Design of a molecular strategy to analyse the subcellular distribution of α_1-adrenoceptors in recombinant and native systems.

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A thesis submitted for the degree of Doctor of Philosophy (Sept, 2001)

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Acknowledgements.

You hear horror stories about PhD supervisors, but it must be said that Prof. McGrath, who must be the busiest man on the planet, always made time for me as a PhD student. I have learnt a lot from his lateral thinking, which in the latter part of my PhD assisted me greatly in my approach to science. I have thoroughly enjoyed the last 4 years in his lab, in terms of my broadened knowledge and the numerous skills that I have attained.

To my colleagues, Janet, John, and Craig, who were subject to my continuous string of questions, well they do say, 'If you don't ask, you don't get!' They were always available to assist and sacrificed valuable time to help me out for which I am eternally grateful.

To Joyce, whose door was always open, and from whom an 'on tap' supply of both technical and personal support was always given.

To Simon, whose ears have no doubt been given a well-deserved break during the last 9 months whilst I've been writing-up from home! Cheers for everything Si!

I must say that working in 440 has been less daunting than first anticipated. This has been due to the friendly, 'chilled-out' atmosphere that always fills the lab, well, unless Anne K (debt collector) is on the warpath! Jillian, Ann, Anne, Angela, Melissa, and Darren have contributed to making life in 440 the place to be in the WMB.

To my parents, special thanks for their love and support during the 8 years that I have studied at Glasgow University. In the last year, they have put up with my constant mood swings and 'flying off the handle' at the slightest thing, not to mention the papers strewn all over their spare room!

To my sister Fiona, who on a regular basis asks 'what's your PhD about again?' The fact that I didn't really have much of a clue myself until my final year is beside the point!

To Bob, who helped me out on several occasions with computing problems.

To my grandparents, all four of them, who always encouraged me to work hard, even as far back as primary school. 'It will be worth it in the end'. How many times have I heard that! MANY years later, I've finally reached a point where I'd like to think my 'education' is finally over, although I will never stop learning.

A special thank-you must go to Clare, who started her PhD in Prof. McGrath's lab at the same time as myself. We have stuck together and been a constant support to each other through thick and thin

over the last 4 years, some people thinking we were joined at the hip! Aside from being a work colleague, Clare has become one of my closest friends. She has kept me sane during a time that I would describe as the toughest 4 years of my life. I owe a lot to her. Thank-you doll!

Other special friends that played a part in maintaining my sanity are Carol and Jill. Both of them can't wait to have their friend back after months of 'drought' and no 'girly nights out'. Last, but by no means least, Marie, who has been a traitor for the last 19months, leaving me and heading south, but alas, she has seen sense and is returning home to sunny Glasgow very soon.

There are so many people that deserve a mention, and I could go on and on. I would like to thank everyone who has supported me in whatever shape or form. Thanks x

I would like to dedicate this thesis to my late Grandfather who supported me financially through all my University years. His generosity has allowed me to get where I am today, and for this reason, I cannot thank him enough.

Declaration.

The work contained within this thesis is entirely my own. Some of the figures represent work that was assisted by others:

Figures 1.10-1.13, 1.21, 1.27-1.29, 2.14 Dr John Pediani

Figure 1.22b Dr Susan Currie

Figure 3.11 Dr Ian Montgomery

This work has not been presented in whole or as part of any other degree course.

Publications:

Woollhead, A.M. and McGrath, J.C. (1999) Validation of fluorescent ligand binding to recombinant mouse and human α_{1b}-adrenoceptors. *J. Physiology.* **521. P**, 63-64P.

McGrath, J.C., Arribas, S.M., Deighan, C., McGrory, S.P., MacKenzie, J.F., Macmillian, J.B., McGee, A., Pediani, J., **Woollhead, A.M.**, Daly, C.J. (2001) Remodelling of resistance artery adventitia is ubiquitos to hypertensive and heart failure models and is under adrenergic influence. 8th International Symposium on Mechanisms of Vasodilation, Boston, USA.

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Summary of results.

Chapter 1.

1. The distribution of α_{1b}-ARs stably expressed in NCB20 cells was predominantly associated with the plasma membrane. However, in some instances, a predominantly intracellular, non-perinuclear location was observed.
2. α_{1b}-ARs were distributed in a similar manner in cells at both high (8000fmol/mg) and lower (4000fmol/mg) receptor densities.
3. FLAG tagging to the N-terminus of the α_{1b}-AR resulted in the construct having lower affinity for prazosin than wildtype receptors indicating that the 8amino-acid tag has restricted access to the ligand-binding pocket.
4. GFP-tagging to the C-terminus of the α_{1b}-AR resulted in the construct having equivalent affinity for prazosin as the wildtype receptor, demonstrating that such fusion proteins do not perturb prazosin binding to α_1-ARs.
5. Both GFP- and FLAG-tagged α_{1b}-ARs had lower affinity than wildtype receptors for QAPB in radioligand binding studies, yet their affinities were similar to that of wildtype in confocal analysis on live cells.
6. Each construct was demonstrated to be functional using the Ca^{++} indicator Fura-2 and both fluorimetric and imaging analysis set-ups.
7. QAPB binding to α_{1b}-ARs in live cells was validated using a GFP-tagged α_{1b}-AR cell line.

8. Coupled with calcium imaging, a GFP-tagged α_{1b}-AR cell line allowed the study of the distribution and active properties of α_{1b}-ARs in response to agonist.

9. α_{1b}-ARs have a basal level of movement between the plasma membrane and a non-perinuclear, intracellular compartment when stably expressed in NCB20 cells.

10. In response to agonist stimulation, and subsequent release of Ca^{++} from intracellular stores, α_{1b}-AR movement ceases, and resumes once Ca^{++} levels returned to baseline.

11. During prolonged (35minutes) agonist exposure, there was an obvious overall shift in the density of α_{1b}-ARs residing at the plasma membrane to the intracellular compartment.

12. QAPB binding to α_{1b}-ARs stably expressed in NCB20 cells initiated alterations in the cellular shape and rapid vesicular movement within the cell. Such observations suggest that QAPB enters via an endocytic pathway in the absence of agonist.

13. QAPB binding to α_1-ARs does not elicit an intracellular Ca^{++} response.

Chapter 2.

1. α_1-AR subtype expression in hepatocytes is heterogeneous and displays a unique form of plasticity: During prolonged culture, an increase in α_1-AR density was observed. In both wildtype and knockout cells these sites were sensitive to RS100329 (α_{1A}-AR-selective) and BMY7378 (α_{1D}-AR-selective).

2. α_1-AR expression undergoes ontogenic modulation during the life cycle of the mouse.
3. In the wildtype, there is a predominantly α_{1B}-AR population which shows a decrease in density between 3 and 4 months, likely to represent post-maturational decline, as seen in the rat.
4. In the knockout, at 3months, only a small population of α_1-ARs exists compared to the substantial α_{1B}-AR population in the wildtype. Up-regulation of an α_{1A}-AR population between 3 and 4months is likely to be a compensatory mechanism to replace the α_{1B}-AR subtype.
5. By 4months, a population of similar α_1-AR density is reached in both strains, yet belong to different subtypes.
6. In both wildtype and knockout cultured hepatocytes, α_1-ARs are distributed throughout the cytoplasm of the cell. Minimal expression was associated with the plasma membrane.

Chapter 3.

1. QAPB bound specifically, and with high affinity, to α_1-ARs present on freshly dissociated (carotid artery) and cultured (carotid artery and thoracic aorta) smooth muscle cells.
2. In freshly dissociated cells, QAPB binding was predominantly intracellular; this was consistent in both wildtype and knockout cells.
3. In cultured vascular smooth muscle cells, QAPB binding varied considerably between individual cells, in both strains, and was influenced by the cellular morphology.

4. RS100329 (α_{1A}-AR-selective) was able to reduce the specific binding of QAPB in some of the cells analysed, from both aorta and carotid artery.
5. BMY7378 (α_{1D}-AR-selective) was able to block the specific binding of QAPB in the majority of knockout cells studied, from both strains, and from both vessels. Less of an effect was observed in the wildtype.
6. In knockout smooth muscle cells (carotid artery and aortic), a predominantly α_{1D}AR population was expressed, whereas a mixture of all three is likely in the wildtype carotid artery.
7. Observations were made that wildtype carotid artery smooth muscle cells appeared to grow at a much slower rate than knockout cells.
8. Using the transfection reagent Tfx™-50, it was possible to express GFP-tagged α_{1b}-ARs in knockout aortic SMCs *in situ* under the control of both a viral, and a mouse α_{1B}-AR-specific promoter.

General Introduction

Historical background

The starting point for the development of the concept of the adrenoceptor (AR) system was in 1895, when Oliver and Schaffer demonstrated that in vivo injection of adrenal gland extract caused a rise in arterial blood pressure (Oliver and Schaffer, 1895).

Following the subsequent isolation of adrenaline (AD) as the active compound, it was shown by Dale (1913) that AD causes 2 distinct types of effect, namely vasoconstriction in certain vascular beds and vasodilation in others (Rang and Dale, 1991). Dale also showed that the vasoconstrictive component disappeared if the animal was injected with an ergot derivative prior to AD causing a drop, as opposed to a rise, in arterial pressure. Due to a disagreement with Langley concerning these results, Dale refused to interpret this as the existence of two distinct classes of receptor species. Subsequent to this, it was thought that this variation in effect was due to the release of different endogenous catecholamines called sympathins (sympathin I for inhibitory and sympathin E for excitatory), each one causing a different effect at the same receptor (Cannon and Rosenbleuth, 1933).

Later, pharmacological work beginning with that of Ahlquist in 1948 clearly showed that several subclasses of adrenoceptor exist in biological systems. Ahlquist found that the rank order of potency for various catecholamines including noradrenaline (NA), adrenaline, and the synthetic catecholamine isoprenaline (ISO) fell into two distinct patterns, depending on the tissue being studied. He postulated the existence of two classes of receptor, alpha (α) and beta (β), defined in terms of agonist potencies: α: NA>AD>ISO and β: ISO>AD>NA.

It was then recognised that certain ergot alkaloids, which Dale had studied, acted as selective α-AR antagonists and that Dales' reversal experiments with such compounds reflected the β-AR effects of AD after α-AR blockade. Although compounds were available at that time to block α-ARs, it was not until 1958 that a compound became available to block β-ARs (Moran and Perkins, 1958; Powell and Slater, 1958) and so confirmed Ahlquist's classification. The use of these selective antagonists also suggested the existence of further subdivisions of both α- and β-ARs. Shortly afterwards, β-ARs were further subdivided into β_1- and β_2-ARs depending on their location and polarity (Lands et al., 1967).

Brown and Gillespie set the ball rolling for subtypes of α-ARs in 1957. Their work and subsequent work in other laboratories looked towards the existence of pre- and post-junctional α-ARs, leading to the subclassification of α-ARs into α_1-postjunctional and α_2-prejunctional sites based on anatomical and pharmacological properties (Langer, 1974).

Problems were created however when several groups demonstrated the presence of α_2-ARs postjunctionally on vascular smooth muscle (Drew and Whiting, 1979; Docherty et al. 1979; Timmermans et al., 1979) and also on various other tissues (Pettinger et al., 1976). This classification was thereafter clarified as exclusively pharmacological regardless of anatomical location (Starke and Langer, 1979; Berthelsen and Pettinger, 1977).

Pharmacological Characterisation

With the development of additional pharmacological tools as well as new techniques for studying drug-receptor interactions such as radioligand binding assays, it became apparent that the situation was significantly more complicated

with further subdivisions of the α_1-ARs. Accumulating evidence of heterogeneity from several groups led McGrath, (1982) to propose a further subdivision of α_1-ARs into α_{1A}- and α_{1B}-ARs. These functional studies, and others (Ruffolo, 1985) indicated that postjunctional responses mediated by α_1-ARs could not be explained adequately on the basis of a single population of receptors. The first clear evidence for the existence of pharmacologically distinct subtypes of α_1-ARs was presented by Morrow and Creese, (1986) who showed evidence for receptor heterogeneity in rat brain. They found that the antagonists WB4101 and phentolamine competed for [^{3}H]-prazosin labelled sites with high and low affinities, suggesting that the prazosin was labelling a heterogeneous population of receptors. The high affinity sites were termed α_{1A}, while the low affinity sites were termed α_{1B}.

Further ligand binding studies in other brain regions, vas deferens and spleen (Docherty, 1979; Han, 1987a; Gross, 1989) confirmed the previous classification of α_{1A} and α_{1B} subtypes showing high and low affinity, respectively for WB4101. From this study they also concluded that the contraction induced by the α_{1B}-AR was independent of extracellular calcium while that induced by the α_{1A}-AR relied on the opening of dihydropyridine sensitive calcium channels. However, this proposal of the source of calcium as a subtype discriminating feature did not hold for all tissues studied. A later publication demonstrated that both subtypes could stimulate IP_3 to a similar extent from cells in culture (Han et al. 1990). It has since been shown that all the cloned receptors can induce the accumulation of IP_3 (Schwinn et al. 1991; Minneman and Esbenshade, 1994; Cotecchia et al. 1995; Theroux et al. 1996).

Further to this, Han et al. (1987b), went on to use both the alkylating agent CEC and WB4101. Again they found there were high and low affinity sites in rat brain for WB4101, and that CEC seemed to inactivate only the low affinity WB4101 sites, although, quantitatively the number of sites inactivated by CEC compared to the low affinity WB4101 sites did not correlate well. Later work attributed this to an incomplete access of CEC to intracellular sites due to variations in experimental conditions (Minneman et al., 1988b).

In more recent studies, it has been recognised that the selectivity of CEC against the α_{1B}-AR may depend upon its access to α_1-AR subtypes rather than any differences in the structure of the α_{1A}- and α_{1B}-AR. In COS-7 cells expressing α_1-AR subtypes, the α_{1a}-AR seems to localise mainly intracellularly but the α_{1b}-AR localised at the cell surface such that CEC inactivated a lower proportion of α_{1a}-AR in intact cells, but nearly the same proportion as α_{1b}-AR when membrane preparations were incubated with CEC (Hirasawa et al., 1997). The α_{1d}-AR can also be inactivated by CEC. Since then, complications in the use of this site-directed alkylating agent and controversies over its selectivity in inactivating the recombinant α_1-AR subtypes (Michel et al., 1993; Hirasawa et al., 1997) have pushed it into disfavour. Such work has indicated that subtype-specific subcellular localisation may constitute a new class of pharmacological properties in addition to ligand binding (Tsujimoto et al., 1998). This point will be addressed later.

The advent of further subtype selective antagonists assisted the current proposal of α_1-AR subclassification. (+)-niguldipine and 5-methylurapidil (5-MU), were found to be selective for the α_{1A}-AR subtype (Graziadei et al. 1989; Hanft and Gross, 1989; Gross et al. 1988), with 5-methylurapidil having an $\approx$70 fold selectivity for the α_{1A}-AR subtype as opposed to only a 20-30 fold selectivity

exhibited by WB4101 in radioligand binding experiments (Hanft and Gross, 1989).

Molecular studies

This classification however, was complicated by the cloning of the cDNAs or genes for the three distinct α_1-AR subtypes, encoding at least one and possibly two subtypes not recognised previously on the basis of pharmacological or radioligand binding studies. Due to a limited number of subtype-selective antagonists, there was some confusion as to the identity of the cloned receptors in relation to their native counterparts.

The first of these receptors to be cloned was the hamster α_{1b}-AR (Cotecchia et al., 1988), isolated from DDT_1 MF-2 hamster smooth muscle cells. Expression and pharmacological analysis of this construct in COS-7 cells showed low affinity for WB4101 and phentolamine, and proved to be associated with the accumulation of IP_3. These findings combined with functional and binding data from the native α_{1B}-AR, indicated that the cloned receptor represented the hamster α_{1B}-AR. A second receptor, which was identified soon after, was derived from a bovine brain cDNA library utilising a probe derived from the hamster α_{1b}-AR sequence (Schwinn et al., 1990). Analysis of this clone showed somewhat different results to that found with the α_{1b}-AR subtype. It had 10-fold higher affinity for the antagonist WB4101 and phentolamine, together with a high affinity for the agonist oxymetazoline. Although this pharmacological profile supported the classification of this receptor as an α_{1A}-AR, the sensitivity of the receptor to CEC (previously associated with α_{1B}-AR), together with failure to detect mRNA expression in rat tissues with binding profiles consistent with the α_{1A}-AR, led

authors to believe that this was in fact a novel adrenoceptor, and was thereafter named, α_{1c}-AR. A third clone was identified from a rat cerebral cortex library (Lomasney et al. 1991), with a high affinity for WB4101 and a distribution in rat tissues corresponding to the native α_{1A}-AR from binding studies. It was therefore assumed that this clone represented the α_{1A}-AR. Perez et al. (1991) soon after identified another α_1-AR clone, using solution-phase library screening of a rat brain library. Based on evidence that it did not correspond to any of the criteria for the other two characterised clones, it was considered to be a novel α_1-AR and was named the α_{1d}-AR. With the exception of two codons within the sequence of the α_{1a}- and α_{1d}-AR, it was soon after believed that these were in fact encoding the same receptor, generally accepted to be the α_{1d}-AR (Minneman and Esbenshade, 1994).

A point was reached where, although the cloned α_{1b}-AR had a functionally defined counterpart, the cloned α_{1d}- and α_{1c}-AR did not, and similarly the pharmacologically defined α_{1A}-AR was without a cloned partner. However, after much deliberation, it became generally accepted that the α_{1c}- clone should be renamed the α_{1a}- and that this cloned receptor corresponded to, and encoded for, the native α_{1A}-AR (Heible et al. 1995). Originally, this possibility was dismissed because of the sensitivity of the bovine α_{1c}- clone to inactivation by CEC and failure to detect mRNA for this receptor subtype in the rat by northern blotting techniques, using a bovine sequence as the probe (Schwinn et al. 1990). However, alterations in the experimental conditions under which sensitivity to CEC was examined were found to influence results. Subsequent studies in the human, bovine (Schwinn et al. 1995) and a rat homologue (Laz et al., 1994) showed

reduced sensitivity to CEC under specified experimental conditions. The recent cloning and expression of the rat homologue of the bovine α_{1c}- clone has clearly shown that the rat α_{1c}-AR has the expected pharmacological characteristics of the pharmacologically defined α_{1A}-AR. For example, Forray et al. (1994a) showed that the rat homologue exhibited higher sensitivity to the α_{1A}-AR selective antagonist (+)-niguldipine compared to the bovine α_{1c}-clone. Also with the arrival of more sensitive technology in this field, namely RNAse protection assays and *in situ* hybridisation studies, mRNA for the α_{1c}-AR clone was thereafter found more widely distributed in tissues of the rat, compared to previous studies (Price et al.1993, 1994a). Functional correlations were made thereafter by several groups showing that in fact the α_{1c}-AR clone represented the native α_{1A}-AR (Blue et al., 1995; Testa et al., 1995; Ford et al., 1994; Faure et al., 1994b).

Following the cloning and analysis of the human receptors (Ramarao et al., 1992; Hirasawa et al., 1993; Forray et al., 1994b; Esbenshade et al., 1995; Schwinn et al., 1995; Tseng-Crank et al., 1995), and identification of more selective drugs (Goetz et al., 1995), a general consensus was reached (Ford et al., 1994; Heible et al., 1995).

Confusion in the nomenclature and pharmacological identity of the cloned α_{1d}-AR was soon resolved with the arrival of the α_{1D}-selective antagonist BMY-7378 (Goetz et al., 1995). It became apparent that the cloned α_{1d}-AR did in fact have a functional partner, and continuing studies show an indispensable role of this α_1-AR subtype in the contraction of a wide variety of vessels in different species (Piascik et al., 1995, 1997; Kenny et al., 1995; Testa et al., 1995; Buckner et al., 1996; Deng et al., 1996; Saussy et al., 1996; Villalobos-Molina et al., 1996, 1997; Ibarra et al., 1997; Hrometz et al., 1999).

Eventually, the uncertainty surrounding the subclassification of α_1-ARs was eliminated, and the IUPHAR Committee (Heible et al., 1995) recommended the following nomenclature. The native α_{1A}-AR corresponds to the cloned α_{1a}-AR, which is located on human chromosome 8 and was previously designated as α_{1c}-AR or $\alpha_{1A/c}$-AR. The native α_{1B}-AR corresponds to the cloned α_{1b}-AR, which is located on human chromosome 5 and was always designated as α_{1b}-AR. The native α_{1D}-AR corresponds to the cloned α_{1d}-AR, which is located on human chromosome 20 and was previously designated as α_{1a}-AR, α_{1d}-AR or $\alpha_{1a/d}$-AR. Subscripts in capital and lower case designate pharmacologically defined and cloned receptors, respectively. The genes and/or cDNAs encoding the three subtypes have been cloned in rats and humans (Heible et al., 1995), and species homologs for some subtypes have additionally been cloned in hamsters, bovine, rabbits and mice (Cotecchia et al., 1998; Schwinn et al., 1990; Alonso-Llamazares et al., 1995; Miyamoto et al., 1997; Suzuki et al., 1997; Xiao et al., 1998).

α_{1L}-adrenoceptor pharmacology

There is functional evidence for further heterogeneity, particularly for a subtype with a low affinity for prazosin (Muramatsu et al., 1990; García-Sáinz et al., 1992a; Heible et al., 1995; Ford et al., 1996; Leonardi et al., 1997; Smith et al., 1997; Shannon Kava et al., 1998; Hirai et al., 2001). However, to date, these putative additional subtypes have resisted identification by biochemical and/or molecular techniques. Therefore, the possibility remains that additional adrenoceptor subtypes may exist. This variability led to the proposal for division of the class into α_{1H}-AR and α_{1L}-AR, based on sensitivity to prazosin (Flavhan and Vanhoutte, 1986; Muramatsu et al., 1990). When the recombinant α_1-ARs

were identified, it was found that all three; α_{1a}-AR, α_{1b}-AR and α_{1d}-AR subtypes had 'α_{1H}' characteristics, based on radioligand binding assays with transfected cells. Antagonists were identified, such as RS17053 (Ford et al., 1996), which had high potency and selectivity for the α_{1A}-AR subtype, but about 100-fold lower potency in functional assays reflecting 'α_{1L}' characteristics. Other antagonists appear to show the opposite selectivity pattern (Heible and Ruffolo, 1996). Based on functional assays using cells transfected with the recombinant α_{1a}-AR, and on the effect of receptor environment on antagonist affinity, it has been proposed that the α_{1L}-AR represents an affinity state of the α_{1a}-AR (Williams et al., 1996; Ford et al., 1997). This may explain why efforts to clone a discrete protein having α_{1L}-AR characteristics have been unsuccessful.

Splice variants

Three splice variants of the human α_{1A}-AR have been cloned which differ in length and sequence of their C-terminal domains (Hiraswa et al., 1995), and additional splice variants and truncated products have been reported (Chang et al., 1998). However, no pharmacological or signalling differences are observed on expression of these different splice variants. The truncated products do not bind radioligands, and their biological significance is still obscure (Chang et al., 1998).

