal or blockade of extracellular Ca^{2+} is coincidental?. This is supported by a recent communication by Downing et al, 1986, who showed that there was no correlation between sensitivity of individual vascular smooth muscles (in this case arteries) to verapamil and their susceptibility to hypoxia, since the verapamil sensitivity order was femoral artery > mesenteric artery >rat aorta, while the sensitivity to hypoxia lies in the order rat aorta > femoral artery > mesenteric artery. These authors further suggested that differences in time course of the effect of verapamil and hypoxia on the femoral artery indicate that the effects of hypoxia on isolated rat arteries cannot be wholly attributed to a decrease in the utilisation of external calcium. However our results do not eliminate an effect of hypoxia on Ca^{2+} entry, availability or action, at a site separate from the dihydropyridine binding site. A hypothesis to explain all the results would however require that Ca^{2+} influx was no longer determined by extracellular $[Ca^{2+}]$ in the supra-physiological range nor could be facilitated by Bay K (or CGP). This would suggest that Ca^{2+} is already entering the cell as fast as is possible because those channels which are still operational are fully

facilitated.

In comparing hypoxic induced relaxation with that induced by nifedipine 10nM or 30nM, while Bay K could reverse the relaxation due to nifedipine in normoxia, it could not modify that due to hypoxia. Nifedipine - induced relaxation is due to inhibition of Ca^{2+} influx caused by closure of the potential operated channels. Reversal of this effect by Bay K is due to increased Ca^{2+} influx allowed by increased open time of the Ca^{2+} channels.

Additional Ca^{2+} on hypoxic relaxation

Increasing concentrations of $[Ca^{2+}]_o$ have been shown to reverse the effects of Ca^{2+} antagonists in several vascular smooth muscles, rat portal vein included (Jetley & Weston, 1980; Mikkelsen, 1985; Mikkelsen et al, 1986). We tested this versus hypoxic relaxation.

The relaxations obtained in the presence of 12.5mM Ca^{2+}, showed that nifedipine -induced relaxations were significantly reduced more than were those to hypoxia. The reversal of nifedipine - induced by additional Ca^{2+} is consistent with the observation made with the Ca^{2+} facilitator Bay K . It is however interesting that that relaxation to hypoxia was reversed to a smaller extent by additional Ca^{2+}.

Sigurdsson & Grammp (1981a) showed that hypoxic

depression of force by the rat portal vein was reversed either by increasing $[K^+]_o$ or decreasing $[Na^+]_o$. The effect of increased K^+ was explained on the basis of facilitation of extracellular Ca^{2+} into an intracellular compartment which, in itself (Droogmans et al, 1977) or by raising intracellular Ca^{2+} (Mayer et al, 1972) may result in an increased activation of the contractile system. Reduced $[Na^+]_o$ on the other hand could increase Ca^{2+} influx by a diminished competition between Na^+ and Ca^{2+} for the same membrane channels (Droogmans & Casteels, 1979). Alternatively, an increased intracellular calcium could be achieved by reduction of Ca^+ efflux via the Na-Ca exchange mechanism in the presence of a decreased transmembrane electrochemical gradient for Na^+ (Reuter et al, 1973; Blaustein, 1977). The effect of increased $[K^+]_o$ or decreased $[Na^+]_o$ could be equated to the addition of excess Ca^{2+} on hypoxic relaxation, in which there was a slight reversal of hypoxic relaxation.

Sigurdsson & Grampp proposed that the mediator function of Ca^{2+} might be insufficient in hypoxia because of insufficient release of Ca^{2+} from intracellular stores or changed Ca^{2+} affinity of the regulatory system of the contractile apparatus. However the possibility of insufficient influx of Ca^{2+} in connection with the discharge activity was ruled out because the configuration of the spikes was unchanged (Sigurdsson & Grampp, 1981). The inability of Ca^{2+} to function as a

mediator as explained by them could arise in hypoxia because of intracellular acidification and /or accumulation of metabolites. (Whether this hypothesis put forward by Sigurdsson & Grammp, 1981, is true or not is debatable). However, Ighoroje (1987), suggested that intracellular acidification in fact promotes vasoconstriction. Hellstrand et al, (1977) showed that in the rat portal vein metabolic activity was higher in hypoxia than in normoxia, with the lactate production 2.7 times higher in hypoxia, thus leading to accumulation of metabolites and acidosis (Sigurdsson & Grammp, 1981). Spedding and co-workers noted that palmitoyl carnitine, an acyl carnitine, is a lipid metabolite that accumulates during ischaemic conditions. This metabolite can directly activate Ca^{2+} channels in smooth muscles and avian heart cell aggregates in a way similar to Bay K 8644 (Bigaud & Spedding, 1986; Duncan et al, 1986; Mir & Spedding, 1986; Spedding & Mir, 1987).