To date, four isoforms of the human α_{1a}-AR have been identified. These have been named $\alpha_{1a\text{-}1}$-, $\alpha_{1a\text{-}2}$-, $\alpha_{1a\text{-}3}$-, $\alpha_{1a\text{-}4}$- and they all diverge in sequence at their carboxy termini (Chang et al., 1998; Hirasawa et al., 1995). It has been shown that when all four isoforms are expressed in cell lines and IP_3 accumulation measured, all the isoforms display α_{1L}-AR pharmacology (Ford et al., 1997; Chang et al.,

1998). Interestingly, a recent study indicated that human-cloned α_{1a}-AR isoforms display α_{1L}-AR pharmacology in functional studies (Daniels et al., 1999).

The α_{1L}-AR is mainly associated with mediating NA-induced contraction of the lower urinary tract of man (Muramatsu et al., 1994, 1995; Ford et al., 1996) and animals, for example rabbit (Leonardi et al., 1997; Shannon Kava et al., 1998; Deplanne and Galzin, 1996). In addition, a pharmacological profile consistent with this subtype has been reported in a variety of blood vessels (Muramatsu et al., 1990, 1995; Smith et al., 1997; Van der Graaf et al., 1996a).

Multiple subtypes

Why are there so many α_1-AR subtypes? Emerging evidence suggests that different subtypes and splice variants have different patterns of tissue expression, coupling efficacy and regulation. Knowledge on the functional relevance of subtypes and splice variants is limited, and even less is known about the physiological significance of the truncated forms of α_{1A}-ARs. There is evidence that for other GPCRs, co-expression of a functional 'full' receptor and a truncated isoform may result in a decreased response of the system i.e., the ability of a 'full' receptor to generate a response is negatively modulated by the truncated isoform (Okuda-Ashitaka et al., 1996; Schöneberg et al., 1995; Le Gouill et al., 1999). It is tempting to speculate whether expression of such truncated receptors play a role in a physiological context, modulating cell responsiveness.

Species Differences in Tissue Distribution

α_1-ARs are present in many tissues and play important roles in the maintenance of homeostasis. These receptors participate in many essential physiological

functions, such as sympathetic neurotransmission, myocardial inotrophy and chronotrophy, modulation of hepatic metabolism, uterine contraction, regulation of water and electrolyte metabolism, modulation of vascular tone, and contraction of smooth muscle in the genitourinary system. It has been shown that the genes for the three subtypes of α_1-AR are expressed in discrete, tissue-specific patterns in the same organism (Price et al., 1994a). Knowledge of α_1-AR subtypes that participate in a given function has essential physiological importance and could allow for specific therapeutic intervention.

In the liver, α_1-ARs have numerous roles; modulate glycogenolysis, gluconeogenesis, and synthesis of urea, fatty acid metabolism, to name but a few. It is interesting that one α_1-AR subtype seems responsible for all these actions within the one species, yet great variation is recognised between species: rabbit (García-Sáinz et al., 1992b), dogs (García-Sáinz et al., 1995c), cats (García-Sáinz et al., 1996a) and humans (García-Sáinz et al., 1995d) mainly express α_{1A}-ARs whereas expression of the α_{1B} subtype has been detected in the livers of a fish (*Ichtalurus punctatus*) (García-Sáinz et al., 1995b), and rodents such as rats, mice and hamsters (García-Sáinz et al., 1994). Coexpression of α_{1A} and α_{1B}-ARs has been observed in the liver of monkeys (García-Sáinz et al., 1996b).

It is tempting to speculate that the differences observed between subtypes may be due to experimental age differences between species. Evidence does exist to suggest that α_1-AR subtype modulation can occur with age (Gurdal et al., 1995a, b; Xu et al., 1997; Ibarra et al., 1997; Rudner et al., 1999; Shen et al., 2000; Yamamoto et al., 2001). This will be discussed in chapter 2.

Although this principle also applies to many other organs and tissues, it is at present best exemplified in the liver. Therefore caution should be made when

extrapolating data from non-human models, as the information may be misleading. Human liver mainly expresses α_{1A}-ARs at both mRNA and receptor protein levels (García-Sáinz et al., 1995d; Price et al., 1994b). The four splice variants have been detected with marked predominance of the $\alpha_{1A\text{-}1}$-AR isoform at the mRNA level (Chang et al., 1998). Similarly, α_{1A}-AR mRNA predominates in the following human tissues: heart (Price et al., 1994b) (splice variants $\alpha_{1A\text{-}4}$ and $\alpha_{1A\text{-}1}$); cerebellum (Price et al., 1994b); cerebral cortex (Price et al., 1994b), and prostate (Price et al., 1994b; Forray et al., 1994b; Faure et al., 1994b) ($\alpha_{1A\text{-}4}$ isoform) (Chang et al., 1998). α_{1B}-AR mRNA is present in high concentrations in human spleen and kidney, while α_{1D}-AR mRNA predominates in human aorta (Price et al., 1994b). However, mRNA levels do not necessarily correlate with receptor protein levels and also functional response. Species variations and these limitations make it necessary to study all these aspects in human tissue. It is obvious that information regarding functional roles, tissue distribution, and density of the different α_1-AR subtypes is far from complete in the normal healthy human being. Even less is known about all these aspects under conditions such as growth or ageing and in different disease states.

Subtype selective ligands: current status

Increasing effort is currently being put into the design of subtype-selective compounds in order to achieve tissue or organ selective α_1-AR blockade. Although a number of antagonists selective for the native and cloned $\alpha_{1A/a}$-AR and $\alpha_{1D/d}$-ARs are now available, e.g., WB4101, (+)-niguldipine, 5-MU, tamsulosin, Rec 15/2739, RS-17053, KMD-3213, Ro 70-0004 and RS-100329,

B8805-033 and L-771,688 (SNAP 6368) for the α_{1A}-AR subtype (Han et al., 1987b; Gross et al., 1988; Boer et al., 1989; Forray et al., 1994b; Eltze et al., 1996; Ford et al., 1996; Testa et al., 1997; Williams et al., 1999; Murata et al., 1999; Chang et al., 2000), or BMY-7378, MDL 73005EF and cystazosin for the α_{1D}-AR subtype (Goetz et al., 1995; Saussy et al., 1996; Bolognesi et al., 1998), the lack of antagonists sufficiently selective for the α_{1B}-AR is a persistent problem in α_1-AR characterisation. CEC was originally identified as an α_{1B}-AR selective alkylating agent (Han et al., 1987b), however, complications in the use of site-directed alkylating agents and controversies over its selectivity in inactivating the recombinant α_1-AR subtypes (Michel et al., 1993; Hirasawa et al., 1997) has omitted its use in several laboratories. Presently, spiperone is the only antagonist known for retaining a moderate selectivity (approximately 5-fold) for native or cloned $\alpha_{1B/b}$-AR over α_{1A}-ARs both in radioligand binding and functional experiments, whereas its selectivity for α_{1B}-AR over α_{1D}-AR is at the most, by a factor of three (Michel et al., 1989; Blue et al., 1995; Kenny et al., 1995; Schwinn et al., 1995; Eltze et al., 1999). The presumed α_{1B}-AR selectivity of other antagonists, e.g., risperidone (Sleight et al., 1993) and cyclazosin or its (+)-entantiomer (Giardiná et al., 1995, 1996), initially found in binding studies, however, could not be confirmed in subsequent functional experiments (Eltze, 1996; Stam et al., 1998). In contrast, the quinazoline compound, L-765,314 (Patane et al., 1998), has been shown to be α_{1B}-AR selective both in binding and functional experiments (Chang et al., 1998). Recently, a novel α_{1B}-AR selective compound with no direct chemical resemblance to other α_1-AR antagonists, AH11110A, has been characterised through binding experiments with native or cloned α_1-AR subtypes in different animal tissues (rat, hamster and bovine) and

human tissues, displaying an approximately 10- and 20-fold selectivity to α_{1B} relative to α_{1A} and α_{1D}-ARs, respectively, and an affinity rank order at these subtypes of $\alpha_{1B}>\alpha_{1A}>\alpha_{1D}$ (King et al., 1994; Giardiná et al., 1996; Saussy et al., 1996). Presently, AH11110A is listed as the only recommended selective antagonist for this subtype (see Alexander and Peters, 2000), but it also has functional affinity at α_{1D}-ARs in rat aorta (King et al., 1994). Further proof of its general utility has recently been quashed by a report by Eltze et al. (2001), who claim that this compound also interacts with α_2-ARs, and thus may be unsuitable for α_1-AR subtype characterisation, at least in functional studies involving smooth muscle (Eltze et al., 2001).

Development of α_{1A}-AR selective antagonists has been the most successful. The first α_{1A}-AR selective antagonist to be identified was WB4101 (Morrow and Creese, 1986), which has about 20-fold higher affinity for the α_{1A}-AR than α_{1B}-AR subtype. This limited selectivity, combined with an intermediate affinity for the α_{1D}-AR subtype, has limited its usefulness in differentiating between subtypes. Subsequently, 5-MU (Gross et al., 1988) and (+)-niguldipine (Boer et al., 1989) were identified as α_{1A}-AR selective antagonists. These drugs had a greater selectivity between α_{1A}-AR and α_{1B}-ARs (80-500 fold) and a low affinity for the α_{1D}-AR subtype, making them useful for distinguishing subtypes in radioligand binding assays (Han and Minneman, 1991). However, each of these drugs has other properties that limited their use in functional experiments. 5-MU is a partial agonist at serotonin 5-HT_{1A} receptors, while (+)-niguldipine is a dihydropyridine Ca^{++} channel antagonist. A derivative of (+)-niguldipine, SNAP 5089 was developed which is highly α_{1A}-AR selective but has a much lower

affinity for voltage-gated Ca^{++} channels (WetZel et al., 1995). Interest in this area has been strong, since α_{1A}-AR selective antagonists may have significant therapeutic advantages in treatment of BPH (Nagrathnam et al., 1998). The moderately α_{1A}-AR selective antagonist tamsulosin has been introduced for this purpose (reviewed in Hieble and Ruffolo, 1996).

The partial serotonin 5-HT_{1A} receptor agonist BMY-7378 has been shown to be a selective antagonist at the α_{1D}-AR subtype, with about 100-fold higher affinity for the α_{1D}-AR than the α_{1A} or α_{1B}-AR subtype (Goetz et al., 1995). This drug has proven useful in clarifying functional roles for the α_{1D}-AR subtype (Piascik et al., 1995). Buspirone, a close structural analogue of BMY-7378, has recently been reported as another compound able to functionally discriminate α_{1D}-AR from the other known α_1-AR subtypes in various tissues (Eltze et al., 1999).

There is a clear need for additional selective agonists and antagonists, particularly for the α_{1B}-AR and α_{1D}-AR subtypes. Such compounds would be very useful in allowing quantitative evaluation of the distribution of subtypes by radioligand binding assays, and in defining their functional roles. However, the lack of clearly defined potential therapeutic applications for such compounds reduces the likelihood of their identification.

Structure-function relationship of the α_1-AR subtypes

α_1-ARs belong to the superfamily of GPCRs. All GPCR sequences share the presence of seven hydrophobic regions that are believed to form a bundle of α-helical transmembrane domains (TMDs), connected by alternating intracellular and extracellular loops (Khorana et al., 1992). Mutational analysis of several GPCRs has revealed that the TMDs contribute to the formation of the ligand-

binding pocket, whereas the amino acid sequences of the intracellular loops seem to mediate receptor-G protein coupling (Khorana et al., 1992; Savarese et al., 1992). Agonist binding to a GPCR is believed to induce a conformational change in the receptor that can result in its productive coupling to G proteins, thus leading to intracellular events. In spite of site-directed mutagenesis and biophysical studies on different GPCRs, which have provided many insights into the structure-function relationships of these proteins, a consistent structural description of the molecular changes underlying the process of receptor activation is still lacking.

The TMDs of the three α_1-ARs show 65-75% of amino acid identity when compared among each other. Potential sites of phosphorylation by protein kinase C (PKC) (Houslay et al., 1991) and protein kinase A (PKA) (Clark et al., 1988) are present in the intracellular domains of all three receptor subtypes, suggesting that protein phosphorylation might play a role in receptor regulation. Such phosphorylation events and their potential regulatory purpose will be discussed later.

Structural determinants of selectivity

Several studies have focused on the molecular interactions of the endogenous catecholamines, AD and NA, with different AR subtypes. The majority of work done on α_1-ARs has focused on the α_{1b}-AR subtype which is thought to be prototypic of this group of receptors. However, the ligand-binding pocket must be distinct in each of the different receptor subtypes, since they can discriminate between a wide variety of synthetic agonists and antagonists (Hwa et al., 1995). Using oxymetazoline, cirazoline and methoxamine as α_{1A}-AR selective agonists, Hwa et al. (1995) used site-directed mutagenesis to identify critical residues in

α_{1a}-AR and α_{1b}-ARs responsible for apparent differences in agonist binding potencies. They found that conversion of Ala^{204} to valine in the TMD V and Leu^{314} to methionine in the TMD VI of the α_{1b}-AR subtype increased the affinity of these selective agonists until they were similar to their affinities for the α_{1a}-AR subtype. The reciprocal double mutant in the α_{1a}-AR decreased the affinities of these agonists so that they were similar to the α_{1b}-AR. Hwa et al. (1995) developed a model suggesting that these two residues are critically involved in subtype-selective agonist binding, and may interact structurally within the receptors. Similar studies with antagonists and the use of α_{1a}/α_{1b}-AR chimeras allowed identification of residues involved in subtype-selective antagonist binding (Zhao et al., 1996). This study suggested that TMD VI and a portion of the second extracellular loop are critically important in subtype-selective antagonist binding. In particular, three adjacent residues located on the extracellular loop of TMD V appeared to be fully responsible for the higher antagonist affinity for α_{1A}-ARs. This suggests that α_1-AR antagonists may bind near the surface of the receptor, rather than deep within the TMDs, like agonists. Involvement of TMD II in specifying the selectivity of niguldipine derivatives between the α_{1A}-AR and α_{1D}-AR subtypes was also reported by Hamaguchi et al. (1996). Ongoing studies in Cotecchia's laboratory and others, are investigating the interaction pattern of different antagonists at α_1-AR subtypes. Other than these studies, little is known about the molecular determinants of the pharmacological differences between the α_1-AR subtypes. Additional information would clearly be useful in helping to predict structures of additional subtype-selective drugs.

Consequences of agonist activation of α_1-ARs

Agonist activation of a GPCR not only results in the G protein-dependent activation of effector systems, but also sets in place a series of molecular interactions that allow for 1) feedback regulation of G protein coupling, 2) receptor endocytosis, and 3) signalling through G protein-independent signal transduction pathways (Lefkowitz, 1993; Ferguson et al., 1996; Ferguson and Caron, 1998; Krunpnick and Benovic, 1998; Hall et al., 1999; Luttrell et al., 1999; Schöneberg et al., 1999). Such work has intimated that the functional activity of GPCRs extends far beyond the traditional model of: receptor→ G protein→ effector.

GPCR activity represents a coordinated balance between molecular mechanisms governing receptor signalling, desensitisation, and resensitisation. Receptor desensitisation, the waning of GPCR responsiveness to agonist with time, represents an important physiological 'feedback' mechanism that protects against both acute and chronic receptor over-stimulation.

Three families of regulatory molecules are known to contribute to the GPCR desensitisation process: second-messenger-dependent protein kinases, G protein-coupled receptor kinases (GRKs) and arrestins (reviewed by Lefkowitz, 1993; Ferguson et al., 1996; Ferguson and Caron, 1998; Krupnick and Benovic, 1998). It is now recognised that the same regulatory molecules that contribute to agonist-stimulated receptor desensitisation (GRKs and β-arrestins), initiate and regulate GPCR endocytosis, intracellular trafficking, and resensitisation (e.g., Tsuga et al., 1994; Ferguson et al., 1995, 1996; Zhang et al., 1997; Oakley et al., 1999).

In addition to signalling via heterotrimeric G proteins, it is now recognised that GPCRs act as scaffolds promoting the formation and compartmentalisation of G

protein-independent signal transduction complexes. A growing number of proteins have been identified that bind GPCRs and either couple GPCRs to G protein-independent signal transduction pathways or alter G protein specificity and agonist selectivity (reviewed by Hall et al., 1999).

GPCR-mediated signal transduction can be attenuated (desensitisation) with relatively fast kinetics (within seconds to minutes after agonist-induced activation). This process is characterised by functional uncoupling of receptors from heterotrimeric G proteins, which occurs without any detectable change in the total number of receptors present in cells or tissues (Clark, 1986). A highly conserved mechanism of rapid desensitisation involves ligand-dependent phosphorylation of receptors by second messenger-dependent protein kinases namely PKA and PKC, GPCR kinases (GRKs) (Benovic et al., 1990) and some receptors with tyrosine kinase activity (Hadcock et al., 1992). GRK-mediated receptor phosphorylation promotes the binding of β-arrestins, which disrupt the interaction between receptors and G proteins (Lefkowitz et al., 1998a). This rapid process, for certain GPCRs is associated with a process called sequestration, which involves a physical redistribution of receptors from the plasma membrane to intracellular membranes (e.g. Fonseca et al., 1995). This process of GPCR internalisation is thought to promote de-phosphorylation of receptors by bringing them near to an endosome-associated phosphatase. De-phosphorylation and subsequent recycling of receptors back to the plasma membrane contributes to a reversal of the desensitised state (resensitisation), which is required for full recovery of cellular signaling potential following agonist withdrawal (Lefkowitz et al., 1998b).

Differential regulation of α_1-AR subtypes; functional significance

Differential regulation within a family of receptors is frequently associated with the susceptibility of members to be modified by phosphorylation. Subtypes of α_2-AR and β-ARs seem to be subject to desensitisation according to their susceptibility as kinase substrates (Liggett, 1993, 1998; Kurose and Lefkowitz, 1994). Limited information is available about the phosphorylation of α_1-AR subtypes.

Two main types of desensitisation of GPCRs are now recognised, namely homologous (reduced response in agonist-activated receptor only), and heterologous (reduced response in agonist-activated receptor, other receptors or distal signalling components unrelated to the original stimulus).

Regulation of adrenoceptors by phosphorylation, and functional consequences, have been studied extensively with the β_2-AR. With view to the three known α_1-AR subtypes, namely α_{1a}, α_{1b} and α_{1d}-AR, information has been limited, most of which is based on data from the hamster α_{1b}-AR subtype. However, recent information concerning the phosphorylation state of the other two subtypes has important functional regulatory implications for this group of receptors (Vázquez-Prado et al., 2000; García-Sáinz et al., 2001). One of the important physiological consequences of this regulatory feature is its contribution to changes in the subcellular localisation of α_1-AR subtypes and cellular responsiveness to their activation, (McCune et al., 2000). Therefore, I have discussed what is currently known about phosphorylation of α_1-ARs in some detail.

α_{1b}-AR homologous desensitisation

Leeb-Lunderberg et al. (1987) first observed attenuation of agonist-induced α_{1B}-AR response in hamster DDT_1 MF-2 cells. This desensitisation of receptor response was accompanied by a marked decrease in the number of surface receptors, termed internalisation (Leeb-Lundberg et al., 1987). Several groups have demonstrated that agonist stimulation markedly increases receptor phosphorylation using the hamster α_{1b}-AR transfected into rat-1 fibroblasts (Lattion et al., 1994; Diviani et al., 1996, 1997; Vázquez-Prado et al., 1997). Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitisation, inferring a critical role of this domain in the regulatory function of this α_1-AR subtype (Lattion et al., 1994). The phosphorylation events involved are understood to be instrumented by G protein receptor kinases (GRKs) 2 and 3 (Diviani et al., 1996) and the phosphorylation sites involved in this GRK-mediated desensitisation of α_{1b}-ARs located in the carboxyl tail are Ser^{404}, Ser^{408}, and Ser^{410} (Diviani et al., 1997). In addition to this, studies have shown that different receptors may be under different levels of control by these protein kinases (Iacovelli et al., 1999). Second messenger-dependent PKC is believed to have no influence on agonist-dependent phosphorylation of the hamster α_{1b}-AR (Lattion et al., 1994).

Interestingly, there are great differences in agonist-induced internalisation of α_{1b}-ARs, probably depending on the cell type. Thus, in human embryonic kidney 293 (HEK 293) cells, stably expressing hamster α_{1b}-ARs, it was observed that NA induced a rapid and striking internalisation of cell surface receptors (Fonseca et al., 1995). In contrast, the agonist-stimulated α_{1b}-AR internalisation that is observed in rat-1 fibroblasts is much slower and involves only a relatively small

proportion of the total surface receptors (Lattion et al., 1994; Vázquez-Prado et al., 1997). In DDT_1 MF-2 cells, agonists induce ~30% α_{1B}-AR internalisation in 30minutes (Leeb-Lundberg et al., 1987; Cowlen and Toews, 1988). It has been observed that surface-sorting and agonist-promoted internalisation of α_{1B}-ARs in this cell type are two independent processes involving different components of the cellular endocytic machinery. Basal surface-sorting was sensitive to breveldin A, an inhibitor of vesicular transport, and that agonist-promoted internalisation was sensitive to actin depolymerisation agents such as cytochalasin D and mycaloide B (Hirasawa et al., 1998). Real-time optical monitoring of agonist-mediated internalisation of α_{1b}-ARs tagged with GFP, in mouse αT3 cells, indicated that the receptor internalised and redistributed in intracellular compartments, was within minutes of exposure to agonist (Awaji et al., 1998). Although PKC does not appear to play a role in the agonist-mediated phosphorylation of α_{1b}-ARs (Lattion et al., 1994), it seems to do so in receptor internalisation (Fonseca et al., 1995; Awaji et al., 1998). Using constitutively active α_{1b}-AR mutants, it has been observed that when different receptor domains are mutated, divergent effects on receptor phosphorylation and internalisation take place. Thus agonist-independent activity of α_{1b}-AR mutants does not necessarily correlate with any of the parameters (Mhaouty-Kodja et al., 1999). Recently, Wang et al. (2000) have identified distinct carboxyl-terminal domains that mediate the internalisation and down-regulation of the hamster α_{1b}-AR. They have generated and characterised receptor constructs that should prove useful for elucidating in greater detail the molecular mechanisms regulating both the expression and the localisation of α_{1b}-ARs (Wang et al., 2000).

Expression of arrestin proteins attenuated α_{1b}-AR-mediated PIP_2 hydrolysis and a dominant negative arrestin mutant inhibited adrenoceptor internalisation, in agreement with what has been observed with other GPCRs (Diviani et al., 1996; Mhaouty-Kodja et al., 1999).

All of these studies have been done using the hamster α_{1B}-AR endogenously expressed in DDT_1 MF-2 cells or transfected cell models. García-Sáinz et al. (1999a) have recently observed agonist-mediated phosphorylation of the human α_{1b}-AR.

Heterologous desensitisation

It has been observed that many GPCRs are desensitised via feedback regulation by second-messenger-stimulated kinases, such as PKA and PKC. This type of desensitisation is classed as heterologous, since any activator that can increase cAMP or DAG has the potential to induce the phosphorylation and desensitisation of any GPCR containing the consensus phosphorylation sites for PKA and PKC. Other signalling proteins containing such consensus sites can also be phosphorylated, and, as a consequence, their function can be modified. Similarly, a single receptor type can be phosphorylated and desensitised by multiple protein kinases (Nambi et al., 1985). In addition to second-messenger-activated kinases, some receptors with endogenous protein tyrosine kinase activity have been shown to induce the phosphorylation in tyrosine residues of GPCRs, such as β_2-ARs, and lead to desensitisation (Hadcock et al., 1992; Baltensperger et al., 1996; Karoor and Malbon, 1996). In the case of α_{1b}-ARs, there is evidence that PKC plays a key role in heterologous desensitisation/phosphorylation. Reported heterologous phosphorylation events involving α_{1b}-ARs are discussed later.

It has been observed *in vitro* that PKA can phosphorylate α_{1B}-ARs purified from DDT_1 MF-2 cells (Bouvier et al., 1987) on the third intracellular loop of the mouse α_{1B}-AR fused to glutathione-S-transferase (Alsonso-Llamazares et al., 1997). However, there is no evidence suggesting that such PKA-mediated phosphorylation might take place *in vivo* and less is known on its possible consequences.

α_{1b}-AR phosphorylation by phorbol esters

It was first shown using rat hepatocytes that activation of PKC with phorbol esters blocked α_{1B}-AR action (Corvera and García-Sáinz, 1984; Corvera et al., 1986; García-Sáinz et al., 1985a). This finding was confirmed by similar studies using DDT_1 MF-2 cells (Leeb-Lundberg et al., 1985). Purified α_{1B}-AR from DDT_1 MF-2 cells has been shown to be phosphorylated by PKC and this phosphorylation was increased by agonist (Bouvier et al., 1987). This phenomenon has been further explored, and investigators have observed the ability of phorbol esters to induce the phosphorylation of this adrenoceptor subtype using transfected rat-1 fibroblasts (Lattion et al., 1994; Diviani et al., 1997; Vázquez-Prado et al., 1997). Also the sites of phosphorylation involved are contained within the carboxyl-terminal (Lattion et al., 1994), and have since been identified as Ser^{394} and Ser^{400} (Diviani et al., 1997). Data also suggests the existence of another, uncharacterised site of action of PKC out with the carboxyl domain (Diviani et al., 1997). Recent studies by García-Sáinz et al. (1999a) show that the human α_{1b}-AR expressed in mouse fibroblasts is also phosphorylated in response to activation of PKC by phorbol esters. Such phosphorylation is associated with blockade of α_{1b}-AR-mediated increases in cytosolic Ca^{++} and phosphoinositide hydrolysis in whole

cells; phosphorylation is also associated with inhibition of adrenergic-stimulated [^{35}S]GTPγS binding in membranes, which suggests receptor-G protein uncoupling (García-Sáinz et al., 1999a). The actions of phorbol esters were blocked by PKC inhibitors and by overnight treatment with phorbol esters that lead to PKC down-regulation (García-Sáinz et al., 1999a). Yang et al. (1999) have studied the human α_{1b}-ARs expressed in rat-1 fibroblasts and observed that treatment for 24 hours with phorbol esters did not change receptor density or cause functional desensitisation. The action of phorbol esters on α_{1b}-ARs seems to involve several processes. It has been observed that the blockade of the receptor function is very rapid, which is consistent with receptor phosphorylation and uncoupling from G proteins. Such uncoupling has been detected as a loss in the ability of GTP and hydrolysis-resistant analogues to modulate agonist-binding affinity, i.e., conversion of the receptors to the low affinity state (Jagadeesh and deth, 1988; Beeler and Cooper, 1993) and also as a decreased α_{1B}-AR agonist-mediated [^{35}S]GTPγS binding (García-Sáinz et al., 1999a; Alcántara-Hernández et al., 2000; Vázquez-Prado et al., 2000).

When transfected into rat-1 fibroblasts, the α_1-AR subtypes are differentially regulated by activation of PKC (Vázquez-Prado et al., 1996). Activation of PKC with phorbol esters blocked the actions on α_{1b} and α_{1d}-ARs whereas those mediated through the α_{1a}-ARs were not altered (Vázquez-Prado et al., 1996).

Activation of PKC not only induces the blockade/desensitisation of α_1-ARs, but also alters the subcellular distribution of these receptors in some cells (Lynch et al., 1985; Cowlen and Toews, 1988; Jagadeesh and Deth, 1988; Beeler and Cooper, 1995; Fonseca et al., 1995). Fonseca et al. (1995) showed that PMA induces α_{1b}-AR internalisation to endosomes when stably transfected in HEK 293

cells. This effect is also induced by agonists and blocked by the PKC inhibitor, staurosporine, suggesting a physiological role of PKC in agonist-induced receptor internalisation (Fonseca et al., 1995). The lysosomotropic agent, chloroquine, which inhibits receptor recycling (Mellman et al., 1986) has been shown to have a similar action as PMA (Covera and García-Sáinz, 1984) on the inhibition of α_{1B}-AR action in hepatocytes, and the involvement of PKC seems likely (García-Sáinz et al., 1998a).

Heterologous α_{1b}-AR phosphorylation

Receptors coupled to G_q increase PIP_2 hydrolysis, generating DAG and IP_3. Such DAG can activate PKC and putatively induce α_{1b}-AR phosphorylation and desensitisation. Vázquez-Prado et al. (1997) have shown that activation of endothelin ET_A receptors endogenously expressed in rat-1 fibroblasts stably expressing transfected α_{1b}-ARs induced a marked and rapid phosphorylation and desensitisation of these receptors (Vázquez-Prado et al., 1997). Such effects were only partially blocked in the presence of either an inhibitor of protein kinases or protein tyrosine kinase, yet completely abolished in the presence of both (Vázquez-Prado et al., 1997). Analysis of the residues involved in the phosphorylation events revealed phospho-serine and phospho-threonine, but no phospho-tyrosine (Vázquez-Prado et al., 1997). Intracellular Ca^{++}seems to play a small role in the α_{1b}-AR phosphorylation induced by endothelin (García-Sáinz et al., 1999b) and is insensitive to the phosphatdylinositol-3-kinase (PI3-K) inhibitor; wortmannin (Vázquez-Prado et al., 1997). Collectively, these data suggest an indirect phosphorylation event that does not involve PKC.

In addition to endothelin, it has been observed that bradykinin can also induce α_{1b}-AR phosphorylation (Leeb-Lundberg et al., 1987; Medina et al., 1998). The receptor phosphorylation induced by bradykinin in rat-1 fibroblasts stably expressing α_{1b}-ARs was of small magnitude as compared to that induced by endothelin and no evidence of desensitisation was observed in whole cells (Medina et al., 1998).

Receptors for lysophosphatidic acid are expressed endogenously in rat-1 fibroblasts. Fibroblasts stably expressing the α_{1b}-AR undergo a rapid, intense and sustained α_{1b}-AR phosphorylation as a result of activation of LPA receptors (Casas-González et al., 2000). The effect of LPA on α_{1b}-AR was blocked by pre-treatment with pertussis toxin (Casas-González et al., 2000), the protein kinase inhibitor; staurosporine, wortmannin, but not blocked by the tyrosine kinase inhibitor; genistein. Collectively these data suggest that LPA mediates its actions on the α_{1b}-AR via G_i and subsequent phosphorylation is mediated by PKC through activation by PI3-kinase (Casas-González et al., 2000). Functional consequences of LPA treatment of rat-1 fibroblasts stably expressing α_{1b}-ARs suggest that such phosphorylation events are associated with receptor-G protein uncoupling (Casas-González et al., 2000).

The effect of EGF on the phosphorylation of the α_{1b}-AR has also been studied recently by del Carmen Medina et al. (2000). Similar observations were made to those found with LPA, indicating a similar sequence of events leading to the growth factor-induced functional uncoupling of the α_{1b}-AR (del Carmen Medina et al., 2000).

Phosphorylation of α_{1a}- and α_{1d}-ARs

Much information is known about the phosphorylation/desensitisation of the α_{1b}-AR, but only recently has any work been done on the α_{1a} and α_{1d}-ARs (Vázquez-Prado et al., 2000; García-Sáinz et al., 2001). García-Sáinz et al. (2001) have shown that rat α_{1d}-ARs, stably expressed in rat-1 fibroblasts, are phosphorylated in the basal state and that such phosphorylation is increased in response to NA and PMA. In addition, they have shown that there is cross-talk with other receptors endogenously expressed in rat-1 fibroblasts, i.e., activation of the receptors for endothelin, bradykinin and LPA induces α_{1d}-AR posphorylation (García-Sáinz et al., 2001). NA and PMA mediated phosphorylation of α_{1a}-AR stably expressed in rat-1 fibroblasts but was much less than that observed for the α_{1b}-AR (Vázquez-Prado et al., 2000). Limited effects of phorbol esters were also noted in relation to functional response on the α_{1a}-AR subtype (Vázquez-Prado et al., 2000). The role of these phosphorylations in a physiological context is as yet unknown.

Role of protein phosphatases

The phosphorylation state of a receptor results from the balance between the activities of the protein kinases and protein phosphatases that act on it. However, little is know about the role(s) of protein phosphatases in receptor phosphorylation and function. It has been suggested that endocytosis via clathrin-coated vesicles is crucial for resensitisation of some GPCRs (Zhang et al., 1997). Receptors proceed from these vesicles to endosomes where they are de-phosphorylated and resensitised by a mechanism that is proposed to involve a conformational change in the receptor brought about by acidification of the endosomal compartment

(Zhang et al., 1997). In this regard, Pitcher et al. (1995) have shown previously that a latent oligomeric form of phosphatase 2A actively de-phosphorylates the β_2-AR *in vitro* and Shih et al. (1999) have reported that phosphatase 2A and 2B are associated with β_2-ARs. It has also been observed that okadaic acid, an inhibitor of phosphatases, induces both augmentation and inhibition of β_2-AR-mediated stimulation of cAMP accumulation (Clark et al., 1993). Inhibition of the ser/thr phosphatase, calcineurin, enhances desensitisation and phosphorylation of adipocyte β_1-AR (Bahouth et al., 1996).

In the case of α_1-ARs, Alcántara-Hernández et al. (2000) have reported that a complex interplay seems to exist between PKC and phosphatases to modulate α_{1b}-AR phosphorylation and function. Their studies with phosphatase inhibitors suggest that phosphatases-1, 2A and 2B could be involved in modulating receptor phosphorylation *in vitro*. Their data also suggest that inhibition of phosphatases increases the phosphorylation state of α_{1b}-ARs, and that this effect seems to involve PKC. In spite of inducing an intense receptor phosphorylation however, okadaic acid alters α_{1b}-AR actions to a much lesser extent than the direct activation of PKC by PMA (Alcántara-Hernández et al., 2000).

Subcellular distribution and phosphorylation

It is tempting to speculate that the susceptibility to phosphorylation may play a role in determining the subcellular distribution of α_1-ARs under basal, agonist-stimulated and cross-talk-regulated conditions. This subject is addressed in detail later.

Signal Transduction

It has been known for some time that α_1-ARs are coupled to phosphoinositide turnover/calcium signalling (Fain and García-Sáinz, 1980). However, it soon became clear that this signalling pathway did not completely explain all α_1-AR actions (Morgan et al., 1984; García-Sáinz et al., 1985) and that activation of α_1-ARs stimulates multiple pathways (Morgan et al., 1984; García-Sáinz et al., 1985b; Cotecchia et al., 1990; Perez et al., 1993). Such multiple signalling seems to involve different G proteins and to depend on the repertoire of signalling molecules expressed by particular cell types (Perez et al., 1993).

Relative coupling efficiencies

The three cloned α_1-AR subtypes have been found to have different coupling efficiencies to PLC through the $G_{q/11}$ family to increase intracellular calcium, suggesting variations in intrinsic activities among receptor subtypes (Vázquez-Prado et al., 1996; Theroux et al., 1996; Chen et al., 1999; Ruan et al., 1998). In general, it has been observed that the α_{1a}-AR subtype is more effective that the α_{1b}-AR subtype, and that the α_{1d}-AR is usually the least effective of the three (Vázquez-Prado et al., 1996; Theroux et al., 1996; Chen et al., 1999; Ruan et al., 1998).

Schwinn et al. (1991) first reported that the α_{1a}-AR was more efficient than the α_{1b}-AR in activating IP_3 formation. Perez et al. (1993) studied the coupling of expressed α_{1b}-AR and α_{1d}-ARs to different signalling pathways in different cell lines. Their studies showed that both subtypes coupled to PIP_2 hydrolysis through

a PTX-insensitive G protein, although the coupling efficiencies of the two subtypes were not directly compared.

A systematic comparison of recombinant human α_1-ARs expressed at similar densities in human embryonic kidney 293 cells found that agonist-occupied α_1-AR subtypes showed substantial differences in their ability to increase both IP_3 accumulation and intracellular Ca^{++} ($\alpha_{1a}>\alpha_{1b}>\alpha_{1d}$; Theroux et al., 1996). Similar results were obtained in human SKNMC neuroepithelioma cells, where different maximal responses were obtained following activation of each subtype, both for IP_3 formation ($\alpha_{1A}>\alpha_{1B}\geq\alpha_{1D}$) and Ca^{++} release ($\alpha_{1A}>\alpha_{1B}>\alpha_{1D}$). Taguchi et al. (1998) also compared the coupling of α_1-AR subtypes to these responses, and examined activation of PKC in rat-1 fibroblasts. A similar order of coupling efficiency was observed for the three subtypes for IP_3 formation ($\alpha_{1a}\geq\alpha_{1b}>\alpha_{1d}$) and Ca^{++} ($\alpha_{1a}>\alpha_{1b}>\alpha_{1d}$) responses, although the receptors were expressed at higher and variable densities.

G protein coupling

α_1-ARs belong to the family of $G_{q/11}$-coupled GPCRs, which initiate signals by activating PLC-dependent hydrolysis of PIP_2. This enzyme generates the second messengers IP_3, which releases Ca^{++} from intracellular stores, and DAG, which synergises with Ca^{++} to activate PKC (Minneman, 1988a; Hieble et al., 1995). The $G_{q/11}$ family of G proteins contains four α subunits, α_q and α_{11} which are co-expressed in most cells, and α_{14} and α_{16} which show much more restricted distribution. Coupling of individual α_1-AR subtypes to different α subunits of the $G_{q/11}$ family has been examined by transient over-expression of both receptor and

G protein α subunits in monkey kidney COS-7 cells. These studies showed that all three α_1-AR subtypes could couple to PLC through α_q and α_{11}, only α_{1a} and α_{1b} subtypes coupled to α_{14}, and only α_{1b} coupled to α_{16} (Wu et al., 1992). Coupling of all three α_1-AR subtypes to both G_q and G_{11} has been supported by studies from Wise et al. (1995). These investigators showed that agonist treatment of rat-1 fribroblasts expressing α_{1a}, α_{1b} or α_{1d}-ARs accelerated degradation of both α_q and α_{11}. These studies suggest that individual receptors can activate multiple G protein α subunits, but also support some specificity in G protein activation. Studies utilising cells that normally express three of the four $G_{q/11}$ α subunits compared to cells with each of the three α subunits knocked out have shown that when one of the family members is removed, the remaining members can fully compensate for its absence (Xu et al., 1998). More recent studies have suggested that signalling specificity may be conferred by interaction with RGS (Regulators of G Protein Signalling) proteins (Xu et al., 1999a). The domains of the α_{1b}-AR involved in coupling to $G_{q/11}$-mediated PLC activation were first studied by Cotecchia et al. (1990). Using chimeric α_{1b}/β_2-AR constructs, these investigators demonstrated the critical importance of the third cytoplasmic loop in coupling to IP_3 formation, and the relative unimportance of the C-terminal tail. This is similar to results with many other GPCRs, and is supported by studies showing that over-expression of the third cytoplasmic loop of the α_{1b}-AR inhibits coupling of this receptor, but not other receptors, to IP_3 formation (Luttrell et al., 1993). Recombinant α_{1b}-ARs have also been reported to couple to other G proteins, particularly $G\alpha_0$ (Blitzer et al., 1993) and $G\alpha_s$ (Horie et al., 1995) following over-expression, although the functional relevance of this coupling is not yet clear. Studies with specific G

protein antibodies have supported the hypothesis that native α_{1B} (but not α_{1A} or α_{1D}) ARs can also couple to $G\alpha_0$ in rat aortic smooth muscle (Gurdal et al., 1997), suggesting a functional role for this coupling.

A high-molecular-weight class of G proteins, named G_h, has been described (Das et al., 1993; Nakaoka et al., 1994; Baek et al., 1993). These proteins seem to be multifunctional with both transglutaminase activity and signalling activities (Nakaoka et al., 1994). There is evidence that such proteins may mediate α_1-AR activation of PLC (Das et al., 1993; Nakaoka et al., 1994; Baek et al., 1993). It has been reported that activation of G_h occurs only by transfected α_{1b} and α_{1d} subtypes, and not α_{1a} (Chen et al., 1996), and is independent of its transglutaminase activity. However, the role of G_h in cellular responses to catecholamines is still obscure. Studies involving G_h/Tissue Transglutaminase II knockout mice indicate both that G_h is functionally coupled to α_1-ARs, and that other GTP-binding transglutaminases are unable to substitute for G_h in α_1-AR-mediated signalling (Nanda et al., 2001). Surprisingly, since α_{1B}-ARs have been reported to play a significant role in maintaining normal vascular tone, and hence in blood pressure homeostasis (Cavalli et al., 1997), it seems odd that there is a lack of change in either blood pressure or parameters of left ventricular function in G_h knockout mice. This would suggest either that G_h in the vasculature contributes minimally to α_{1B}-AR-mediated vasoconstrictor responses, or that in the absence of G_h, vascular tone can be maintained by compensatory signalling pathways (Nanda et al., 2001). Interestingly, cardiac-specific over-expression of G_h results in mild hypertrophy and ventricular fibrosis, as well as impaired cardiac function (Small et al., 1999), a phenotype that is consistent with that obtained by over-

expression of either the wild-type α_{1b}-AR (Akhter et al., 1997) or a constitutively active α_{1b}-AR mutant (Milano et al., 1994b).

Coupling to other second messenger systems

A variety of other signalling pathways have also been shown to be activated by α_1-ARs (Minneman, 1988). These include cAMP accumulation, Ca^{++} influx, arachidonic acid release, phospholipase D activation and mitogenic responses:

cAMP accumulation There is clear evidence that cAMP accumulates in response to α_1-AR activation (Morgan et al., 1984; Cotecchia et al., 1990; Perez et al., 1993; Nomura et al 1993; Chen et al., 1999) and that this cAMP accumulation induces phosphorylation of the cAMP response element and activates transcription (Lin et al., 1998). α_1-AR-mediated cAMP accumulation results from direct receptor activation of G_s and possibly via indirect pathways secondary to phosphoinositide turnover (Horie et al., 1995).

Ca^{++} influx The mechanisms by which Ca^{++} influx is increased following α_1-AR activation are not always completely clear. As with most $G_{q/11}$ linked receptors, activation of α_1-ARs often activated a considerable Ca^{++} influx which is not voltage-gated and not blocked by dihydropyridine Ca^{++} blockers (Minneman, 1988). In rat portal vein myocytes, for example, α_1-ARs cause Ca^{++}-activated Ca^{++} influx that replenishes intracellular Ca^{++} stores through a mechanism involving α_{11} (Macrez-Leprêtre et al., 1997). Similarly, activation of α_1-ARs in rat aorta also activates considerable Ca^{++} entry subsequent to the initial increase in intracellular Ca^{++} (Noguera et al., 1997). However, α_1-AR activation also increases dihydropyridine-sensitive Ca^{++} influx (presumably through voltage-

gated channels) in some tissues but not others. Zhang et al. (1998) showed that activation of α_1-ARs potentiated L-type Ca^{++} currents in rat ventricular myocytes in a PKC dependent manner, suggesting that such effects may be secondary to increases in intracellular Ca^{++} and activation of PKC. However, α_1-ARs may also couple directly to activation of Ca^{++} channels in some cells. In rat medullary thyroid carcinoma 6-23 cells, activation of α_{1b}-like ARs increased dihydropyridine-sensitive Ca^{++} influx in a manner independent of release of intracellular Ca^{++}, activation of PLC and/or activation of PKC (Esbenshade et al., 1994). Since this increase in intracellular Ca^{++} was blocked by removal of extracellular Ca^{++}, and occurred even in the presence of thapsigargin, it suggested that there might be a direct coupling between these receptors and voltage-dependent Ca^{++} influx.

Arachidonic Acid Other signalling pathways have also been shown to be activated by α_1-ARs. Burch et al. (1986) showed that stimulation of α_1-ARs in a rat thyroid cell line increased release of arachidonic acid (AA), which in turn mediated a NA-stimulated cell replication. Other studies have shown that stimulation of α_1-ARs can cause AA release in many cells (Kanterman et al., 1990; Blue et al., 1994), either through activation of PLA_2 (Kanterman et al., 1990; Nishio et al., 1996; Xing and Insel, 1996) or phospholipase D (Ruan et al., 1998). Some evidence suggests that such increases in AA release require extracellular Ca^{++} but are independent of increases in intracellular Ca^{++} (Kanterman et al., 1990), suggesting a role for PLA_2. Studies in rabbit aortic smooth muscle cells support the involvement of a pertussis toxin sensitive G protein (Nishio et al., 1996), while other studies provide evidence for the involvement of PKC or MAPK pathways. Perez et al, (1993) directly compared

activation of AA release by α_{1b} and α_{1d}-ARs after over-expression in two different cell lines. Their studies found that both subtypes can cause this response in both cell lines, but by different mechanisms. In COS-7 cells, AA release was mediated through PLA_2 activation, involved a PTX-sensitive G protein, and required Ca^{++} influx through dihydropyridine-sensitive channels. In CHO cells, however, slightly different mechanisms were observed. Even though CHO cells lack voltage-sensitive Ca^{++} channels, both subtypes were still able to activate AA release. Therefore α_{1b}- and α_{1d}-AR subtypes can couple to PLA_2 activation via a PTX-sensitive pathway in CHO cells, and a single α_1-AR subtype can activate AA release through different mechanisms in different cells.

More recent studies have shown that AA release occurs downstream of MAPK pathways. In Madin-Darby canine kidney cells, immunoprecipitation studies combined with specific inhibitors showed that an 85-kD cytosolic PLA_2 was activated by α_1-AR stimulation. PD 98059, a specific inhibitor of MAPK pathways, which also inhibited AD-promoted AA release, blocked this activation. Down-regulation or inhibition of PKC also blocked MAPK activation and AA release. Therefore, α_1-ARs appear to regulate AA release through phosphorylation dependent activation of PLA_2 by MAPK, subsequent to activation of PKC in these cells (Xing and Insel, 1996). Whether this represents a general mechanism for α_1-AR activation of PLA_2 it is not yet known.