These lines of evidence suggest that there may be increased intracellular Ca^{2+} activation as a result of hypoxia via intracellular acidification/metabolites and that the Ca^{2+} channels may already be fully facilitated, again by accumulation of metabolites.

A hypothesis to explain Bay K 's ineffectiveness on hypoxic relaxation

In our previous experiments we showed that Bay K could facilitate potential operated channels to a similar extent from 16% to 1%O_2. However, in 0%O_2 even though the control responses to KCl were not depressed compared with those at 16%, Bay K could not facilitate the responses much. We suggested that it could be that the control responses to KCl were already facilitated at 0%O_2 therefore rendering Bay K ineffective. The facilitated effect in hypoxia could be linked to the Fig 7.11 shows a schematic representation of an hypothesis that could explain the events that occur during hypoxia. accumulation of acyl carnitine -like metabolites.

Ca^{2+} antagonists on re-oxygenation contraction.

Administration of nifedipine during hypoxic relaxation produced a relaxation similar in magnitude to the control relaxation to nifedipine without hypoxia (Table 7.2). Failure of further relaxation by nifedipine during hypoxic relaxation is consistent with the hypothesis that nifedipine is relatively resistant in hypoxic conditions.

The blockade of the re-oxygenation contraction by either nifedipine or verapamil suggests that the re-oxygenation

nifedipine or verapamil sensitive Ca^{2+} channels. Ebeigbe (1982), showed a correlation between a reduction of response of the rabbit aorta to 1uM NA during hypoxia and a concomitant decrease in ^{45}Ca uptake. A corresponding recovery of the responses following re-oxygenation was closely related to an increase in ^{45}Ca uptake of the preparations. Downing et al (1985), using the rat aorta, showed that while the recovery of contraction on re-oxygenation was dependent entirely on a verapamil (10uM) sensitive calcium entry, the depression of NA contractions by acute hypoxia appeared to be due to a decrease in both a verapamil -sensitive and a verapamil-insensitive entry of Ca^{2+}.

Does it mean then that re-oxygenation contraction can be blocked by Ca^{2+} antagonists, while the same contraction is only slightly enhanced, though with increased baseline tension, by a near maximal concentration of Bay K 0.3uM? While the contractile spikes superimposed on the hypoxic relaxation were further reduced by the Ca^{2+} antagonists, Bay K caused increased frequency of spikes without increasing baseline tension.