PLD activation Activation of phospholipase D (PLD) also appears to be involved in the action of α_1-ARs (Ruan et al., 1998; Balboa and Insel, 1998; Llahi et al., 1992). Such activation seems to be induced by increases in calcium or PKC activation. Most evidence suggests that it is the increase in cytosolic Ca^{++} rather

than in PKC that mediates PLD activation, through, so far, poorly defined mechanisms (Balboa et al., 1998; Llahi et al., 1992). Interestingly, activation of PKA due to cAMP accumulation may modulate PLD and AA release (Ruan et al., 1998). Balboa and Insel. (1998) studied activation of PLD stimulated by AD in Madin-Darby canine kidney cells. They found that although AD stimulated PKCα and PKCε, this was not associated with activation of PLD. Furthermore, blocking PLC activation with neomycin did not significantly decrease AD-stimulated PLD activity. Chelation of extracellular Ca^{++} markedly inhibited PLD activation, suggesting a role for Ca^{++} in PLD activation by α_1-ARs. Ruan et al. (1998) studied α_1-AR-mediated stimulation of arachidonic acid release by activation of PLD in rat-1 fibroblasts. After over-expression, α_{1a}-ARs increased AA release, cAMP levels, and PLD activity. The increase in AA release was attenuated by inhibition of PLD, and by increases in cAMP caused by forskolin or addition of a non-hydrolyzable analogue of cAMP. They also showed that all three subtypes of ARs were able to activate PLD in these cells, although to different extents ($\alpha_{1a}>\alpha_{1b}>\alpha_{1d}$). Therefore, all three α_1-AR subtypes appear to be able to activate PLD, although the mechanisms may differ in different cell types.

Mitogenic responses Signalling to the nucleus has become a major area of research in recent years. It is clear that α_1-AR activation may result in mitogenesis and that, as previously mentioned, constitutively active mutants are tumorigenic (Allen et al., 1991), that is, α_1-AR genes could be considered as protooncogenes. An initial action, in response to agents that induce cell growth and proliferation, is the expression of early genes such as c-fos and c-jun expression. It has been shown that α_1-AR activation induces c-fos and c-jun expression in a large variety

of cells including cardiac myocytes (Iwaki et al., 1990), rat aorta, cultured vascular smooth muscle cells (Okazaki et al., 1994), hepatocytes (González-Espinosa and García-Sáinz, 1996), and cells transfected with different receptor subtypes (Theroux et al., 1996; García-Sáinz et al., 1998b; Hu et al., 1996). All three subtypes seem to be able to induce c-fos and c-jun expression (Chen et al., 1999; García-Sáinz et al., 1998; Hu et al., 1996). Signalling via increased intracellular calcium concentration and activation of PKC seems to participate, but important differences have been observed (Chen et al., 1999; Iwaki et al., 1990; Okazaki et al., 1994; González-Espinosa and García-Sáinz, 1996; García-Sáinz et al., 1998; Hu et al., 1996).

α_1-AR stimulated DNA synthesis and activation of MAPK involved activation of PI3-K via a PTX-sensitive G protein (Hu et al., 1996). Tyrosine phosphorylation of multiple cellular proteins has been observed (Xin et al., 1997). It is interesting that differences in the signalling pathways of α_{1a}-AR and α_{1b}-ARs have been observed involving PI3-K, $p21^{ras}$ and MAPK (Hu et al., 1999a). Neither calcium nor PKC seems to mediate α_{1a}-AR activation of MAPK in transfected PC12 cells (Berts et al., 1999).

Myocytes α_1-AR agonists stimulate hypertrophy in cultured neonatal rat ventricular myocytes (LaMorte et al., 1994), and over-expression of a constitutively active mutant of the α_{1B}-AR in cardiac myocytes induces myocardial hypertrophy in transgenic animals (Milano et al., 1994b). Other studies have shown that α_1-ARs can activate various MAPK pathways in many cells and tissues, and may play an important role in regulating cell growth (Ramirez et al., 1997; Spector et al., 1997; Alexandrov et al., 1998; Lazou et al.,

1998). Zechner et al. (1997) studied the role of α_1-AR stimulated MAPK in myocardial cell hypertrophy. Treatment of myocardial cells with the α_1-AR agonist phenylephrine induced the hallmark features of cardiac cell hypertrophy: increases in cell size, sarcomeric organization, and induction of certain cardiac-specific genes. Phenylephrine, in the presence of propranolol to block β-ARs, also stimulated the dual-phosphorylation of p38 MAPK, extracellular signal regulated protein kinase, and to a lesser extent, c-Jun-NH_2 terminal kinase. They further showed that a specific p38 inhibitor was able to block all the hypertrophic responses stimulated by phenylephrine, suggesting that α_1-ARs can affect cardiac cell hypertrophy by activation of p38 MAPKinases.

Ramirez et al. (1997) also studied the effect of α_1-AR activation on cardiac cell hypertrophy, specifically transcriptional activation of atrial natriuretic factor gene expression by α_1-AR stimulation and over-expression of Ras. Treatment with phenylephrine activated extracellular signal regulated kinases and c-Jun-NH_2 terminal kinase. However overexpression of Ras activated only c-Jun-NH_2 terminal kinase and caused little increase in extracellular signal regulated kinase activity. This study suggests that a pathway involving Ras and c-Jun-NH_2 terminal kinase is involved in α_1-AR regulated atrial natriuretic factor gene expression.

Hepatocyes Spector et al. (1997) studied the role of α_1- and β_2-AR stimulated MAPK in quiescent and regenerating rat hepatocytes. α_1-ARs activated all three arms of MAPK pathway in quiescent cells. However, p38 MAPK played a more important role than extracellular signal regulated kinases, since a specific p38 inhibitor effectively inhibited cell proliferation.

Smooth muscle cells Different subtypes of α_1-ARs may play different roles in controlling growth, differentiation, and cell fates through selective activation of one or more MAPK pathways. Chen et al. (1995) studied the regulation of vascular smooth muscle growth by AR subtypes. Although all three subtypes of ARs are expressed in these cells, only the α_{1B}-AR subtype appeared to be involved in smooth muscle cell growth. Blocking the other subtypes actually potentiated the response. These authors proposed that prolonged stimulation of α_{1B}-ARs induces hypertrophy of arterial smooth muscle cells, and other ARs attenuate this response. Studies by Xin et al. (1997) showed that NA caused rapid and transient activation of extracellular signal regulated protein kinases and increased protein synthesis and cell size. These responses were inhibited by the selective protein kinase inhibitor, PD 98059. These data combined with pharmacological analysis suggest that smooth muscle cell growth induced by NA may be mediated by α_{1D}-ARs that couple to activation of extracellular signal regulated kinase cascades (Xin et al., 1997).

PC12 cells Minneman et al. (2000) stably transfected PC12 with α_1-ARs to define pathways linking these receptors to mitogenic responses. Nerve growth factor (NGF) acts through tyrosine kinase receptors in these cells to activate MAPK pathways and cause the cells to differentiate into a neuronal phenotype. The mechanisms involved include receptor autophosphorylation, binding of Shc and Grb2, and activation of Sos, Ras, and Raf (Davis, 1993). Stimulation of α_{1a}-ARs in PC12 cells also activate MAPK pathways and causes differentiation (Williams et al., 1998). This response involves the tyrosine kinase C (Berts et al., 1999). Also, different α_1-AR subtypes activate different mitogenic responses

(Zhong and Minneman, 1999). This group directly compared the coupling of α_1-AR subtypes to mitogenic responses in PC12 cells. They expressed each subtype under control of an inducible promoter, and compared their coupling to common second messenger responses as well as their ability to activate various MAPK pathways. At similar receptor densities, coupling to IP_3 and intracellular Ca^{++} was similar to that observed in other cell lines ($\alpha_{1a}>\alpha_{1b}>\alpha_{1d}$). For mitogenic responses, the α_{1a}-AR subtype was able to strongly activate all three arms of the pathway (extracellular signal regulated kinase, c-jun-NH_4-terminal activated kinases, and p38 MAPK), the α_{1b}-AR was weaker in stimulating these responses, and the α_{1d}-AR only weakly activated extracellular signal regulated kinases.

Subsequent experiments using reporter constructs (AP1, SRE, CRE, NFκB, and NFAT) to examine transcriptional responses, have revealed a pattern of coupling efficiencies similar to that observed previously with second messenger and MAPK responses ($\alpha_{1a}\geq\alpha_{1b}>\alpha_{1d}$). Comparison of the three human subtypes showed that the α_{1a}-AR activated all five reporters, the α_{1b}-AR showed smaller effects, and the α_{1d}-AR was ineffective. α_{1a}-AR activation caused a much wider array of transcriptional responses than growth factor receptor activation. The transcriptional respones to α_{1a}-AR activation were not linearly related to second messenger generation, and could not be predicted from the known G protein coupling specificity of the receptors. As inhibitors of a variety of second messenger, MAPK, and tyrosine kinase pathways, all block differentiation in response to NE, but not NGF, it appears that α_{1a}-AR-induced differentiation requires a complex interplay of signals. This supports the hypothesis that GPCR signalling pathways are not linear cascades resulting in second messenger

formation and activation of specific targets, but must involve networks of interacting signals.

Alexandrov et al. (1998) showed that activation of p38 MAPK by α_1-ARs in rat-1 cells had a negative effect on activation of extracellular signal regulated kinases. However, the significance of this regulation and its consequences are not known.

Other studies have compared the efficiencies of the three α_1-AR subtypes in activating mitogenic responses. Siwik and Brown (1996) studied the regulation of protein synthesis by α_1-AR subtypes in cultured rabbit aortic vascular smooth cells. Competition binding studies with subtype selective antagonists showed that these cells express mainly α_{1B}-ARs (75%) relative to α_{1A}-ARs (25%). These subtypes increased protein synthesis in proportion to their relative abundance. Therefore, α_{1B}-ARs predominated in coupling to metabolic responses, in contrast to reports that contractile reponses in the same tissue are preferentially mediated by α_{1A}-ARs. Wenham et al. (1997) studied the coupling of three α_1-AR subtypes in cultured neonatal rat cardiac myocytes. Although mRNA transcripts for all subtypes were detected, binding sites for only the α_{1A}- and α_{1B}-ARs were detected. This study showed that phenylephrine-induced activation of MAPK cascades appeared to be mediated by a subtype resembling most closely the pharmacological profile of the α_{1B}-AR. These studies support the idea that mitogenic responses may be caused by multiple α_1-ARs.

Therefore, clear differences exist among the three α_1-AR subtypes in their ability to activate various mitogenic responses, although the functional significance is not yet known.

Keffel et al. (2000) have recently investigated the differential coupling of the human α_1-AR subtypes to growth promotion and inhibition in CHO cells.

Maximum elevation of intracellular calcium by the three subtypes occurred with the rank order of $\alpha_{1a}>\alpha_{1d}>\alpha_{1b}$. In contrast, activation of the ERK, JNK and p38 forms of MAPK occurred with the rank order $\alpha_{1d}>\alpha_{1a}>\alpha_{1b}$. α_{1a}-AR stimulation inhibited basal and growth factor stimulated [^{3}H]-thymidine incorporation by 74%, and this was extenuated by p38 inhibition. In contrast, α_{1d}-AR stimulation enhanced cellular growth by136%, and this was blocked by two distinct inhibitors of ERK activation. Previous work in rat-1 fibroblasts (Ruan et al., 1998; Schwinn et al., 1995; Taguchi et al., 1998) or CHO cells (Horie et al., 1995), has demonstrated that the three subtypes of α_1-ARs, activate the same signalling pathways, but are capable of doing so with different efficiencies. Keffel et al. (2000), have shown that, in terms of cell growth, when expressed in CHO cells, each subtype is capable of exerting quantitatively similar, yet opposite effects. α_{1d}-AR and α_{1a}-AR can cause stimulation and inhibition of growth, respectively. Their data has also demonstrated that a given receptor subtype, e.g., human α_{1a}-AR can cause stimulation and inhibition of ERK forms of MAPK in CHO cells and rat-1 fibroblasts, respectively, despite similar expression levels.

It is clear that α_1-ARs can activate a variety of MAPK pathways to regulate various cellular functions. The mechanisms involved in this regulation are likely to depend on the particular cellular environment and phenotype. The multiple pathways may also interact with each other in unexpected ways.

Hu et al. (1999b) have characterised phosphorylation of tyrosine proteins as an early event in the α_1-AR-induced protein kinase signalling cascade, which may be responsible for regulation of catecholamine stimulation of mitogenic effects in human vascular smooth muscle cells. Activation of tyrosine phosphorylation by α_1-ARs was independent of the activation of PKC but dependent on intracellular

Ca^{++} in these cells, suggesting that the intracellular Ca^{++} plays a critical role in α_1-AR signaling pathways not only for smooth muscle contraction but also for cell growth.

G protein independent pathways

In recent years, several reports have appeared in the literature describing various physiological consequences of receptor stimulation that do not seem to be mediated by G protein activation (reviewed in Hall et al., 1999). Novel techniques have revealed associations of such receptors with a variety of intracellular partners other than G proteins. In some cases, these partners are known receptor-interacting proteins, such as arrestins and GRKs, which were thought previously to be involved only in receptor desensitisation. In other cases, they are novel partners that were not known previously to interact with hepatahelical receptors.

No such interactions have yet been reported for α_1-AR subtypes. However, it has been reported that the consensus motif for the binding of the immediate-early gene Homer to metabotrophic glutamate receptors is found in the C-terminal tail of the α_{1d}-AR, but not the α_{1a}-AR or α_{1b}-AR (Tu et al., 1998). If such interactions occur in the α_1-AR family, it might provide additional understanding of the biological importance of the C-terminal splice variants which have been reported (Hirisawa et al., 1995; Chang et al., 1998).

***Transcriptional regulation of* $\alpha_{1B/b}$-ARs**

In order to understand the molecular mechanisms involved in the regulation of α_1-AR subtype expression, several groups have focused on control at a transcriptional level, in particular the α_{1b}-AR. Of interest is the regulation of this

subtype under a variety of physiological and pathological conditions. Therefore, the rat α_{1b}-AR gene has been cloned, and its regulatory elements identified (Gao et al., 1993, 1994). To determine potential transcriptional control elements, extensive analyses have been carried out on the untranslated region of the rat (Deng et al., 1994b; Kanasaki et al., 1994; Gao et al., 1993, 1994 and 1995) and human (Ramarao et al., 1992) α_{1b}-AR gene. The human and rat α_{1b}-AR genes share certain features in their upstream and downstream regulatory regions. The first 500bps upstream from the translation start site are 87% homologous (Kanasaki et al., 1994). Both species contain a cAMP response element (Ramarao et al., 1992; Kanasaki et al., 1994; Gao et al., 1993) and a common transcriptional start site that is thought to encode the 2.7 kb α_{1B}-AR sequence found in most tissues and in other species (Gao et al., 1995). They also share a homologous polyadenylation site (Graham et al., 1996).

However, both species also contain some notable differences and unique features. The human α_{1b}-AR gene does not contain TATA or CAAT boxes, but does contain a second putative polyadenylation site (Ramarao et al., 1992). The human α_{1b}-AR gene also possesses consensus Sp1 binding sites consistent with promoters of other housekeeping genes (Ramarao et al., 1992). In contrast, the rat α_{1b}-AR gene contains a transcriptional promoter site with putative TATA and CAAT box (Gao et al., 1995) and recognition sites for liver-specific transcriptional factors. The rat α_{1b}-AR gene also contains AP-1 and AP-2 binding sites (Gao et al., 1993), as well as thyroid and glucocorticoid response elements (Gao et al., 1993).

Gao et al. have reported the most striking difference between rat and the human α_{1b}-AR. The α_{1b}-AR gene found in rat liver contains three different transcriptional

promoters, a feature not found in the human α_{1b}-AR gene (Gao et al., 1995). Gao et al. (1994) believe that the three promoters (termed P1, P2 and P3) account for the presence of three different mRNA transcripts (2.3, 2.7 and 3.3kb) found in different tissues. The 3.3-kb transcript was detected only in the liver and brain (Gao et al., 1994; McGehee et al., 1990, 1991). The 2.3-kb transcript was found in DDT_1 MF-2 hamster smooth muscle cells (Hu et al., 1993b) and in extremely low levels in the liver (Gao et al., 1993). The 2.7-kb species has been detected in various amounts in a number of rat tissues (Lomasney et al., 1991; McGhee et al., 1990, 1991), and is believed to be the most prevalent α_{1b}-AR species (McGhee et al., 1991). Gao et al. (1995) have expanded their α_{1b}-AR transcriptional characterisation with a DNase I footprint analysis of the middle transcriptional promoter (P2, which leads to the 2.7-kb message), using rat liver extracts. Three protected areas were found and termed footprints I, II and III. Two distinct DNA-binding proteins were found in the area defined by footprint I: aCP-1 trancription factor and a unique protein that Gao and colleagues labelled as an α-adrenergic receptor transcription factor (α-ARTF).

The α-ARTF was present in the brain, liver, spleen, kidney and lung, with notable absence in the heart. Expression of a pCAT reporter construct containing sections of the P2 promoter in DDT_1 MF-2 cells demonstrated that the deletion of areas containing each of the three defined footprints inhibited P2 promoter activity (Gao et al., 1994, 1995). Because the 2.7-kb transcript is found in the heart, Gao et al. postulate that its transcription may be mediated by heart-specific transcription factors, and tissue-specific differences in transcriptional regulation could account for the ability of hyperthyroidism to produce up-regulation of the α_{1B}-AR in the

heart and lung, with simultaneous downregulation of the α_{1B}-AR in the liver (Lazar-Wesley et al., 1991).

Subsequent experiments have identified the sequence specific factors that interact with the dominant P2 promoter, including NF1, CP1, AP2, and CREB (Gao et al., 1995, 1997; Chen et al., 1997). Further data have shown that NF1 and Sp1 are the major transcription factors involved in controlling the P2 promoter in liver and in DDT1 MF-2 smooth muscle cells, respectively (Chen et al., 1997). NF1 has been reported to act as a transcriptional silencer, e.g., gene for collagen (Rossi et al., 1988) and activator e.g., p53 gene (Furlong et al., 1996). This, however, is the first report for NF1 acting either as a positive or as a negative regulator for the same gene in a cell-specific manner. Further studies in rat liver have shown that a non-DNA binding protein interacts with NF1 and suggests that its activity may be modulated by cell-specific cofactors (Gao et al., 1998). Identification of these cofactors should provide researchers with the key to understanding the underlying mechanisms involved in the transcriptional activity of the α_{1B}-AR gene. Dao et al. (1998) have since further characterised the P1 promoter and identified its three sequence-specific binding sites. One of these has been identified as an SV40 core enhancer-like sequence element, which plays a positive regulatory role in P1 promoter activity. The protein binding to this core sequence is cell-type specific, which may explain the tissue-restricted expression of 2.3kb α_{1B}-AR mRNA (Dao et al., 1998).

Zuscik et al. (1999), have since cloned, sequenced and analysed a 3.4kb mouse α_{1B}-AR promoter fragment. Sequence analysis and comparison with known rat and human α_{1B}-AR promoter sequences has led these authors to hypothesise that, whereas there is substantial similarity between the three, sufficient divergence has

occurred that shows some level of species specificity. This group have also confirmed that this mouse promoter fragment is able to regulate reporter gene expression in response to the competency of a given cell to express α_{1B}-ARs. Regulation of promoter function has also been observed by cAMP and hypoxia (Zuscik et al., 1999).

The transcriptional elements for the α_{1A}-AR and α_{1D}-AR have not been so extensively characterised as those for the α_{1B}-AR subtype. Promoter region analysis by Faure et al. (1994b) revealed three different α_{1A}-AR transcripts (5.0, 3.9 and 3.0-kb) and two α_{1D}-AR transcripts (3.0 and 1.7-kb) in the human prostate. There is evidence that the human α_{1A}-AR is alternatively spliced, with the detection of three transcripts that differ only in their C-terminal tail (Hirasawa et al., 1995). In contrast, Laz et al. (1994) detected only a 4.0-kb in the heart and kidney. A single 3.0-kb fragment for the α_{1A}-AR has been detected in various tissues studied, with the exception of the prostate (Lomasney et al., 1991).

Hamster DDT_1 MF-2, rat and rabbit smooth muscle cells have also been extensively used to study the transcriptional regulation of the α_1-ARs. Treatment of these smooth muscle cell lines with various hormones has been shown to upregulate the levels of the α_{1B}-AR and α_{1D}-AR. In DDT_1 MF-2 hamster vas deferens smooth muscle cells, glucocorticoids increase the levels of the α_{1B}-AR with no change in mRNA stability (Sakaue et al., 1991). Other hormones that increase α_{1B}-AR mRNA in DDT_1 MF-2 cells are testosterone and aldosterone, but not β-estradiol or progesterone (Sakaue et al., 1991). Hu et al. (1993) found that 4h treatment with the protein synthesis inhibitor cyclohexamide could induce up-regulation of α_{1B}-AR mRNA in DDT_1 MF-2 cells. Up-regulation occurred even

with the addition of puromycin, another inhibitor of protein synthesis. This provided evidence that cyclohexamide exerts its actions by binding directly to transcriptional elements rather than by an indirect mechanism involving protein synthesis (Hu et al., 1993a). In contrast, Bouvier et al. (1987) and Cowlen et al. (1987, 1988) have reported that short-term treatment (minutes) of DDT_1 MF-2 cells with cyclohexamide causes down-regulation of the α_{1B}-AR. Increasing cAMP levels with forskolin or the cAMP analogue CPT cAMP has been shown to increase the abundance of α_{1B}-AR and mRNA (Morris et al., 1991). Also, these treatments did not change the stability of the mRNA for α_{1B}-AR indicating an effect on gene expression (Morris et al., 1991). Interestingly, the same study showed that the activation of β_2-AR also caused up-regulation of the α_{1B}-AR by a cAMP-dependent mechanism. Schachter and Wolfe (1995) have since reported similar findings. More recent work by Gao et al. (1997) indicates that both the CRE and the AP2 element in the P2 promoter contribute to basal as well as cAMP-induced transcription of the α_{1B}-AR in this cell type. These findings subsequently initiated investigation of α_{1B}-AR mRNA regulation in rat pineal gland, as mammalian pineal function is regulated by NE acting through α_{1B}-AR and β_1-ARs. In this study, Coon et al. (1997) observed an increase in α_{1B}-AR mRNA that was due to the activation of β-ARs. Second messenger analysis indicated that α_{1B}-AR mRNA is increased by agents that increase cAMP, including dibutyrl cAMP, cholera toxin, forskolin, or vasoactive intestinal peptide (Coon et al., 1997). These authors concluded that α_{1B}-AR mRNA can be physiologically regulated by a β-AR-dependent enhancement of cAMP. They also observed that *in vivo* and *in vitro* changes in α_{1B}-AR mRNA were not

accompanied by similar changes in α_{1B}-AR binding, indicating that turnover of α_{1B}-AR protein is significantly slower than that of α_{1B}-AR mRNA and that post-transcriptional mechanisms must play an important role in regulating α_{1B}-AR binding (Coon et al., 1997).

Hypoxia-sensitive regions of the α_{1B}-AR gene have also been identified that may confer the differential hypoxic increase in α_{1B}-AR gene transcription in aorta, but not in vena cava smooth muscle cells (Eckhart et al., 1997).

Several other reports exist in the literature showing changes in α_{1B}-AR expression due to conditions that are associated with hepatocyte de-differentiation (Bevilacqua et al., 1991; Kost et al., 1992; Kunos et al., 1987, 1995; Rossby et al., 1991). This will be discussed in more detail in chapter 2.

The role of PKC in regulating α_{1B}-AR transcription

Conflicting information about the role of PKC activity on gene transcription has recently been found in different animal species. Using the PKC inhibitors H7 and saurosporine, Hu and colleagues (1993) showed that DDT_1 MF-2 cells utilise PKC to up-regulate the α_{1B}-AR. This upregulation was not dependent on activation of calcium channels. Hu et al. (1993b) also noted that PMA induced up-regulation of α_{1B}-AR mRNA transcription but did not alter mRNA stability, ruling out post-transcriptional regulatory mechanisms in the expression of the α_{1B}-AR. In contrast to these data, Izzo et al. (1994) have shown that activation of PKC with NA in cultured rabbit aortic smooth muscle cells decreased α_{1B}-AR mRNA and α_{1B}-AR receptor levels. The α_{1B}-AR transcription rate increased slightly, but this was outweighed by the increased degradation of the α_{1B}-AR mRNA (Izzo et al.,

1994). One explanation for the opposite effects found in the hamster and rabbit may be the expression of two isoforms of PKC or the expression of different PKC-dependent proteins (Izzo et al., 1994).

Yang et al. (1998b) have demonstrated that MDCK cell α_{1B}-ARs can be down-regulated by phenylephrine and is accompanied by activation of PKC. However, PKC activation does not appear to mediate this agonist-induced down-regulation.

Zhu et al. (1996) reported that exposure of a particular clone of CHO cells stably expressing the hamster α_{1b}-AR, to adrenaline for 24 hours led to an up-regulation of α_{1b}-ARs rather than an expected down-regulation. In further studies, the same group have found that this up-regulation was an atypical response to agonist treatment (Bird et al., 1997). The possibility that PKC activation might be involved was investigated. The PKC inhibitor staurosporine indicated that PKC was not involved, yet surprisingly, this compound induced up-regulation of the α_{1b}-AR. This up-regulation did not involve PKC and could be blocked by the tyrosine kinase inhibitor, genistein. In the other clones tested, agonist and staurosporine induced down-regulation of the α_{1b}-AR. These results suggest that perhaps a novel mechanism can be activated by AD and staurosporine involving tyrosine kinases in this cell type.

Vasoactive receptors appear to be able to modulate each other's expression. Treatment of rat aortic smooth muscle cells with angiotensin II has been shown to increase expression of α_1-ARs with a concomitant increase in both α_{1D}-AR and α_{1B}-AR mRNA (Hu et al., 1995). This process depended on the presence of PKC but not calcium.

TSH was shown to increase expression of the α_{1B}-AR in two rat thyroid cell lines (Kanasaki et al., 1994; Meucci et al., 1994). These effects are apparently cAMP-dependent because deletion of a cAMP response element caused the loss of cAMP sensitivity. Increased α_{1B}-AR mRNA transcription was also noted following treatment with dibutyryl cAMP or theophylline, the increased transcription of α_{1B}-AR mRNA was not accompanied by changes in α_{1B}-AR mRNA degradation (Deng et al., 1994a).

Age, development and changes in the α_1-AR subtypes

Many studies have demonstrated that the number and abundance of different α_1-AR subtypes change with age. This is exemplified in the rat liver. Studies beginning in the early 80's were the first to report such ontogenic modulation of α_1-ARs (McMillian et al., 1983). The α_{1B}-AR is found in low levels in hepatocytes isolated from foetal liver and in increasing amounts in early adulthood (Rossby et al., 1991). Van Ermen and Fraeyman, (1992) have also shown using [^{3}H]-prazosin binding to plasma membranes from livers of 2-4 day, 2-3 month and 24-26 month rats, that an increase in α_{1B}-AR receptor density occurs upon maturation and thereafter a post-maturational decline. Previous studies by Borst and Scarpace, (1990) are in agreement with such work who reported a decrease of 39% in α_1-AR density in 25 month old female rats as compared to those at 5 months. Controversy does exist however in recent work by Shen et al. (2000), using subtype selective antibodies to each of the three α_1-AR subtypes. They reported that the abundance of α_{1B}-AR immunoreactive protein was at least twofold higher in the neonatal (1 week old) than in the adult (2-3 month old) liver. In addition, they have reported that receptor protein for all three

subtypes does exist at both ages, with a slight, but not significantly different increase in α_{1A}-AR immunoreactivity in the adult liver.

Most distribution studies to date have detected only α_{1B}-AR mRNA in the rat liver, with no detection of α_{1A}-AR mRNA (Lomasney et al., 1991; Price et al., 1994a; Rokosh et al., 1994). However, Scotfield et al. (1995), using RT-PCR, detected a minor component of the α_{1A}-AR in the liver.

Rudner et al. (1999) have shown that human vascular α_1-AR subtype distribution is modulated with age (Rudner et al., 1999). Their results reveal age-related increases in mammary artery α_1-AR density (but not saphenous vein) and a switch from α_{1A}-AR predominance in younger adults to $\alpha_{1B}>\alpha_{1A}$ in older patients. In the rat heart, the content of α_{1B}-AR has been shown to decrease with age (Kimball et al., 1991). Aging affects the influence of calcium channel-blockade on potassium-induced contraction of the aorta, with old aortas (>19 months) being more sensitive than younger (2 months) vessels (Wanstall et al., 1989)

Previous studies by Gurdal et al. have demonstrated that α_1-AR function differs with age (Gurdal et al., 1995a, b). The same group used a combination of radioligand binding, Northern and Western blot analysis to show that the distribution of α_1-AR subtypes changes with age in rat aorta (Gurdal et al., 1995a, b). They demonstrated that young aorta (1 month) contains more mRNA for the α_{1B}-AR and more receptor, with a decrease in α_{1B}-AR with age (24 months) and no change in α_{1D}-AR mRNA or receptor. They also provided evidence that the abundance and functional contribution of the α_{1A}-AR may increase with age.

Other studies suggest age decreases all α_1-ARs (Xu et al., 1997). Age-related changes seem to be vessel specific, with rat renal α_{1B}-AR mRNA declining without change in mesenteric/pulmonary α_1-ARs (Xu et al., 1997). Similarly, age does not uniformly affect the expression of the three α_1-AR subtypes in different tissues (Shen et al., 2000). Studies by this group have shown that α_{1B}-AR proteins were less abundant in the heart and the brain of neonatal rat than of the adult, while the reverse was true in the liver. α_{1A}-AR and α_{1D}-AR did not change significantly with age in the heart, liver or kidney but increased in the brain. It would thus appear that in some tissues such as the heart, both α_{1A}-AR and α_{1B}-AR play an important functional role in the neonatal period while α_1-AR agonists modify cardiac functions in the adult primarily via α_{1B}-AR as previously suggested (Michel et al., 1994; Deng et al., 1996, 1998). Furthermore, age increases functional α_{1D}-ARs in resistance vessels compared with α_{1A}-AR predominance in young rats (Ibarra et al., 1997). Data from clinical studies have demonstrated less blood pressure perturbation in elderly patients with tamsulosin (α_{1A}/α_{1D} -selective antagonist) compared with alfuzosin (non-selective) (Buzelin et al., 1997), suggesting importance of α_{1B}-ARs with aging in resistance vessels of humans.

Yamaoto et al. (2001) have recently reported differences in the expression of α_1-ARs in the bladders of neonatal and adult rats. Pre-junctional, α_{1A}-ARs are functionally expressed in the bladder of both neonatal and adult rats, whereas post-junctional $\alpha_{1B/D}$-ARs are present only in older adult rats (Széll et al., 2000; Yamaoto et al., 2001b).

α_1-ARs and vascular smooth muscle

Heterogeneity of vascular α_1-ARs was recognised quite early (Flavahan and Vanhoutte 1986); however, because of the confusion in identifying pharmacologically defined α_{1D}-ARs in native tissues and the lack of a specific α_{1D}-AR antagonist until 1995, most studies attempted to define relative roles of α_{1A}-AR and α_{1B}-ARs in vasoconstrictor responses to α_1-AR agonists (Tian et al., 1990; Piascik et al., 1991; Aboud et al., 1993; Oshita et al., 1993) although data could not be fully explained by this assumption (Oriowo and Ruffolo. 1992).

The challenge of the functional studies has been to isolate the contribution of each α_1-AR subtype to contraction, because multiple subtypes may co-exist in the same blood vessel and be activated by the non-selective α_1-AR agonists used in such studies. In addition, the literature survey clearly indicates that regional differences in the distribution and density of each subtype may vary across vascular beds within the same species and across species. Another aspect of characterising vascular α_1-ARs has been to try to combine conflicting functional, receptor subtype binding and molecular data in a constructive manner to develop a complete picture of vascular α_1-AR pharmacology. Most isolated blood vessel studies have employed Schild analysis (Arunlakshana and Schild, 1959) to measure the affinity constants (pA_2) of subtype-selective antagonists for vascular α_1-ARs and correlate this with values (pKi) obtained from receptor binding studies of cloned or native α_1-ARs. Another strategy has been to assess the ability of CEC to antagonize NE- or phenylephrine-induced contractions to decide whether α_{1A}-AR (CEC-insensitive) or α_{1B}-AR and α_{1D}-AR (CEC-sensitive) mediate the vascular response (e.g., Ibarra et al., 2000). While thus far, these

strategies have been successfully used to subclassify vascular α_1-AR subtypes, the subtypes identified in some blood vessels have to be considered provisional due to contradictory reports or incomplete characterisation with a number of subtype selective antagonists, i.e. pharmacological classification of receptor subtypes has been determined by an investigator's faith in certain compounds.

Because of their importance in controlling peripheral resistance, it is of great therapeutic interest to know which subtypes are located in which vascular beds and the contribution of each subtype to sympathetically mediated vasoconstriction. However, determining their distribution and correlating this with a functional response is still problematic.

Recent reports have shown that rat arteries express the three α_1-ARs at the mRNA level (Piascik et al., 1995). However, binding and functional studies have indicated the predominance of one or two of the subtypes, and correlation between these different approaches has not been direct. This has greatly complicated the precise definition of the roles of each subtype.

Functional studies have shown that α_{1A}-ARs mediate contraction, at least in part, in a number of rat isolated blood vessels, including tail, renal, and mesenteric arteries (Villalobos-Molina et al., 1996, 1997; Piascik et al., 1997; Ibarra et al., 1997). These results have been correlated with high amounts of mRNA for α_{1A}-ARs in several of these vessels (Piascik et al., 1995), suggesting that this receptor is indeed involved in contraction. The role of α_{1B}-ARs in the contraction of vascular smooth muscle is less clear. α_{1D}-ARs are functionally expressed in arteries such as aorta, iliac, carotid, mesenteric, femoral, and renal (Piascik et al., 1995, 1997; Kenny et al., 1995; Testa et al., 1995; Buckner et al., 1996; Deng et al., 1996; Saussy et al., 1996; Villalobos-Molina et al., 1996, 1997; Ibarra et al.,

1997; Hrometz et al., 1999). Conflicting reports exist regarding the mouse aorta. Using α_{1B}-AR knockout mice, Cavalli et al. (1997) found a reduced respone to phenylephrine suggesting a role for the α_{1B}-AR subtype, whereas Daly et al. (in press), used the same knockout model, in conjunction with selective antagonists, revealing a dominance for the α_{1D}-AR subtype. Yamamoto et al. (2001a) have since confirmed this observation. Contraction in dog and rabbit aorta is reported to be via α_{1B}-AR (Murumatsu et al., 1991; Suzuki et al., 1990).

Thus far, no systematic study of the expression of the α_1-ARs and correlation to function has been done. Piascik and co-workers have used a subtype-specific antibody to show that the α_{1B}-AR can be detected in a series of peripheral blood vessels (Piascik et al., 1997). In spite of this widespread distribution, the receptor was not linked to the activation of contraction in a majority of the arteries in which it was expressed. This observation has led this group to believe that a receptor can be expressed in a blood vessel yet have no role in its contractile response.

Work on the localisation of the α_2-AR has shown that there is a differential cellular localisation of these receptors (von Zastrow et al., 1993). In transfected fibroblasts, the α_{2C}-AR was localised to the cell membrane and intracellular compartments whereas the α_{2A}-AR was found exclusively on the cell membrane. In COS 7 cells transiently transfected with GFP/α_1-AR constructs, the α_{1a}-AR was localised in a perinuclear fashion whereas the α_{1b}-AR was detected throughout the entire border of the cell (Hirasawa et al., 1997). Such work led Piascik's group to determine the distribution of all α_1-AR subtypes in peripheral arteries and therafter assess their functional expression (Hrometz et al., 1999).

Using rat-1-fibroblasts stably transfected with all three α_1-ARs and subtype selective antibodies, Hrometz et al. (1999) have supported the idea that α_1-ARs are expressed not only on the cell surface, but also in intracellular compartments. They have also examined the distribution of α_1-ARs in cells cultured from femoral and renal arteries to determine if differences in receptor localisation could modulate α_1-AR responsiveness in vascular smooth muscle cells. Such work has shown that all three subtypes are expressed. However, the abundant expression of the α_{1B}-AR subtype, associated with the plasma membrane, does not correlate with the subtype responsible for contraction in these blood vessels. Although similarities do exist between recombinant and native systems, there is still no explanation for the lack of correlation between subtype distribution and functional expression. Thus, this evidence indicates that cellular localisation alone, does not modulate contraction, and that perhaps the α_{1B}-AR subtype exists at this location for some other purpose. Piascik et al. (1997) have suggested a contractile role for α_{1B}-ARs in mesenteric arteries only. The possibility arises that α_{1B}-ARs in general may be more functionally relevant in other aspects of vascular function.

The mechanisms controlling the linkage between receptor expression and the regulation of smooth muscle contraction are far from understood. In smooth muscle cells different subtypes could be expressed predominantly in intracellular compartments and be sequestered from the cell surface. In this fashion a receptor could be expressed but not localized to the cell membrane where it can be activated by hydrophilic agonists (Hrometz et al., 1999).

In terms of drug action, distribution of α_1-AR subtypes has several important implications. Pharmacological agents must exert their effects by penetrating the cell membrane, either by diffusion or via transmembrane carriers. NE is taken up

and accumulated by SMCs that express α_1-ARs (Avakian and Gillespie, 1968). The transporter responsible, the extraneuronal monoamine transporter (EMT), formerly known as $Uptake_2$, has been cloned (Grundemann et al., 1998a). A similar carrier is responsible for the uptake of catecholamines in the liver (Grundemann et al., 1998b). The physiological actions of the majority of released neurotransmitters are terminated by their removal from the extracellular space. The blockade of these membrane transport proteins, results in attenuation of metabolism of NE, indicating this to be their physiological function. The functional expression of intracellular α_1-ARs opens the possibility that these transporters function to deliver catecholamines to intracellular receptors. These transporters are regulated by corticosteroids, indicating a possible role in the regulation of α_1-AR-mediated function. It has been reported that glucocorticoids induce expression of α_{1B}-AR (Sakaue and Hoffman, 1991) and β-AR (Collins et al., 1988; Hadcock et al., 1988; Jazayeri et al., 1988) genes in smooth muscle cells, emphasising a possible role for these transporter proteins in AR mediated function.

Transgenics and murine α_1-ARs

Presently, pharmacological analysis of the α_1-AR subtypes is limited by the relatively poor selectivity of antagonists; none of which could be regarded as 'gold standards'. This has led to a lack of consensus on subtyping of α_1-ARs in general. *In vivo* studies to assess specificity of the functional responses mediated by distinct α_1-AR subtypes have been hampered by the fact that the subtype-selective drugs are only moderately selective and might interact with both other adrenergic and non-adrenergic receptors. Thus, the functional implications of α_1-AR

heterogeneity and their physiological relevance remain largely unknown. Recently, targeted gene disruption has been increasingly used to elucidate the *in vivo* functions of receptor subtypes. Thus, to contribute to the understanding of the physiological roles of the α_1-AR subtypes *in vivo* Cotecchia and colleagues have been using gene targeting to create knockout mice lacking the α_{1B}-AR (Cavalli et al., 1997). This α_{1B}-AR knockout mouse model provides a useful tool to clarify the functional specificity of different α_1-AR subtypes. Daly et al. (in press) have used this particular knockout mouse model to demonstrate that the α_{1D}-AR subtype is predominantly responsible for contraction of mouse thoracic aorta and carotid artery. Since the α_{1B}-AR was removed from the equation, a straightforward comparison was permitted with controls. Minor changes in antagonist potency between wildtype and knockout indicate only a small role for the α_{1B}-AR in these vessels.

A full understanding of the functional significance of adrenoceptor heterogeneity awaits the knockout of all AR subtypes as well as the intercross among different knockout models. Studies of these mice have yielded intriguing observations, but their interpretations are limited by the scarcity of knowledge regarding the physiological characteristics of murine α_1-ARs relative to those of other species. In the mouse, studies at the mRNA level have been performed by RT-PCR (Alonso-Llamazares et al., 1995; Cavalli et al., 1997) and northern analysis (García-Sáinz et al., 1994). They have detected α_{1A}-AR mRNA in the heart, lung, liver, spleen, kidney, aorta, adipose tissues and several brain regions, including the cortex and cerebellum. α_{1B}-AR mRNA was detected in all of these tissues, although at somewhat lower abundance in the spleen and adipose tissue. α_{1D}-AR mRNA was detected in all of these tissues, although its presence in liver was seen

in one (Alonso-Llamazares et al., 1995) but not another (Cavalli et al., 1997) study. Radioligand binding studies by Yang et al. (1998a), have detected and characterised α_1-AR subtypes in a variety of murine tissues. The overall pharmacological profile of murine α_1-AR subtypes appears to be similar to that in other species. However, the quantitative and qualitative α_1-AR subtype expression in a given tissue appears to differ considerably between species (Yang et al., 1998a). Several reports in the literature show that expression of α_1-ARs in humans varies quite considerably from animal models.

Introduction to the thesis

Internalisation of GPCRs after activation by agonists is a well-documented phenomenon in recombinant cell lines, although equivalent understanding in native cells is lacking.

Several techniques have been developed over the years to try to map the location of GPCRs within cells. The introduction of foreign epitope tags on to cDNAs encoding receptor proteins has become a standard technique to facilitate the study of receptor dynamics. The technique of 'epitope tagging', first described by Munro and Pelham in 1984, is a recombinant DNA method for making a gene product immunoreactive to an already existing antibody. The process typically involves inserting a polynucleotide encoding a short continuous epitope onto/into a gene of interest and expressing the gene in an appropriate host. Epitope tagging has become a standard molecular genetic method for enabling rapid and effective characterisation and localisation of the protein products of cloned genes. The alternative and more traditional approach; raising antibodies to the encoded protein itself, is usually successful, but it is slow, costly and unpredictable. The

attraction of epitope tagging (typically 6-30 amino acids) is that they do not add new biological activity to the protein, and hence retain structure and function of the native receptor. However, this is not to say that large additions cannot retain normal function, e.g., green fluorescent protein (GFP) (>200 amino acids). More recently, GFP has been introduced for use as an autofluorescent epitope tag. By virtue of its ability to allow the real time visualisation of protein function in living cells, GFP may supplant all previous techniques for studying the cellular regulation of proteins. Tagging of GPCRs with the Green Fluorescent Protein has enabled the direct visualisation of real-time trafficking of GPCRs in living cells. Such analyses have provided crucial insights into the mechanisms involved in controlling GPCR function (reviewed by Milligan, 1999; Kallal and Benovic, 2000). Much effort has gone into developing several GFP variants to try and maximise fluorescence intensity when excited at certain wavelengths. In particular, Stanley Falkow and colleagues at Stanford University (Cormack et al., 1996) have developed EGFP vectors that encode the GFPmut1 variant, which contains the double substitution of Phe^{64} to Leu and Ser^{65} to Thr. Based on spectral analysis, EGFP fluoresces 35-fold more intensely than does wildtype GFP when excited at 488nm (Cormack et al., 1996). This increased sensitivity provided by EGFP improves its use as a bioluminescent reporter and, this is what has been used for all GFP work within this thesis.

However, much of the evidence for this comes from the use of recombinant cell lines. Equivalent experiments with live, native cells have thus far not been explored due to technical limitations.

Attempts made to bridge this gap, have seen the arrival of fluorescent ligands, as a means of monitoring GPCRs. Previous work by Prof. McGrath's group has

demonstrated the fluorescent ligand binding properties of the high affinity ligand, QAPB (BODIPY-FL prazosin), for α_1-ARs (McGrath et al., 1996; Daly et al., 1998). This work has established the technique of fluorescent ligand binding (at nanomolar concentrations) and its application for the study of receptors in both recombinant and native, single, living cells (Daly et al., 1998; McGrath et al., 1999; Mackenzie et al., 2000). The lipophilic nature of this compound allows it to bind to receptive sites irrespective of cellular location, whether associated with the plasma membrane or intracellular locations (Daly et al., 1998; McGrath et al., 1999; Mackenzie et al., 2000). Thus a major gain from the use of this fluorescent ligand is competitive ligand binding at the single cell level, revealing pharmacological data. A further advantage of the use of QAPB is its low level of fluorescence in the unbound state, thus allowing accurate measuments to be made under equilibrium conditions (Daly et al., 1998). Although QAPB is non-selective, the use of selective competitive ligands enables precise identification of the subtype in question (Mackenzie et al., 2000).

The first assessment of α_1-AR subcellular distribution was by Fonseca et al. (1995) who examined the subcellular dynamics of the α_{1b}-AR expressed in HEK 293 cells and the importance of PKC-dependent phosphorylation in the process of desensitisation and internalisation of this receptor. Using antibodies specific for the α_{1b}-AR, they were able to show that the presence of NA caused a redistribution of the α_{1b}-AR on the plasma membrane from a diffuse, uniform pattern to a globular, non-uniform pattern. This group interpreted this change as the visualisation of α_{1b}-AR internalisation. The phenomenon was reversed when NA was removed, and was blocked in the presence of prazosin. Using endosomal-specific antibodies, this group also demonstrated that α_{1b}-ARs were internalised

into endosomes. Stimulation of PKC and PMA mimicked the agonist-induced changes in receptor distribution, while the inhibition of PKC by staurosporine blocked agonist-induced receptor internalisation. Fonseca et al. (1995) concluded that stimulation of the α_{1b}-AR causes the activation of PKC-dependent phosphorylation, which mobilises cellular processes such as receptor mediated endocytosis and recycling of the α_{1b}-AR.

Recent evidence has indicated that α_1-ARs are not exclusively localised at the cell membrane. Using GFP-tagged α_1-ARs, Hirasawa et al. (1997) showed that the α_{1b}-AR was expressed on the cell surface whereas the α_{1a}-AR was expressed in intracellular compartments. Although a significant amount of α_{1b}-AR is located at the plasma membrane, there is a distinct fraction located intracellularly (Awaji et al., 1998; Tsujimoto et al., 1998; Stevens et al., 2000). This however, is substantially more pronounced for the α_{1a}-AR (Awaji et al., 1998; Tsujimoto et al., 1998). Interestingly, intracellular α_{1a}-ARs in LLCPK cells have been shown to be rapidly recruited to the cell surface by treatment with either a high concentration of the α_1-AR selective agonist, phenylephrine, or combinations of subthreshold levels of both phenylephrine and neuropeptide Y (Holtbäck et al., 1999).