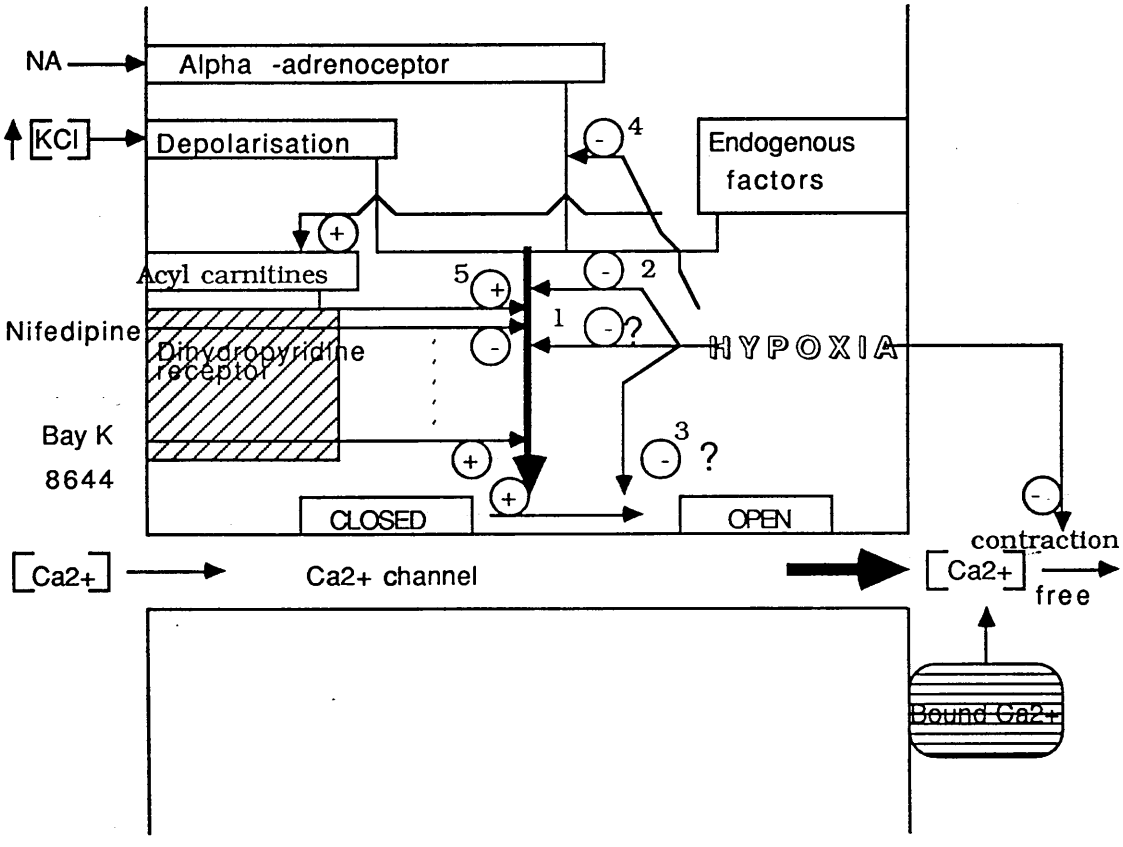
The inability of Bay K to facilitate reoxygenation contraction was reminiscent of Bay K 's ineffectiveness against NA or KCl in 2.5mM Ca^{2+} (See Chapter 4). The reoxygenation contraction is hardly higher than the pre-hypoxia contraction whether Bay K was present or not; or whether it was 2.5mM Ca^{2+} or 0.3mM Ca^{2+}; suggesting that Bay K was incapable of potentiating this size of

Fig 7.11

A schematic representation of hypothetical sites of action of hypoxia and dihydropyridines and their possible interactions.

Hypoxia is given for 4 possible sites of action (1-4) to inhibit Ca2+ channel opening and a 5th (5), via increased acyl carnitines synthesis, to potentiate it. Site (1), a direct inhibition at the dihydropyridine receptor looks unlikely since hypoxia can completely overide the action of Bay K 8644, leaving another point in the activation still leading to channel opening (2) or a direct inhibition of the opening process (3). Site (4), in the specific pathway following alpha-adrenoceptor activation is necessary to account for the greater susceptibility of NA-induced contraction to hypoxia.

It is interesting that hypoxia can override an existing contraction but cannot prevent a new contraction to KCl, i.e why does mechanism (5) dominate in cells which are already hypoxic but not in cells which acutely become hypoxic? Perhaps a pre-existing high level of acyl carnitines facilitate Ca^{2+} and protect against the inhibition of hypoxia. This then leaves the question whether the acute relaxant effect of hypoxia would gradually wane with time as acyl carnitines build up. Alternatively, acyl carnitines might not build up so quickly in a cell which had been pre-contracted.


NA
Alpha -adrenoceptor
KCl
Depolarisation
4
Endogenous factors
Acyl carnitines
5
2
Nifedipine
1
?
HYPOXIA
Dihydropyridine receptor
Bay K 8644
3
?
CLOSED
OPEN
contraction
Ca2+
Ca2+ channel
Ca2+
free
Bound Ca2+

response. The factors therefore limiting the facilitatory effect of Bay K in this tissue are the level of Ca^{2+} and the size of control response (Refer to response in buffered and unbuffered Ca^{2+}, where the higher response in the buffered Ca^{2+} of supposedly equivalent concentration of Ca^{2+} as unbuffered limited the facilitation of Bay K).

GENERAL DISCUSSION AND CONCLUSIONS.

The principal findings in this study are:

(1). The sensitivity of RPV to O_2 has been confirmed. This makes it a useful preparation for studying the effects of O_2 on sensitivity to drugs and predicting drug effects that could be particularly resistant to or sensitive to hypoxic/ ischaemic conditions.

(2). Differences have been observed between drug effects in hyperoxia and in normoxia. Clearly, the use of hyperoxia would not reflect *in vivo* conditions and might be misleading to the interpretation of drug effects.