Significant levels of intracellular α_{1d}-ARs have also been recorded by monitoring their binding by QAPB (Daly et al., 1998).

Piascik and co-workers, using subtype selective antibodies, have also confirmed that the α_{1b}-AR is predominantly located at the cell surface, and α_{1a}-AR and α_{1d}-ARs are located in a perinuclear orientation (Hrometz et al., 1999; McCune et al., 2000). Recent data suggests that a similar distribution pattern of α_1-ARs is found in native systems (Mackenzie et al., 2000; Hrometz et al., 1999; McCune et al.,

2000). Mackenzie et al. (2000) have reported remarkable similarities between the intracellular distribution of $\alpha_{1a/A}$-AR binding sites in recombinant and native systems. They found that in prostatic smooth muscle cells, 40% of the α_{1A}-AR population was intracellular, particularly around the nucleus, which could represent binding in the Golgi and may include both newly synthesised and recycling stores of receptors. Similarly, in vascular smooth muscle cells, α_{1A}-AR and α_{1D}-ARs are localised in a perinuclear fashion similar to that observed in α_1-AR transfected fibroblasts (Hrometz et al., 1999; McCune et al., 2000). This group also reported the α_{1B}-AR to be associated with the cellular periphery in vascular smooth muscle cells, consistent with studies in recombinant cells (Hrometz et al., 1999; McCune et al., 2000). These data argue that the cellular localisation of α_1-ARs is not an epiphenomenon of experiments in recombinant cell lines, but rather is characteristic of cells natively expressing these receptor subtypes.

An increasingly active area of interest, with regard to the localisation of α_1-ARs in native systems, is the apparent lack of correlation between the subcellular distribution of α_1-AR subtypes and their role in contraction of blood vessels. GPCRs are thought of, traditionally, to mediate their effects whilst located at the plasma membrane, thus allowing exposure to the extracellular environment.

The use of pharmacological approaches and antisense oligonucleotide technology has provided evidence to suggest that the activation of smooth muscle contraction is caused by a single receptor in any given artery and that the α_1-AR subtype responsible varies throughout the vasculature (Piascik et al., 1995, 1997; Hrometz et al., 1999). Regarding the expression of the α_{1B}-AR subtype in particular, some studies have approached this question by quantifying mRNA levels in several

different rat blood vessels. However, such work has shown no significant difference in α_{1B}-AR mRNA content between various vessels (Guarino et al., 1996). Experiments using antibodies raised against the α_{1B}-AR (Fonseca et al., 1995) and antisense technology have complemented the earlier mRNA studies (Piascik et al., 1997; Hrometz et al., 1999). However, from a functional perspective, only the mesenteric resistance artery exhibits an antisense-evoked reduction in contractile responses to agonist (Piascik et al., 1997; Hrometz et al., 1999). These findings have led this group to explore possible mechanisms to account for the observation that expression in blood vessels is not sufficient to link an α_1-AR to contraction. They have focused on subcellular localisation of α_1-AR subtypes as a factor that modifies the ability of these receptors to regulate cellular function. Examination of the cellular localisation and signalling properties of the α_{1b}-AR and α_{1d}-ARs expressed in rat-1 fibroblasts has been informative. Data show that the α_{1b}-AR exhibits expected GPCR activity regarding cellular localisation, agonist-mediated internalisation, and coupling to second messengers (McCune et al., 2000). In their native environment, α_{1B}-ARs are situated at the cellular periphery in smooth muscle cells derived from renal and femoral arteries. However, inhibition of the expression of only one, the α_{1A}-AR in renal and the α_{1D}-AR in femoral arteries, reduced the contractile response to agonist (Hrometz et al., 1999). This evidence indicates that cellular localisation alone is not a control point in modulating contraction, and the possibility arises that α_{1B}-ARs in general may be more functionally relevant in other aspects of vascular function

The subcellular distribution of α_{1d}-ARs is not consistent with the traditional model. In unstimulated fibroblasts, the α_{1d}-AR was detected in a perinuclear orientation and co-localised with arrestin 2 in a compartment containing the

transferrin receptor, an endosomal marker. This, combined with intracellular signalling data have provided strong evidence that the α_{1d}-AR is constitutively active and, as a result, is localised to intracellular compartments involved in receptor recycling (McCune et al., 2000). The cellular localisation of a constitutively active mutant (A293K, Kjelsberg et al., 1992) of the α_{1b}-AR transiently expressed in COS 1 cells, showed a significant degree of α_{1b}-AR internalisation and suggested that perhaps receptor internalisation in the absence of agonist is a property common to constitutively active α_1-ARs. Association of constitutively active α_{1b}-AR mutants with arrestin 2 has also been demonstrated (Mhaouty-Kodja et al., 1999). In contrast, Stevens et al. (2000) have recently reported that stable expression of constitutively active mutants (CAMs) of the α_{1b}-AR in HEK293 cells, with mutations D142A or A293G, are heavily concentrated at the plasma membrane, with little evidence of an intracellular loci (Stevens et al., 2000). Another constitutively active mutant of the α_{1b}-AR, that has had a small segment of the distal region of the third intracellular loop replaced with the equivalent segment of the β_2-AR, stably expressed in HEK293 cells, shows a substantially greater fraction of sites located intracellularly compared with that seen in the wild-type receptor. In addition, the pattern of distribution, rather than being perinuclear, was present in small punctate vesicles distributed throughout much of the cytoplasm (Stevens et al., 2000). However, considerably lower levels of expression than the other constructs, can possibly explain this observation, and suggests that perhaps only a fraction of the receptors in clones with high expression levels become relocated to the plasma membrane, leaving the 'excess' at their site of synthesis in the Golgi apparatus.

The α_{1d}-AR is somewhat of a mystery, and has not been studied to the same extent as the other two subtypes in molecular terms, although its functional role in large blood vessels is well documented (Kenny et al., 1995; Martinez et al., 1999). Theroux et al. (1996) showed that this receptor was weakly coupled to second messenger pathways. Yang et al. (1997) were unable to detect a significant degree of expression of the α_{1D}-AR in a variety of rat tissues. These findings are consistent with the observations made by McCune et al. (2000), that the receptor is predominantly expressed in intracellular compartments due to constitutive activity. As a result, the α_{1D}-AR is not as responsive to agonist activation as the other subtypes of the α_1-AR. Studies with either transfected HEK 293 or SK-N-MC cells failed to demonstrate any constitutive function of the α_{1d}-AR (Theroux et al., 1996), yet expressed at similar receptor densities to the fibroblasts. However, previous studies also using stably transfected α_{1d}-AR fibroblasts showed constitutive activation of the calcium response (García-Sáinz and Tórres-Padilla, 1999c). The α_{1D}-AR also binds most agonists but not antagonists with higher affinity than the other two α_1-AR subtypes. This phenotype is a hallmark of constitutive activity (Samama et al., 1993). Constitutive activity is also associated with constitutive phosphorylation and, therefore, densensitization. The traditional model of GPCR signalling indicates that the receptor is de-phosphorylated and recycled back to the cell surface. García-Sáinz et al. (2001) have shown that the α_{1d}-AR is phosphorylated in the basal state, and this increases in response to agonist and PMA. The internalised pool of α_{1d}-ARs may represent the equilibrium component in the recycling process due to its constitutive nature.

The possibility that the α_{1D}-AR is constitutively active in cells where it is endogenously expressed would be very interesting. However, data obtained in a

stably transfected cell line may not accurately represent the localisation and signalling characteristics of the native α_1-ARs expressed in vascular smooth muscle cells. The α_{1D}-AR is localised in a perinuclear orientation in vascular smooth muscle cells similar to that observed in fibroblasts, suggesting constitutive activity in cells that endogenously express this α_1-AR subtype (McCune et al., 2000).

The mechanisms controlling the linkage between receptor expression and the regulation of smooth muscle contraction are far from understood. In smooth muscle cells different subtypes could be expressed predominantly in intracellular compartments and be sequestered from the cell surface.

Recent technical advances are creating new ways to analyse expression of α_1-AR subtypes in native systems. As mentioned previously, Zuscik et al. (1999), have recently cloned, sequenced and analysed a 3.4kb mouse α_{1B}-AR promoter fragment. This promoter is capable of activating transcription of reporter gene expression in response to the competency of a given cell to express α_{1B}-ARs. Given the lack of highly selective ligands for this α_1-AR subtype, similar promoter-reporter constructs could be valuable tools when screening numerous cells/tissues for α_1-AR competency. Therefore, using GFP as a valuable reporter tool, a construct designed to drive the cell/tissue-specific expression of the α_{1B}-AR gene could prove invaluable in the screening of native cell lines for α_{1B}-AR expression. This, combined with pharmacological and functional data available, should hopefully ease this currently elusive situation. If the subtype location is common in various tissues/cells, it could indicate a possible cell-specific

functional location of the α_{1B}-AR, although, as is evident, contraction may not be the main functional role of this subtype.

The ultimate objective of the project was to study two related fundamental aspects of receptor biology in which recent advances using recombinant cell lines have not yet been translated into an equivalent understanding in tissues, i.e., receptor subcellular distribution and internalisation.

I was given the task of designing a molecular strategy to analyse the subcellular distribution of α_1-ARs in recombinant and native systems, in an attempt to uncover the similarities and differences between the two. It was also anticipated that I would further this and demonstrate receptor distribution within the walls of resistance arteries.

The α_{1b}-AR was chosen as a starting point for two reasons: firstly, it is under continuous study at a molecular and cellular level; secondly, Prof. McGrath's group have validated their techniques on the recombinant receptors (McGrath et al., 1996) and several groups have identified tissues whose pharmacology suggests the presence of α_{1B}-ARs, although their functional role is less well defined. Most importantly an α_{1B}-AR knockout mouse has been successfully generated, displaying a diminished pressor response to α_1-AR agonists (Cavalli et al., 1997) suggesting an important role of the α_{1B}-AR subtype in cardiovascular functions.

Fortunately, at the start of the project, a colony of α_{1B}-AR knockout mice (Cotecchia et al., 1997) was established in our laboratory, which was invaluable to me in my attempts to uncover the pharmacological nature of this α_1-AR subtype. Also, a recently developed technique enabling the analysis of α_1-AR subcellular distribution within live cells (McGrath et al., 1996) allowed me further scope.

Chapter 1.

The development of recombinant α_{1b}-adrenoceptor cell lines.

Chapter 1

Introduction

The general aim of this project was to develop a molecular method in order to localise, identify and quantify α_1-AR subtypes within heterogeneous tissues, where ligand binding is inappropriate. The two species of interest were human (to relate to clinical issues) and mouse (to enable the use of genetic manipulation). Despite obvious differences, many basic aspects of development, physiology, and GPCR function are well conserved between mice and humans and, in many instances, the development of mouse models to aid in the study of human disorders shows promise and may serve as a convenient experimental platform.

Traditional Pharmacological methods rely on the use of available drugs to identify populations and subtypes of receptor, hence every subtype having an anatomical location and a putative selective ligand. Molecular biology has allowed the isolation and cloning of genes for receptor subtypes for which there are no defined subtype-selective ligands or native tissue. Therefore, it is necessary to combine multiple technologies in an attempt to understand structure, function, location and regulation of individual receptor subtypes at both a subcellular and tissue level.

One of the fundamental issues facing pharmacologists is the ability to dissect out the mechanistic and functional differences between α_1-AR subtypes. The ability to do so is critical, for in many cases the need to discriminate between related subtypes is of therapeutic significance.

Studies of α_1-AR subtypes in tissues where they are endogenously expressed are frequently complicated by the presence of many different cell types and coexisting receptor subtypes. The analysis of individual subtypes in this situation is also

frustrated by the lack of reliable subtype-selective agents. This is best illustrated by the lack of antagonists that have a high affinity for the α_{1B}-AR subtype and can distinguish it from both α_{1A}- and α_{1D}-ARs. So, from a therapeutic perspective, the ultimate goal is to develop highly selective agents for each α_1-AR subtype.

The use of cell lines that endogenously express known complements of α_1-AR subtypes, or are stably transfected with receptor cDNAs, provides valuable tools for studying the pharmacological properties and functional characteristics of individual receptor subtypes. Cell lines expressing a single AR subtype can be identified and used to study their signalling properties in the absence of other subtypes. Different receptor subtypes can be expressed in the same cell line, allowing direct comparison of their pharmacology and signalling properties in the same cellular phenotype.

Using established cell lines expressing cDNAs makes it possible to obtain high levels of receptor expression thereby permitting more detailed pharmacological analysis, as well as second messenger generation. In most cases, heterologous expression is carried out in cell types that are null for α_1-ARs to allow the analysis of single α_1-AR subtypes in a defined system, and in addition can provide easy access to human receptors outside their native tissues. Unfortunately, ligand binding and intracellular signalling mechanisms are affected by cell type, receptor density and the presence and relative amounts of particular signalling elements in the chosen host cell line. Therefore, caution must be taken when interpreting results from such heterologous expression systems (Perez et al., 1993; Zhu et al., 1996; Bird et al., 1997). One potential way around this is the current development of lines of transgenic knockout mice, which are lacking a particular subtype (Smiley, 1998). α_{1B}-AR knockout mice are currently being studied in our laboratory.

Another important consideration is post-translational modifications. For example, some receptors require glycosylation events that are crucial for their functional expression within the cell. An example of this is vasoactive intestinal peptide 1 receptor, where glycosylation of two out of four sites is necessary and sufficient to ensure correct delivery of the receptor to the plasma membrane when expressed in COS 7 cells (Couvineau et al., 1996). Therefore, nothing can be predicted with any certainty about the suitability of an expression system for a particular receptor but some general guidelines can be drawn from the literature.

The promoter present also influences the choice of expression system, which must be active in the cell line to be transfected. Different vectors with different promoters may achieve their maximal expression in different cell lines. All expression systems used in this chapter harboured the CMV promoter that can achieve high-level constitutive expression. The same mammalian cell line was also used throughout. Another important factor to be considered in choosing a vector is the selectable marker(s) encoded by the vector, which provide antibiotic resistance for obtaining stably transfected cells. The mouse α_{1b}-AR was subcloned into the vector pRK-5 (figure 1.3), which lacked an antibiotic resistance marker to allow for expression in mammalian cells, therefore, co-transfection with pBabe^{Hygro} (figure 1.3) to allow for selection with hygromycin was employed. All other expression vectors used in this chapter contained the G418 marker to allow for selection using geneticin (figures 1.6 and 1.23).

The first step in my project was to obtain the cDNAs for the mouse (Prof. S. Cotecchia, Switzerland) and human (Pfizer, U.K.) α_{1b}-ARs. A host cell line to express the recombinant receptors was thereafter decided. Several criteria were taken

into consideration including lack of endogenous α_1-ARs, suitable morphology for confocal analysis, and autofluorescence levels. Several cell lines were examined and mouse neuroblastoma x embryonic hamster brain NCB20 cells were chosen, supplies of which were gifts from Prof. Graeme Milligan, University of Glasgow and Merck, U.K. This cell line has previously been used to express β-ARs (Milligan et al., 1995), and also shown to endogenously express α_2-ARs (Gleason and Hieble, 1991).

Stable cell lines were generated expressing both species of α_{1b}-AR in the same cell type and at similar receptor densities. A human α_{1b}-AR cell line with a receptor density double that found in the mouse α_{1b}-AR cell line was also generated, as a comparison.

The specific objective, initially, was to compare the binding properties of α_{1b}-ARs from the two species, first by radioligand binding using ^{3}H-prazosin and then by fluorescent ligand binding using QAPB (structure, figure 1.4). The functionality of the recombinant receptors were assessed by measuring intracellular Ca^{++} levels in response to agonist stimulation. The details of these studies are contained within this chapter.

The next logical step was to validate the binding of QAPB to α_1-ARs. QAPB has previously been demonstrated as a competitive antagonist (Daly et al., 1998). Binding and functional antagonism data show that, despite the modification of the molecule to incorporate the fluorescent tag, it retains the properties required of a high affinity pharmacological 'antagonist' ligand (Daly et al., 1998). Although the actions of this compound are specific to α_1-ARs, at nanomolar concentrations, the non-specific component increases in cells densely populated with α_1-ARs introducing artefact, and therefore not allowing a true representation of the location of receptive sites.

In order to validate the binding of this fluorescent compound, the rapidly developing technique of 'tagging' GPCRs created a couple of potentially useful options to us (see general introduction). The idea behind using 'tagged' receptors was that it would allow us to visualise the exact location of individual receptors and thereafter validate QAPB binding with co-localisation studies. The construction of a FLAG™ Epitope (DYKDDDDK) tagged mouse α_{1b}-AR is detailed in this chapter. The original description of the antibody to this sequence and its use as an epitope tag was in 1988 (Hopp et al., 1988). The FLAG™ system relies on the fusion of the coding sequence of the octapeptide (AspTyrLysAspAspAspAspLys) to the coding sequence of the target receptor and expression in a suitable vector. The expressed FLAG fusion protein can be detected with anti-FLAG monoclonal antibodies, and the receptor visualised using a fluorescent conjugate. The sequencing, expression, pharmacological and functional analysis of this construct is also detailed within this chapter. Western blot analysis and immunofluorescence studies of the expressed receptor protein are also discussed.

Coincidentally, at this time, an EGFP-tagged human α_{1b}-AR construct became available to us, a gift from Gozoh Tsujimoto, Japan. This provided me with an ideal way of locating α_{1b}-ARs, in real time, and on intact cells. Problems associated with non-specific binding of the fluorescent ligand and antibodies were removed from the equation when using GFP as it requires no additional substrates and can therefore be monitored non-invasively in living cells. It also gave me further scope with the development of a system that would allow analysis of agonist-induced internalisation, a reported phenomenon of this subtype of α_1-AR (Fonseca et al., 1995; Hirasawa et al., 1997). The search for the mechanism of internalisation of the

α_{1b}-AR has occurred at a rapid pace because of the importance of internalisation in receptor function and regulation. All the work associated with the expression, pharmacological and functional analysis of the GFP-tagged construct expressed in NCB20 cells is described in this chapter.

Tagging techniques are known to have an artifactual effect on receptor folding and sorting. Hence, all experiments were performed with wildtype receptors in parallel whenever possible.

Chapter 1

Methods

Manipulation of DNA

LB (Luria –Bertani) plates and LB broth

LB broth was made using the following ingredients: 10g tryptone (Sigma), 5g yeast extract (Sigma) and 10g NaCl were added to ~800mls of deionised water (dH_2O)$^-$, pH was thereafter adjusted to 7.0. Volume was adjusted to 1Litre, divided between 3 × 500ml bottles, autoclaved and subsequently stored at 4°C until required. For agar plates, LB broth was made as above and bacto-agar added at 15g/L prior to autoclaving. LB agar was cooled to ~45°C and either 50μg/ml ampicillin (Sigma) or 25μg/ml kanamycin (Sigma) added prior to pouring. LB Agar was then poured into 100mm plates and allowed to set. Plates were stored, inverted at 4°C until required. These plates allow the growth of bacteria harbouring plasmids that carry ampicillin/kanamycin resistance genes. Plates were streaked with bacteria using an inoculating loop which was sterilised by flaming and cooling.

Protocol to make competent bacteria

E. coli (DH5α™) bacteria were streaked out on a minimal plate (see above for plate preparation) and grown overnight at 37°C. 5mls of LB broth was inoculated with a single colony from this plate and grown up overnight. This was then added to a further 100mls LB broth and grown until the optical density at 550nm was 0.48. This took 1.5-1.75 hours. Cells were then chilled on ice for 5mins followed by centrifugation at 2-3k for 10mins at 4°C. Each pellet was gently resuspended in 20mls of buffer 1 (1M KAc, 1M $RbCl_2$, 1M $CaCl_2$, 1M $MnCl_2$ and 80% glycerol; pH5.8 with acetic acid, filter sterilised and stored at 4°C). Cells were then returned to

ice for a further 5mins and spun as before. Each pellet was then gently resuspended in 2mls of buffer 2 (100mM MOPS; pH6.5, 1M $CaCl_2$, 1M $RbCl_2$ and 80% glycerol; pH6.5 with concentrated HCl, filter sterilised and stored at 4°C), chilled on ice for a further 15mins, divided into 100µl aliquots and stored at -80°C until required.

Competent HB101 cells (Promega) were also used for some of the work within the thesis. HB101 has long been a popular strain for propagating plasmids that do not allow α-complementation.

INVαF' competent cells (supplied with cloning kit, Invitrogen) were used in conjunction with pCR®2.1. The genotype of INVαF' offers the following features: Blue/white screening of colonies via α-complementation of the N-terminal portion of β-galactosidase produced by the vector with the product of the ϕ80Δ*lacZ*ΔM15 gene present in the cell line.

Transformations

All transformations involving competent DH5α's™, or competent HB101 cells (Promega) were carried out as follows. An aliquot of competent cells was thawed on ice and 50µl per transformation was gently mixed with 1-10ng of plasmid DNA, left on ice for 15mins, heat shocked for 90secs at 42°C and returned to ice for a further 2mins. 1ml of LB broth (pre-heated to 37°C) was added to each reaction tube and left for 10mins at 37°C then incubated on a horizontal shaker at 225rpm for a further 50mins at the same temperature. Bacteria were then spun down briefly, the LB broth was removed, and the pellet resuspended in 100µl and spread on selective plates. These were then incubated overnight as before.

For transformations using pCR®2.1, LB plates containing 50μg/ml ampicillin were spread with 40μl of 40mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) dissolved in dimethylformamide, and allowed to dry. Meanwhile the ligation mixture was centrifuged and placed on ice. One vial of INVαF' competent cells was carefully thawed on ice and 2μl of 0.5M β-mercaptoethanol (supplied) gently stirred into the cells with the pipette tip. 2μl of ligation mix was also stirred into the thawed cells and the mixture then incubated for 30mins on ice. Cells were then heat-shocked at 42°C for 30secs and placed back on ice for a further 2 mins. 250μl of SOC media (supplied) was added to each vial of cells and placed in a horizontal shaker at 37°C for 1 hour at 225rpm. Two LB plates, streaked with X-gal, were used per transformation. Each plate was spread with either 50 or 200μl of the transformation mixture. Plates were left at room temperature for 15mins, then inverted and placed in an incubator overnight at 37°C. The following morning, plates were removed and placed in a cold room (4°C) for several hours, after which, colonies were selected for further growth and analysis.

Analysis of colonies

Competent cells transformed with all vectors excluding pCR®2.1 were selected in a similar manner. An aliquot of transformed cells was spread on selective plates containing the appropriate antibiotic and incubated overnight at 37°C. Positive colonies were selected and grown-up for further analysis.