(3). The basic excitation-contraction coupling mechanisms remained unchanged within the Po_2 range 95%O_2 - 1%O_2, according to the unchanging pD_2 values for Bay K 8644. It was only at hypoxia (0%O_2) that a radical change occurred. Responses to KCl became bigger and the maximum facilitations by all the Ca^{2+} facilitators tested were depressed. This also applied to NA: although its responses were depressed at hypoxia, there was a proportionate depression of the maximum facilitation.

(4). The contractions to KCl were relatively resistant to nifedipine at hypoxia compared with those at normoxia.

(5). Control responses to KCl were more susceptible to POCA in hypoxia than in normoxia.

(6). Bay K and CGP tested in various ways were ineffective in reversing acute hypoxic vasodilatation.

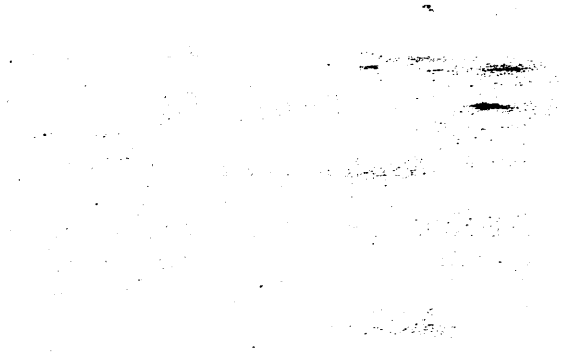
The implication of these principal findings is that the voltage operated channels (VOCs) through which the Ca^{2+} agonists and Ca^{2+} antagonists act are fully facilitated in ischaemic/ hypoxic conditions, this is because of the accumulation of the endogenous acyl carnitines e.g. palmitoyl carnitine. These compounds have been shown to have direct activating effects on Ca^{2+} channels. Consequently, further addition of other Ca^{2+} facilitators be it Bay K or CGP or even PC itself would not cause much facilitation. This would explain why Bay K or CGP was unable to reverse hypoxic vasodilatation. The physiological implication of this is that the hypoxic site of action might be different from the dihyropyridine site of action. Furthermore, pharmacologically, this type of drug i.e. Bay K or CGP cannot be employed in the treatment of hypoxic vasodilatation.

The relative ineffectiveness of nifedipine in hypoxic or ischaemic conditions arises because acyl carnitines interact with the dihydropyridine sites and consequently, they can oppose inhibition due to nifedipine, therefore rendering it ineffective. It is interesting that Spedding & Mir, (1987) predicted a similar finding even though they did not provide direct evidence for the ineffectiveness of nifedipine in ischaemic conditions. The implication of the relative ineffectiveness of nifedipine in ischaemia is that this type of drug may be ineffective in improving blood flow

in ischaemic conditions.

Pharmacologically, administration of POCA would prevent the synthesis of acyl carnitines. This would alleviate the exacerbation of ischaemic conditions caused by acyl carnitines and allow the control of vascular smooth muscle by other drugs.

Researchwise, it would be interesting to see whether facilitation to Ca^{2+} facilitators can be restored in the presence of POCA.



APPENDIX

I. E.G.T.A AND N.T.A BUFFERS

The problem of lack of a suitable Ca^{2+} buffer for the 10uM to 100uM range (near neutral pH) is partially circumvented with an EGTA and NTA mixture (Miller & Smith 1984).

EGTA (Ethylene glycol bis-(B- aminoethyl ether) N,N,N'N'-tetra acetic acid).
EGTA is a tetravalent ion which buffers near luM Ca^{2+} at physiological pH.

The following equation represent the forms in which EGTA occurs during buffering.

$$EGTA^{4-} + H^{+} \underset{}{\overset{k1}{\rightleftharpoons}} HEGTA^{3-} + H^{+} \overset{k2}{\rightleftharpoons} H_2EGTA^{2-} + H^{+}$$

$$\overset{k3}{\rightleftharpoons} H_3EGTA^{-} + H^{+} \qquad \overset{k3}{\rightleftharpoons} H_4EGTA$$

EGTA exists mostly as H_2EGTA^{2-} at pH 6.5 - 7.5. Therefore, the form in which EGTA exists depends on the pH of the medium.