The pCR®2.1 vector contains the lac promoter and the lacZα cDNA which produces β-galactosidase and, in the presence of X-gal, will give blue colonies. If the insert has been successfully cloned into the plasmid, this will disrupt the lacZα cDNA and

the colonies will be white. 15 colonies were selected from each transformation. Each colony was innoculated into 5mls of LB containing 50μg/ml of ampicillin and grown up overnight at 37°C in a horizontal shaker at 225 rpm. Plasmids were then isolated and subjected to restriction digestion to screen for the presence of the insert. Glycerol stock solutions of the positive colonies were made and stored at -70°C until required.

Plasmid isolation

Plasmids were purified using the Wizard™ *Plus* SV Minipreps DNA Purification System from Promega. A 3ml aliquot of overnight bacterial culture was centrifuged at 10,000g for 1 minute to pellet the cells. LB broth supernatant was removed and excess media removed from the pellet. Cells were then resuspended in 250μl of cell resuspension solution (50mM Tris-HCl and 10mM EDTA). 250μl of cell lysis solution was then added (0.2M NaOH and 1% SDS) and mixed by inversion. 5 minutes later, 10μl Alkaline protease solution was added and again mixed by inversion. After no longer than 5 mins, 350μl of neutralisation solution was added (4.09M guanidine hydrochloride; 0.759M potassium acetate; 2.12M glacial acetic acid: pH 4.2) and again mixed by inversion. The lysate was then centrifuged at 13,000g for 15mins at room temperature. Clear lysate was then transferred by decanting into a spin column and centrifuged at 13,000g for a further 2mins. The column was then washed twice with column wash solution (60mM potassium acetate, 10mM Tris-HCl, pH 7.5; 60% ethanol) by spinning the wash through the column in the centrifuge. DNA was then eluted using 100μl of nuclease-free water and stored at -20°C until required.

Primers

The mouse α_{1b}-AR primers were a gift from Susanna Cotecchia, Institute of Pharmacology, University of Lausanne, Switzerland.

Sense-

5' CCACTCTAAGAACTTTCATGAGGACACC 3',

Antisense-

5' ATGCAGCTGCCACTGTCATCCAGAGAGT 3'.

The FLAG/mouse α_{1b}-AR primers were custom made by Cruachem Ltd. West of Scotland Science Park, Glasgow. On arrival, both sets of primers were aliquoted and stored at -20°C until required. Frequently, the ends of insert DNA do not contain suitable restriction enzyme sites required for subcloning into the multiple cloning site (MCS) of an appropriate expression vector. This problem is resolved by using PCR to generate a site at the desired location. For this technique, the restriction enzyme site is designed into the 5'-end of the PCR primer. Because certain restriction enzymes inefficiently cleave recognition sequences located at the end of a DNA fragment, additional bases (at least three) were included in front of the enzyme recognition sites. An additional sequence, Kozak (Kozak, 1983) was also included to increase mRNA efficiency. The coding sequence of the **mouse α_{1b}-AR** was modified by PCR amplification using the amino-terminal primer-

5'AAAGCTAGCGCCACCATGGACTACAAGGACGACGATGACAAG**AATCC**

GGATCTGGACACCGGCC-3',

an NheI restriction site and the FLAG epitope tag were introduced. Using the COOH-terminal primer-

5'TTTTCTAGA**GACCGCGGGCCCTTGAAAATC**-3',

an XbaI restriction site following the stop codon was introduced.

Polymerase chain reaction

All reactions were carried out using a Techne "progene" thermal cycler. Reaction conditions were slightly different for each set of primers used and have therefore been dealt with separately below.

Unless otherwise stated the reaction was carried out in a 50µl volume using the following:

FLAG/mouse α_{1b}-AR primers

5µl of 10×PCR buffer (Promega), 20pmol of each primer, 1.25µl of dNTP mix (10mM, Promega), 3µl $MgCl_2$ (25mM, Promega), 5µl of cDNA and 0.5µl of Taq Polymerase enzyme (5units/µl, Promega). These components were made up to 50µl with sterile deionised water. The cycle programme used for FLAG/mouse α_{1b}-AR primers was as follows: 94°C for 5 minutes followed by 40 cycles comprising denaturation (94°C for 2 minutes), annealing (65°C for 1 minute) and extension (72°C for 2 minutes). This was followed by a further 10 minute extension period at 72°C.

Mouse α_{1b}-AR primers

This reaction was set up in the same manner. The annealing temperature required was slightly lower, at 56°C.

For each primer set used, negative controls were run in parallel. These contained identical ingredients except that the cDNA template was replaced with sterile deionised water. At the end of each reaction, aliquots were electrophoresed on 1% agarose gels as described above, to allow for visualisation of PCR products. In the

case of the FLAG tagged product, the entire PCR product was run on a gel, the amplified band excised, purified from agarose and stored at -20°C until required.

Restriction digests

A variety of restriction digests were carried out throughout this project. Analytical and small scale digests were usually carried out in 10-20µl reaction volumes using ~1µg DNA. All other digests were scaled accordingly and incubated for enzyme-specific time periods and temperatures suggested by the manufacturers. Initially, when attempting to subclone the FLAG tagged product into pcDNA3.1 (+), the PCR product DNA was extracted from the agarose gel, purified, then subject to restriction digestion using restriction enzymes *NheI* and *XbaI* (Boehrinnger Mannheim) in conjunction with the appropriate buffer system. The cleavage sites for both of these enzymes lie within a few bases of the end of the DNA fragment which can cause decreased cleavage efficiency. Therefore the incubation period suggested by the manufacturer, was extended accordingly. Such digests were usually carried out in ~50µl volumes using all the amplified DNA extracted from the gel, enzyme quantities etc, were scaled according to the concentration of DNA in each digest.

Alkaline phosphatase treatment

Calf intestinal phosphatase (CIAP) catalyses the hydrolysis of 5'-phosphate groups (Sambrook et al., 1989). This enzyme was used to prevent recircularisation and religation of the linearised cloning vehicle DNA by removing phosphate groups from both 5'termini. Restriction digested vector DNA was mixed with 3µl of enzyme (CIAP 1unit/µl, promega), 6µl of 10× reaction buffer (Promega), made up to 60µl

with sterile deionised water and incubated for 1hour at 37°C. DNA was 'cleaned up' to remove any traces of CIAP.

DNA clean-up system

Wizard™ DNA Clean-Up System (Promega), was used to clean-up DNA from heat-stable restriction enzymes and alkaline phosphatase treatment according to the manufacturer's instructions.

Agarose gel electrophoresis

Unless otherwise stated, 1% agarose gels were used. In general, the percentage of agarose used depended on the molecular weight of the fragments being separated. Small fragments (<400bp) requiring a higher percentage gel than larger fragments (>3kb). The fragment sizes of interest ranged from ~1-8kb. Agarose was dissolved in 50×TAE buffer (242g Tris Base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA, pH 8.0. pH was adjusted to 7.2 after making up to a final volume of 1 litre with distilled water). Gels were also run in this buffer at 100 volts. Ethidium bromide (0.5µg/ml) was used to stain gels in order to visualise the DNA on a UV transilluminator. Markers (0.07-12.2kb) (Boehringer Mannheim) were used at all times and samples were loaded with one-tenth volume of 10× loading buffer (0.25% bromophenol blue and 40% v/v glycerol in water).

Isolation of DNA from agarose gels

DNA fragments were excised from agarose gels with a clean, sharp scalpel, minimising the removal of any excess agarose. Care was taken to ensure the gel was not over exposed to the UV rays. QIAquick Gel Extraction Kit (QIAGEN) was used

according to the manufacturers instructions. Eluted DNA was aliquoted and stored at -20°C until required.

Quantitation of DNA

DNA was diluted in dH_2O by a factor of 100, and the optical density measured at 260 nm. A concentration of 50µg/µl gives a 260nm absorbance of 1. Therefore the concentration is 50 x O.D. at 260 nm x dilution factor = µg/ml. Pure DNA has a 260:280nm ratio of 1.8 (Sambrook et al., 1989).

Ligations

All ligation reactions, excluding those specific to the TA Cloning® Kit (Invitrogen), were set up aiming for a vector:insert ratio of 1:3, unless otherwise stated. The following components were made up in a final reaction volume of 20µl with autoclaved dH_2O and incubated for 1 hour at room temperature: 100-200ng vector/ligation and the appropriate amount of insert, 1unit T4 DNA ligase (Life technologies, Inc) and 4µl of 5x Ligase Reaction Buffer (Life technologies, Inc). Reactions specific to the TA Cloning® Kit involved the use of fresh PCR product, as degradation of the 3' A-overhangs occurs over time. To allow for a recommended 1:1 ratio to be established, 2µl of the pCR®2.1 vector and an appropriate volume of insert were mixed with 1µl of 10x Ligation Buffer, and T4 DNA Ligase (4.0 Weiss units) (both supplied). The total reaction volume of 10µl was incubated at 14°C overnight.

Cloning of DNA fragments

Attempts were made to subclone the amplified FLAG/mouse α_{1b}-AR fragment into expression vector pcDNA3.1(+) (Invitrogen) using restriction enzymes; *NheI* and *XbaI* (Boehringer Mannheim). Due to experimental difficulties, Invitrogen's Original TA Cloning® kit, employing the vector pCR®2.1 (fig), was ultimately employed for this purpose. The advantages of using this kit include elimination of any enzymatic modifications of the PCR product or the need to use primers containing restriction enzyme sites. This kit is based on the fact that *Taq* Polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearised vector supplied in this kit has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. Thereafter the insert was cut out using *EcoRI* (Promega) and subsequently subcloned into pDNA3.1 (+).

Sequencing

Samples to be sequenced were sent to PNACL, University of Leicester. Sequencing data was analysed using Chromas (version 2.0, beta 3) sequence analysis software.

Analysis of receptor constructs

Stable transfection of NCB20 cells

Cells were grown to 60-80% confluence before transfection. Transfection was performed using DOTAP (Boehringer Mannheim) according to the manufacturers instructions. Mouse-α_{1b}-AR cDNA was subcloned into the expression vector pRK5 (figure 1.3a), and stably transfected clones were generated by co-transfection of

pRK5 with pBABE^{HYGRO} (figure 1.3b) (Morgenstern and Land, 1990) at a ratio of 10:1. All other constructs were expressed in vectors harbouring the G418 resistance marker. To generate cell lines stably expressing the desired construct, 1day post transfection, cells were seeded and maintained in DMEM (Sigma) supplemented with 200μg/ml Hygromycin (Boehringer Mannheim) (mouse-α_{1b}-AR) and 400μg/ml Geneticin (Life technologies, Inc) (FLAG/mouse-α_{1b}-AR, human-α_{1b}-AR and GFP/human-α_{1b}-AR). In both cases, a 'kill curve' was used to determine the concentration required to select for antibiotic resistant cells. Approximately two weeks post-transfection, discrete colonies became visible on the culture dishes, a selection of which were picked, and expanded to allow for further selection. Clonal expression of each subclone was examined initially by Confocal Microscopy and Radioligand Binding, then clones for further study were selected and expanded.

Confocal Microscopy

Two NORAN odyssey confocal laser scanning microscopes were used in the course of this work. The vessel work was performed on an upright (Nikon Optiphot) configuration and the majority of the single cell work was performed on an inverted configuration (Nikon Diaphot) UV CLSM system. The upright microscope is fitted with a mixed gas UV laser (Coherent) and can deliver lines of 364, 488 and 525 nm, with barrier filters of 400, 515 and 529 nm, respectively. This system is optically best suited for UV and green fluorescent stains. The system is physically best suited for fixed cell/vessel work.

Argon Ion CLSM system: The inverted system is fitted with a single argon ion laser (OmniChrome) capable of delivering lines of 454, 488 and 529 nm and available barrier filters of 515, 550 and 610 nm. This system is optically best suited to dual

fluorescence work using green and red stains (i.e., fluorescein and rhodamine type stains). The system is physically best suited to work with single cells mounted on a specialised flow chamber.

Software

Identical parameters (brightness, contrast, laser intensity, excitation and emission wavelengths, slit size and frame averaging) were set in every experiment, unless stated otherwise. The objectives used throughout the project were Nikon x40, oil and Zeiss x40, water, in conjunction with the inverted and upright systems, respectively. The resolution in X and Y for the upright/invert microscopes using a x40 objective was 0.2μm/pixel. The Z resolution used when acquiring z-series was 0.3μm.

Whole cell image analysis

Images were collected and analysed using Universal Imaging's 'Metamorph' software. Cells were grown on coverslips for 24 hours prior to use, unless otherwise stated. Coverslips were mounted in a flow chamber (WPI) and placed on the stage of an invert (Nikon Diaphot) microscope fitted with a Noran Odyssey Laser Scanning Confocal Module. Fluorophores were excited using a 488 nm argon laser and detected with a 515 nm band pass filter. In all experiments, a 15μm slit was used and all other parameters were kept constant. All cells were checked for viability using Propidium Iodide staining prior to every experiment.

Screening transfected subclones for homogeneity

A single concentration of QAPB (1nM) (Molecular probes) was added to a coverslip of cells representative of different transfected subclones. Images were acquired from around the entire coverslip to establish homogeneity.

QAPB

Using cell autofluorescence, a suitable group of cells was selected and the focal plane fixed by locking the focus motor. The system was then set to acquire images (64 frame averages, unless stated otherwise) at 1 minute intervals. After a baseline was established, typically 5-6 minutes, the first concentration of fluorescent ligand was added and allowed to equilibrate (i.e., until no further increase in fluorescence) for at least 5 mins. After equilibration the next concentration of fluorescent ligand was added, without washing, and given time to equilibrate as before. Once saturation had been reached the individual cells were outlined using Metaporph's define-region tool and the fluorescent intensity values representative of each concentration at equilibrium were recorded. Non-specific binding was defined as fluorescent binding in the presence of 10μM prazosin. The composition of the Hepes buffer used in all single cell work was as follows: NaCl 130mM, KCl 5mM, HEPES 20mM, Glucose 10mM, MgCl 1mM, CaCl 1mM.

GFP detection

Using GFP-associated fluorescence, suitable cells stably expressing the GFP-tagged humanα_{1b}-AR construct (a gift from Tsujimoto) were chosen and images acquired. In co-localisation experiments, with QAPB, cells with relatively low GFP expression were selected and analysed as described above.

Immunofluorescence detection

Fixation was performed in 80% acetone for 5 minutes. Cells were subsequently washed briefly in PBS then permeabilized with 0.05% Triton X-100 (Sigma) in PBS for 30 minutes. The primary monoclonal antibody, anti-FLAG® M5 (Sigma-Aldrich) was then applied to cells at 10μg/ml in PBS containing 10% goat serum (DAKO) and 0.05% triton X-100. Cells were subsequently kept in a humidified chamber on a

Gyro-rocker at 10 rev/min for 1 hour at room temperature. Cyanine-conjugated Affinipure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) was diluted 1/200 in PBS containing 2% bovine serum albumin and 0.05% Triton X-100, and applied to cells for 1 hour at room temperature in darkness. Cells were then washed twice with PBS, and coverslips mounted using fluorescent mounting medium (DAKO). After immunocytochemical staining, cells were examined using a NORAN Odyssey CLSM with mixed gas UV laser using a x40 water immersion objective.

Western blotting

Electrophoresis and blotting of protein were performed with the Novex XCell II Mini-Cell and Blot Module under reducing and denaturing conditions. All samples were prepared in 4x Laemmli sample buffer [Tris-base (0.24 M, pH 8.3), glycine (1.9 M) and SDS (35mM)] and were heated at 55-60°C for 5 min before loading. Typically, 10-20 μg of protein was loaded in each lane of a 10% Tris-glycine gel. Electrophoresis was carried out in 4x Laemmli buffer for 1.25 hours at a constant current of 25mA. Proteins were transferred on to nitrocellulose membranes using the Trans-blot semidry transfer cell system (Biorad) for 30 mins at 20V at a limiting current of 500mA in transfer buffer (39mM Glycine, 1.3mM SDS, 20% methanol, 48mM Tris, pH 9.2). Nitrocellulose sheets were blocked in wash buffer (150mM NaCl, 1mM EDTA, 0.1% Triton X-100, 10mM Tris-HCl, pH 7.4) containing 3% BSA. Incubation with the primary antibody (Anti-FLAG M5 Monoclonal Antibody, 1:2000 dilution of the stock, Sigma.) was carried out overnight at 4°C in blotting buffer (100mM $MgCl_2$, 0.5% Tween, 1% Triton X-100, 1% BSA, 100mM Tris-HCl, pH7.4) containing 5% foetal calf serum (Life technologies, Inc). Membranes were

then washed three times for 5 min at room temperature in wash buffer. Secondary antibody (Goat anti-mouse IgG-HRP, 1:2000 dilution, Transduction laboratories) incubations were for 2 hr at room temperature in blotting buffer. The blots were washed a further three times and developed using the ECL detection system (Amersham).

Radioligand binding assays

Production of plasma membrane fractions

Cells were grown to confluence then the cell layer harvested using a rubber policeman followed by centrifugation at 4000rpm for 3 minutes at room temperature. The cell pellet was resuspended in 1ml of Tris-HCl assay buffer (150mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$, 5mM EDTA pH 7.4) and stored at -80°C until the following day. Cells were then homogenised using a pre-chilled teflon-in-glass homogeniser. The homogenate was centrifuged at 4000rpm for 5 minutes at 4°C, the supernatant recovered and centrifuged at 26,000rpm for 30 minutes at 4°C. The resulting membrane pellet was resuspended in 500µl of ice-cold Tris-HCl buffer and sheared by passage through a 26-guage needle. 100µl aliquots were stored at -80°C. Protein concentrations were determined using BCA™ Protein Assay Kit (Pierce) using bovine serum albumin as the standard.

Screening cell lines for endogenous receptor expression

For initial screening, expression of α_1 and α_2-ARs was estimated by measuring specific binding through a range of concentrations (0.05-10nM) of [^{3}H]-prazosin (Amersham) and [^{3}H]-yohimbine (Amersham), respectively.

Screening transfected subclones for receptor expression

Antibiotic resistant subclones were first screened for expression by measuring receptor density with a single concentration (1nM) of [^{3}H]-prazosin. Clones with high specific and low non-specific binding were expanded for further pharmacological and functional analysis.

Saturation analysis

For saturation experiments, membranes (10μg of protein) were incubated in duplicate with increasing concentrations (0.05-10nM) of [^{3}H]-prazosin, in a final volume of 0.5ml of assay buffer (150mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$, 5mM EDTA, pH 7.4), at room temperature for 30 minutes. Non-specific binding was defined as the binding retained on the filter and membranes in the presence of 10μM Phentolamine. Following equilibrium, bound and free ligands were separated by rapid filtration under vacuum through GF/C glass-fibre filters (Whatman, Maidstone, Kent, UK), using a Brandell cell harvester. The filters were washed three times with ice-cold wash buffer (50mM Tris-HCl pH 7.4), transferred to scintillation vials containing Ecoscint™A (National Diagnostics). Filters were soaked overnight and counted.

Competition analysis

For competition experiments, membranes (10μg of protein) were incubated with approximately the K_D concentration of radioligand in duplicate, together with increasing concentrations of competing agent. Inhibition of specific binding of [^{3}H]-prazosin by ligands was analysed to estimate the IC_{50} (concentration of the ligand displacing 50% of specific binding). The inhibitory constant (K_i) was calculated from the IC_{50} by the equation of Cheng and Prusoff (1973). Ligands used in competition analysis included prazosin, QAPB, (R)-A-61603, R enantiomer of A-61603 (Dr. Michael Meyer, Abott Labs), BMY7378 (Research Biochemical Int, RBI). Binding

isotherms from displacement studies were analysed by a non-linear least square parametric curve-fitting programme, GRAPHPAD prism® version 3.0, capable of iterative curve fitting to a single-site model.

Measurement of $[Ca^{++}]_i$

Fluorimetric analysis

Cells were grown on glass coverslips then loaded (15mins at 37°C), with Fura-2 AM (1μM) (Sigma, Dorset, U.K.). A rise or fall in $[Ca^{++}]_i$ causes a corresponding effect in the Fura-2 fluorescence ratio recorded from cells loaded with this dye, and this allows receptor/voltage-mediated changes in $[Ca^{++}]_i$ to be microspectrofluorimetrically monitored (Grynkiewicz et al., 1985). In the present study, Fura-2 fluorescence ratios, (excitation wavelength 340 and 380 nm), were recorded directly at 4 Hz intervals from single cells at room temperature. Data were digitised and recorded directly to computer disk using an interface and associated software (Version 5.2) obtained from Cairn Research Ltd (Faversham, Kent, U.K.).

Calcium imaging

Cells stably expressing the GFP/human α_{1b}-AR were plated onto glass cover slips and grown overnight, then loaded (15 min at 37°C), with Fura-2 AM (1.5 μM). Fura-2 loaded cells were then. mounted in a chamber attached to the stage of an Nikon Diaphot inverted microscope where the cells were superfused with physiological saline solution. An Optoscan monochromator (Cairn Research, Faversham, Kent U.K.), positioned between a 75W Xenon lamp and the epifluorescence port of the microscope, was used to alternate the excitation wavelength between 340, 380 and 488 nm (band pass 10 nm) and to control the excitation frequency. Excitation light was reflected from a custom designed dichroic mirror through a Nikon 40x oil immersion FLUOR objective (NA=1.3). Fura-2/GFP emitted fluorescence light at

515 nm was monitored either by a low noise COHU CCD camera or a photomultiplier tube with a bialkali photocathode. Images acquired with the CCD camera were stored and analysed digitally under the control of Meta Fluor imaging software (Universal Imaging Corp., West Chester, PA, USA). Images acquired for each excitation wavelength were collected every 1 sec; exposure to excitation light was always 80ms/image and the time interval between the acquisition of each images was ~2 msec. Cells were delimited by producing a mask that contained pixel values above a threshold applied to the 380 nm image and time-dependent changes in $[Ca^{2+}]_i$ was calculated from the ratio of two background subtracted images.

Cell culture

NCB20s were maintained in DMEM supplemented with 10% (v/v) foetal bovine serum and 1mM L-glutamine in a 95% air and 5% CO_2 atmosphere at 37°C. Stable cell lines were maintained in the presence of selective antibiotics. Clones expressing the mouse-α_{1b} were grown in media containing 200μg/ml hygromycin. For all other stable cell lines, 400μg/ml geneticin was used as the selective agent.

Freezing cells

Cells are grown to confluence in $75cm^3$ tissue culture flasks, culture media removed and replaced with fresh media containing 10% DMSO. Cells were removed from the bottom of the flask by gentle agitation, aliquoted into 1.5ml cryovials and placed in a 'Mr Frosty' overnight at -70°C. Vials were then removed and filed in cryocanes and frozen down in liquid nitrogen.

Thawing cells

Cell ampoules were removed from cryocanes and placed in an incubator at 37°C for 5mins. The aliquot was carefully added dropwise to 9mls of fresh media, spun down,

resuspended in 5mls of media and added to $25cm^3$ flask, placed in incubator and maintained as normal.