Reaction with Ca^{2+}

$$Ca + EGTA^{4-} \overset{kCa_1}{\rightleftharpoons} CaEGTA^{2-}$$

$$Ca + HEGTA^{3-} \overset{kCa_2}{\rightleftharpoons} CaHEGTA^{-}$$

EGTA also reacts with Mg^{2+} but this not too important under the conditions of the present work. The reactions are given as follows:

$$Mg^{2+} + EGTA^{4-} \overset{kMg_1}{\rightleftharpoons} MgEGTA^{2-}$$

$$Mg^{2+} + HEGTA^{3-} \overset{kMg_2}{\rightleftharpoons} MgHEGTA^{1-}$$

Nitrilotriacetic acid (NTA)

-Buffers Ca^{2+} near 0.1mM

$$NTA^{3-} + H^{+} \overset{k1}{\rightleftharpoons} HNTA^{2-} + H^{+}$$

$$Ca + NTA^{3-} \overset{kCa_1}{\rightleftharpoons} CaNTA^{-}$$

$Ca + 2NTA^{3-} \overset{kCa_2}{\rightleftharpoons} Ca(NTA_2)^{4-}$. (This reaction always occurs, but it is only appreciable when 'free' NTA is high).

The calculation for $[Ca^{2+}]_{free}$ requires knowledge of the total ionic strength of the solution.

At neutral pH, an apparent, overall reaction can be described:

$$H_2EGTA^{2-} + Ca^{2+} \underset{\text{Kapp}}{\rightleftharpoons} CaEGTA + 2H^+$$

where Kapp = K apparent.

The H^+ ions liberated are neutralised by adding NaOH or by the response of the CO_2/HCO_3 system in an open solution.

$$\frac{[H_2EGTA]}{[CaEGTA]} \times \frac{[Ca^{2+}]}{[H^+]^2} = \frac{1}{Kapp}$$

If the pH is adequately buffered, the $[H^+]^2$ is constant and can be cancelled out.

$$\text{Therefore } [Ca^{2+}]_{free} = \frac{[CaEGTA]}{[EGTA_{free}] \times Kapp}$$

Conditions taken into account in these calculations are:
(1). Ionic strength.
(2). pH of the medium. It is important that this is kept constant, because this will shift the equilibrium between the different forms of EGTA and therefore affect its buffering capacity and the $[Ca^{2+}]$.

The recipe of the Ca^{2+} buffers is shown on the table below.

Recipe for E.G.T.A (2.5mM) and N.T.A (2.5mM) Ca2+ buffers (2litres).

	BUFFER 1	BUFFER 6
E.G.T.A	1.9g	1.9g
N.T.A	0.95g	0.95g
NaCl	223ml	223ml
NaOH	15ml	15ml
NaHCO3	49.6ml	49.6ml
KH2PO4	2.4ml	2.4ml
MgSO4	2.4ml	2.4ml
KCl	9.4ml	9.4ml
CaCl2	9.38ml	4.69ml
Glucose	4g	4g

FORMULA FOR BUFFERS 2, 3, 4 & 5

	BUFFER 5	BUFFER 4	BUFFER 3	BUFFER 2
BUFFER 1	13.36ml	36.64ml	82.8ml	167.2ml
BUFFER 6	236.64ml	213.36ml	167.2ml	82.8ml

[Ca] free levels in the buffers.

BUFFER 6	1µM
" " " 5	3µM
" " " 4	10µM
" " " 3	30µM
" " " 2	0.1mM
" " " 1	0.3mM

II. MEASUREMENT OF BATH Po_2

The measurement of the Po_2 of the bath solution was made by a PO_2 electrode connected to a Po_2 meter (Strathkevin instruments model 781.

Calibration of the PO_2 meter.

The zero was set by either
(i) chemical method using sodium borate and sodium sulphite.
or
(ii) 100%N_2.
Usually the chemical method was preferred as it got rid of any residual PO_2 in the bath.

To set the zero reading, the PO_2 electrode is placed in contact with the solution of sodium borate to which a pinch of a sodium sulphite is added and swirled to gradually dissolve. The reading of the meter falls gradually until it reaches zero.

To set the gain, the solution in the bath is washed out and replaced with distilled water kept at 37°C. The bath solution is then rapidly bubbled with air and the PO_2 meter reading goes up. After a while it settles to a steady value usually close to the PO_2 of air determined from the equation below. If the registered PO_2 is outside this value then the gain of the meter is adjusted accordingly.

$$PO_2 \text{ (mm Hg)} = \frac{20.93}{100} \times (b - vp)$$

where b is the barometric pressure and vp is the water vapour pressure, which is usually 47mmHg.

The required gas mixture for the experiments was made from an anaesthetic machine into a Douglas bag. The gas mixture always contained 5%CO_2, the required percentage of O_2 and the balance made up with N_2 gas.

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