Results

Construction of FLAG-tagged mouse α_{1b}-AR

The construction of an oligonucleotide that encoded the FLAG™ epitope (DYKDDDDK) to be inserted into pcDNA3.1 (+) was carried out in two separate stages. Firstly, the coding sequence of the mouse α_{1b}-AR (figure 1.14, nucleotides 724-2269) was modified by PCR amplification. Using the amino-terminal primer (see methods), an *NheI* restriction site and the FLAG™ epitope were introduced. Using the COOH-terminal primer (see methods), an *XbaI* restriction site was introduced. Unfortunately, the amplified product (figure 1.15a), digested with *NheI* and *XbaI*, could not be successfully ligated to pcDNA3.1(+). The activity of these restriction sites in the MCS of the expression vector was assessed (figure 1.15b). The cleavage sites for both of these enzymes lie within a few bases of the end of the amplified fragment, which can cause decreased cleavage efficiency. Therefore the incubation period suggested by the manufacturer was extended accordingly; unfortunately to no further avail. Another consideration for the failure of this ligation was that both enzymes produced compatible ends. Ultimately, Invitrogen's TA Cloning® Kit was employed. Initially *EcoRI* was used to check that the amplified fragment had been successfully ligated to pCR®2.1 (figure 1.16a). Thereafter, *NheI* and *XbaI*, were used to excise the insert from the MCS of pCR®2.1 (figure 1.16b) which was subsequently ligated to the sticky ends of pcDNA3.1(+). The construct was sequenced (figure 1.17) and expressed in NCB20 cells for pharmacological and functional analysis (figures 1.18-1.22).

Stable expression of functional α_{1b}-ARs in NCB20 cells

mouse/human α_{1b}-ARs

NCB20 cells did not contain any detectable ^{3}H-Prazosin binding sites before transfection with α_{1b}-ARs (figure 1.1b). A small population of α_2-ARs was detected using ^{3}H-Yohimbine (K_D 0.93±3.1, B_{max} 15.87±18.73fmol/mg) (figure 1.2, bottom). In contrast, when the cells were transfected with α_{1b}-AR constructs, clones were identified expressing specific binding sites for ^{3}H-Prazosin on crude membrane fractions. All clones expressing the specific ^{3}H-Prazosin binding sites, as identified by hygromycin (mouse α_{1b}-AR) or geneticin (human α_{1b}-AR) resistance, had equivalent affinity for the radioligand, consistent with α_1-ARs, but differed in receptor density. Among the several clones screened, a clone expressing maximum receptor density of the ^{3}H-Prazosin binding sites was chosen from both species, and used for further analysis. Scatchard analysis of the specific binding curve for ^{3}H-prazosin binding to mouse and human α_{1b}-AR membranes produced a K_D of 0.7 and 0.9nM respectively (Figure 1.8, Table 1). Competition studies demonstrated that 0.2nM ^{3}H-prazosin binding was displaced from both mouse and human α_{1b}-AR sites with high affinity by prazosin, with QAPB displacing ^{3}H-prazosin with slightly lower affinity (Figure 1.9, Table 1). Consistent with the pharmacology of recombinant α_{1b}-ARs, both A-61603 (α_{1a}-selective, Knepper et al., 1995) and BMY-7378 (α_{1d}-selective, Goetz et al., 1995) had low affinities for both species of α_{1b}-AR (Figure 1.9, Table 1).

FLAG-tagged/mouse α_{1b}-AR

The clone of NCB20 cells stably expressing the FLAG-tagged mouse α_{1b}-AR, that was selected and used for further analysis, showed a lower affinity for ^{3}H-prazosin, and a receptor density that was a quarter of that in the wildtype clone (figure 1.19, Table 1). Competition analysis revealed that prazosin displaced 2nM ^{3}H-prazosin binding with slightly lower affinity from tagged receptive sites. In addition, a substantial difference in the ability of QAPB to displace ^{3}H-prazosin was also observed at these sites (figure 1.20, Table 1).

GFP-tagged/human α_{1b}-AR

In contrast to the difference in affinity observed when an 8 amino acid (aa) epitope tag is attached to the N-terminus of the α_{1b}-AR, an equivalent affinity of ^{3}H-prazosin for the GFP-tagged (C-terminus, >200 aa) and the wildtype receptor was observed (figure 1.25, Table 1). Similarly, competition analysis demonstrated that unlabelled prazosin displaced 0.2nM ^{3}H-prazosin from all sites with equivalent ability. However, the fluorescently labelled antagonist, QAPB, again showed a much lower ability to displace ^{3}H-prazosin as was observed for the FLAG-tagged sites (Figure 1.26, Table 1).

Fluorescent ligand saturation binding on intact recombinant cells

mouse/human α_{1b}-AR

QAPB-associated fluorescence binding was concentration-dependent in recombinant cells. Incubation with QAPB produced minimal background fluorescence using a working range starting at 0.4 up to a maximum of 30nM in non-transfected cells where α_1-AR expression was negligible (figure 1.1a). In the presence of 1μM prazosin, QAPB binding was significantly inhibited and the

residue was used to calculate 'non-specific' binding. This residual fluorescence seen in transfected cells is found in the same regions in the cell that you find specific binding. QAPB-associated fluorescence seen in control cells (entirely non-specific) has a different distribution (figure 1.1a). This 'non-specific' binding is also greater in transfected cells compared to control cells, and is dependent on the density of receptors within the cell in question. So, although this binding at high concentrations of QAPB is resistant to prazosin (1μM), it is receptor-related, and therefore cannot be termed as non-specific per se. The 'non-specific' component in all cells was almost negligible at low concentrations of QAPB, allowing the subcellular distribution of specific α_{1b}-AR binding sites to be visualised. Receptor capacity of the cell line expressing the mouse α_{1b}-AR was ~4000fmol/mg. Two human α_{1b}-AR cell lines were generated, one with similar and the other with a receptor density double that of the mouse cell line. Intense staining of saturable intensity was observed in regions associated with the plasma membrane, at very low concentrations of QAPB, in both cell lines, at both densities (figures 1.5 and 1.7). In some cells, a distinct intracellular population of binding sites was also apparent (figure 1.5, middle, left), presumably dependent on the stage each cell was at in its cycle. These results indicate that the α_{1b}-AR is located predominantly at the cell periphery. The 'non-specific' binding seen at higher concentrations of QAPB was distributed in a similar manner, since receptor-related, yet resistant to prazosin.

FLAG-tagged mouse α_{1b}-AR construct/wildtype mouse receptor

QAPB binding to FLAG-tagged recombinant cells was highly specific at low concentrations of QAPB, and associated with the plasma membrane as seen in

cells expressing the wildtype receptor (figure 1.18). The receptor density of this cell line, as shown by radioligand binding, was a quarter of that recorded in the mouse α_{1b}-AR cell line, which is consistent with the reduced staining found.

GFP-tagged human α_{1b}-AR construct/wildtype human receptor

In NCB20 cells stably expressing low levels of the pEGFP/human α_{1b}-AR construct, QAPB bound to regions within the cell that represented the receptor, as shown by low levels of pEGFP-expression (figure 1.24e), thus validating the binding of the fluorescent ligand to specific α_1-AR binding sites. GFP-associated fluorescence was associated mainly with the plasma membrane (figure 1.24a), although, as with QAPB binding alone in wildtype cells (figure 1.5, middle, left), some displayed intense fluorescence in an intracellular location (figure 1.24b). Attempts made to co-localise QAPB binding with the GFP-tagged receptors also resulted in a QAPB induced change in the distribution of the receptive sites (figure 1.24d). The image of GFP alone (figure 1.24di) compared to the binding of 2nM QAPB (figures 1.24dii and iii) over 2 and 5minutes respectively, illustrates this. The transient expression of the GFP-tagged construct driven by the mouse α_{1B}-AR promoter in NCB20 cells, which are α_{1B}-AR negative, was significantly low, in that only a couple of cells from a confluent cover slip were able to express the promoter-reporter construct (figure 1.24c).

Lack of specific immunostaining of Anti-Flag ® M5 monoclonal antibody

Immunofluorescence analysis

Anti-Flag ® M5 monoclonal antibody had low specificity in binding to the epitope-tagged mouse α_{1b}-AR stably expressed in NCB20 cells. Intense non-

specific immunostaining was observed on both transfected and non-transfected cells (figure 1.22a). A possible explanation could be cross-reactivity between the mouse monoclonal antibody and the NCB20 cell line, both of which are of mouse origin.

Western blot analysis

A ladder of immunoreactive bands was observed in Western blots using plasma membranes or whole cells from both non-transfected and FLAG-tagged cells (figure 1.22b). Each band corresponded in size to one another in each preparation, indicating that the mouse monoclonal antibody was binding to the same cellular proteins in both control and FLAG-tagged blots. This result also suggests cross-reactivity as the limiting factor in these experiments.

Functionality of recombinant cell lines

Both wildtype (figures 1.10, 1.12) and conjugated (figures 1.21, 1.27) α_{1b}-ARs stably expressed in NCB20 cells were found to be functional, as a phenylephrine (10μM)-induced intracellular free calcium respone was observed. Both fluorimetric analysis (FLAG-tagged construct) and calcium imaging (wildtype and GFP-tagged constructs) were used to demonstrate this. It was noted that within a patch of cells (figure 1.12), individually, cells responded in a quantitatively dissimilar manner, presumably influenced by slight differences in their expression levels.

Using the GFP-tagged human α_{1b}-AR cell line, and alternating the excitation wavelength between 340, 380 and 488nm, Fura-2/GFP emitted fluorescence light at 515nm was monitored allowing time-dependent changes in $[Ca^{++}]_i$ and GFP

intensity to be recorded in response to agonist (figure 1.28a). An unlabelled cell, situated alongside a cell expressing GFP-tagged human α_{1b}-ARs, acted as a control, and as expected, did not elicit an $[Ca^{++}]_i$ in response to the α_1-AR selective agonist, phenylephrine (figure 1.28a). The possibility of GFP photobleaching was assessed, and the overall GFP-intensity of the cell did not change during a 3minute control experiment (figure 1.28c). In unstimulated cells, small oscillations of movement of receptors was observed between the membrane and the intracellular cluster (figure 1.28d). In response to agonist stimulation, during the period of time when Ca^{++} was being released from intracellular stores, the movement of receptors appeard to plateau. After a couple of minutes, movement resumed and an overall increase in the GFP intensity inside the cell and a concomitant decrease associated with the plasma membrane was observed during agonist stimulation (38minutes) (figure 1.28f). This movement of GFP-tagged human α_{1b}-ARs stably expressed in NCB20 cells can be seen the movie in figure 1.28b.

QAPB associating with mouse/human α_{1b}-ARs

When QAPB bound to α_{1b}-ARs stably expressed in NCB20 cells, it was possible to monitor the movement of the receptive sites by following the fluorescent ligand, acquiring images approximately every 2msec. Using the experimental set-up described in methods (*calcium imaging*), it was possible to record 5nM QAPB binding to mouse (figure 1.11a,b) and human (figure 1.13a,b) α_{1b}-ARs over 54 and 41minutes respectively. The outcome of such experiments suggested that QAPB enters cells via an endocytic pathway, as extensive movement of vesicle-

like structures was observed within the cell. The binding of QAPB to α_1-ARs does not elicit a calcium response (figure 1.29).

Discussion

The main aim of this chapter was to stably express recombinant α_{1b}-ARs in a mammalian cell line, to assess their functional and pharmacological characteristics including their subcellular distribution in live single cells using the fluorescent ligand, QAPB. It is thought that the binding of QAPB to α_1-ARs may be altered post-fixation. Validation of the specific binding of QAPB to α_1-ARs was therefore imminent in both fixed and live cells. The re-distribution of α_{1b}-ARs in response to agonist was also of interest.

Radioligand binding assays using plasma membrane fractions eliminate some non-specific binding but they do not reflect the true binding of ligands to receptors on intact, live cells. The fluorescent ligand approach, using confocal laser scanning microscopy (CLSM), allows an estimation of both affinities and specific binding to receptor subtypes on single, live cells. In addition, the subcellular distribution of receptor populations can be assessed.

Initially, NCB20 cells were screened for α-ARs. Confocal and radioligand binding analysis revealed a small population of α_2-ARs, but null for α_1-ARs (figures 1.1 and 1.2). Subsequently, NCB20 cells were stably transfected with mouse/human α_{1b}-ARs, receptor binding capacities of individual clones were examined (by radioligand binding), homogeneity (confocal microscopy) and functional properties (using $[Ca^{++}]_i$ response) were also assessed.

Cell lines demonstrating low non-specifc and high specific binding of ^{3}H-prazosin to plasma membranes bearing mouse and human α_{1b}-ARs were used as a model. It was investigated whether the transfected cells showed the pharmacological characteristics similar to other established recombinant α_{1b}-AR cell lines. Both species of α_{1b}-AR were found to have binding profiles similar to those reported

previously for 'α_{1b}-AR cells' (Kenny et al., 1995; Mackenzie et al., 2000). They displayed relatively low affinities for the α_{1A}-AR selective agonist, A-61603 as well as for the α_{1D}-AR selective antagonist, BMY7378, suggesting a homogeneous population of α_{1b}-ARs.

Both cell lines bound QAPB (figures 1.5 and 1.7) and ^{3}H-prazosin (figure 1.8) with high specificity and affinity. The distribution of QAPB binding was predominantly associated with the plasma membrane, although this did vary, presumably dependent on the stage each cell was at in their cycle. Interestingly, expression of the α_{1b}-AR at both 4000 and 8000fmol/mg showed no difference in the distribution other than intensity of ligand binding at low concentrations of QAPB. It is currently thought that populations of recombinant α_{1b}-ARs found intracellularly represent excess receptors, either at their site of synthesis (Golgi apparatus) or site of degradation (lysosomes). This line of work is currently being investigated in our laboratory and will be discussed later.

Recombinant mouse and human α_{1b}-ARs were functional as shown by release of Ca^{++} from intracellular stores in response to 10μM phenylephrine (figures 1.10 and 1.12).

In order to validate the binding of QAPB and to assess possible alterations in ligand binding post-fixation, the FLAG™ epitope (DYKDDDDK) was attached onto the N-terminus of the mouse α_{1b}-AR (figure 1.17). The construct was thereafter stably expressed in NCB20 cells. From radioligand binding studies using plasma membranes, the non-specific component in both wildtype and FLAG-tagged cells was the same, taking into consideration the difference in expression levels. However, the affinity of prazosin was 30% lower for the FLAG-tagged receptors, probably due to steric interference. With respect to

QAPB binding on living cells, the concentration at which QAPB bound did not differ between the wildtype and FLAG-tagged cells indicating that perhaps this experimental set-up is more sensitive to ligand-receptor interactions as the cells are alive.

The practicability of immunoreactive approaches depends on specific detection of FLAG-fusion proteins with no or little cross-reactivity to cellular proteins. Unfortunately, analysis of the expression and activity of the epitope by Western blot and immunofluorescence studies showed that the monoclonal antibody bound non-specifically to cellular proteins within both control and transfected cells (figure 1.22). A possible explanation for the lack of specificity could be cross-reactivity as both the antibody and the cell line were of mouse origin. A report in the literature has revealed that monoclonal anti-FLAG antibodies react with a new isoform of rat Mg^{++} dependent protein phosphatase β (MPPβ). The occurrence of MPPβ enzymes in various tissues illustrates that anti-FLAG antibodies have to be used with caution when FLAG-tagged proteins are to be investigated in mammalian cells (Schäfer and Braun, 1995). The next stage with this line of investigation will be to express the construct in a non-rodent cell line e.g. HEK 293 to try and reduce cross-reactivity.

This work is of importance to our group as the possibility that fixation changes the binding of QAPB needs further investigation. The immunofluorescence method is ideal for us to locate the receptor, post fixation, and prior to QAPB binding.

In an attempt to validate the binding of QAPB to α_1-ARs in live cells, the construction of a GFP-tagged human α_{1b}-AR cell line held the potential for co-localisation of QAPB with GFP-associated fluorescence. GFP-tagged human α_{1b}-

ARs were stably expressed in NCB20 cells and pharmacological analysis revealed that C-terminal fusion proteins do not perturb normal ligand binding. Addition of this protein (>200aa) to the C-terminus of the human α_{1b}-AR did not alter the ligand binding affinity of ^{3}H-prazosin (figure 1.25) or QAPB (live cells only) (figure 1.24e) nor affect its functionality (figure 1.27).

α_{1b}-ARs have a basal-level of movement between the plasma membrane and their intracellular location. In the absence of agonist ligand, GFP-tagged α_{1b}-ARs are predominantly located at the plasma membrane, with a distinct intracellular population situated in a non-perinuclear orientation (figure 1.28a, syto 13 stain). In response to agonist, during the period of time when $[Ca^{++}]_i$ was being released from intracellular stores, this movement appeared to cease (figure 1.28e) but resumed once calcium levels returned to baseline. During the prolonged period of agonist exposure (35minutes), a symmetrical movement of receptors from the membrane to an intracellular location was observed with an overall increase in receptor density situated intracellularly (figure 1.28f).

GFP fluorescence is usually very pH stable but the variant EGFP is more pH sensitive than wt GFP (pH 7.0-11.5 compared with ~pH 5.5-12 for wt GFP). When interpreting EGFP-associated fluorescence experiments, one must be cautious since movement of EGFP-tagged receptors to intracellular compartments (pH < 7.0) may result in artifactual changes in fluorescence.

Attempts were made to co-localise QAPB binding to GFP-tagged receptors. Unfortunately, co-localisation of the two fluorophores was hampered as both were excited at the same wavelength. By choosing patches of cells expressing low levels of GFP, QAPB bound to similar regions within the cell. Although not ideal experimental conditions, it was illustrated in the cells chosen here, that intense

QAPB binding was associated with the plasma membrane in one cell yet predominantly intracellular in the other. These cells were taken from the same clone, plated onto cover slips at the same time, and incubated for a similar time period. The images from these experiments emphasise the point made previously, that the subcellular distribution of α_{1b}-ARs in recombinant cell lines is influenced by the stage individual cells are at in their cell cycle, not solely dependent on their receptor density. Another example of this was seen in figure 1.5, where NCB20 cells stably expressing mouse α_{1b}-ARs also displayed differences in their distribution within the same clone.

As an alternative method, attempts were made to subtract QAPB-associated fluorescence from GFP-associated fluorescence in the same cell. Interestingly, when I tried to subtract the images, the cell in question appeared to have changed shape upon QAPB binding, and receptors initially present around the outskirts of the cell, appeared to move inside (figure 1.24d). This finding was investigated further. The association of 5nM QAPB with mouse (figure 1.11) and human (figure 1.12) α_{1b}-ARs was monitored, and images captured every 2msec over 54 and 38minutes respectively. In the patch of cells expressing mouse α_{1b}-ARs, QAPB bound to receptors that moved around inside the cell in vesicular-like structures (figure 1.11b). So, in the absence of agonist, this preliminary data suggets that QAPB (α_1-AR selective antagonist) enters cells through a predominantly endocytic mechanism and not solely via membrane penetration. It should also be noted that QAPB does not induce the release of $[Ca^{++}]_i$ (figure 1.29).

Prazosin is lipophilic, so it was logical to assume QAPB was also lipophilic and could pass through the cell membrane by diffusion. QAPB appeared to access and

bind both receptors on the plasma membrane and intracellularly at the same instant with confocal analysis, as could be seen when 1nM QAPB equilibrated with human α_{1b}-ARs over one minute intervals (figure 1.7b). However, this theory is now debateable as some preliminary studies in our laboratory using a more sensitive system, suggest that QAPB enters cells via an endocytic pathway. Such studies show that QAPB co-localises with a lysosomal marker in acidified vesicles, suggesting that QAPB enters cells via an endocytic mechanism as opposed to diffusion through the membrane. In fact, QAPB binding has been blocked in the presence of sucrose/concanavalin A, inhibitors of clathrin-mediated endocytosis (Pediani, manuscript in preparation). However, limited information is available for antagonist binding and basal endocytosis. Most of the work has been with agonists.

In unstimulated cells, receptors are reported to be slowly endocytosed from the cell surface to endosomes (Koenig and Edwardson, 1997). All nucleated cells use endocytosis to continuously remodel their plasma membranes and membrane endocytic recycling acts as a mechanism to balance the internalisation of membrane components. This recycling process is assumed to be constitutive. Cells also use the endocytic pathway to deliver newly synthesised receptors from the golgi complex to the cell surface (Koenig and Edwardson, 1997). This phenomenon would explain the movement of vesicular structures seen when QAPB associated with α_{1b}-ARs, also the basal level of movement of GFP-tagged receptors in unstimulated cells between the membrane and inside the cell.

Tolbert and Lameh. (1998) used an epitope-tagged (EYMPME) human muscarinic cholinergic subtype 1 receptor, transefected into HEK 293 cells, together with an antibody to the epitope (anti-EE), to study internalisation. In their

studies, they found that the antibody to the epitope tag could induce internalisation of the tagged-receptor in the absence of agonist. This study is an example of an atypical ligand interacting with a GPCR in a manner distinct from that of an agonist, capable of inducing internalisation, without initiating an intracellular signal. This group have also shown that the path of internalisation induced by the antibody is identical to that resulting after agonist treatment.

Similarly, Roettger et al. (1997) have shown that an antagonist of the cholecystokinin receptor is capable of mediating its internalisation in the absense of both receptor phosphorylation and second messenger signalling.

Such reports, including the preliminary done work in our laboratory, suggest that the signal for internalisation is independent of second messenger production. Perhaps GPCRs are capable of adopting different conformations, allowing internalisation, with only one (agonist-activated) capable of inducing an intracellular response.

Such fluorescence microscopical methods combined with GFP-tagged receptor cell lines can provide valuable information on the distribution and functional response ($[Ca^{++}]_i$) of receptors in the absence or presence of ligands.

Another construct became available to us during this time, similarly tagged with GFP, except the receptor was under the control of a promoter specific to the mouse α_{1B}-AR gene. In this chapter, the fidelity of this construct, transiently expressed in NCB20 cells, which were previously shown to be α_{1B}-AR negative, was assessed. Minimal GFP-expression was detected in these cells, confirming the fidelity of this promoter.

In summary, the present study shows that the ligand binding properties, pharmacological characteristics and intracellular transduction mechanism of α_{1b}-ARs stably expressed in NCB20 cells appear to be similar to those defined in other recombinant α_{1b}-AR cell lines. Thus, they can be a useful model system for further characterisation of this α_1-AR subtype. The methods developed in this chapter were used to study the pharmacological properties of α_1-ARs in native cells.

Internalisation of a cell surface receptor from the plasma membrane into intracellular compartments, such as vesicles, endosomes, or lysosomes, constitutes one of several processes, which regulates cell surface receptors. The mechanisms by which such receptor internalisation occur is beginning to unfold.

Several lines of evidence indicate that current models of GPCR signalling are insufficient to account for all the intracellular signals associated with GPCR activation. It is now known that many GPCRs regulate MAPK cascades, leading to activation of the ERKs, Jun amino-terminal kinase/stress-activated protein kinase, and p38 MAPK, which function as transcriptional regulators. These Ras-dependent signals are frequently dissociated from the regulation of classical G-protein effectors, such as PLC. Rather, mechanisms whereby GPCRs regulate tyrosine protein kinase activity and induce the formation of mitogenic signalling complexes are involved (see reviews by Luttrell et al., 1999; Hall et al., 1999).

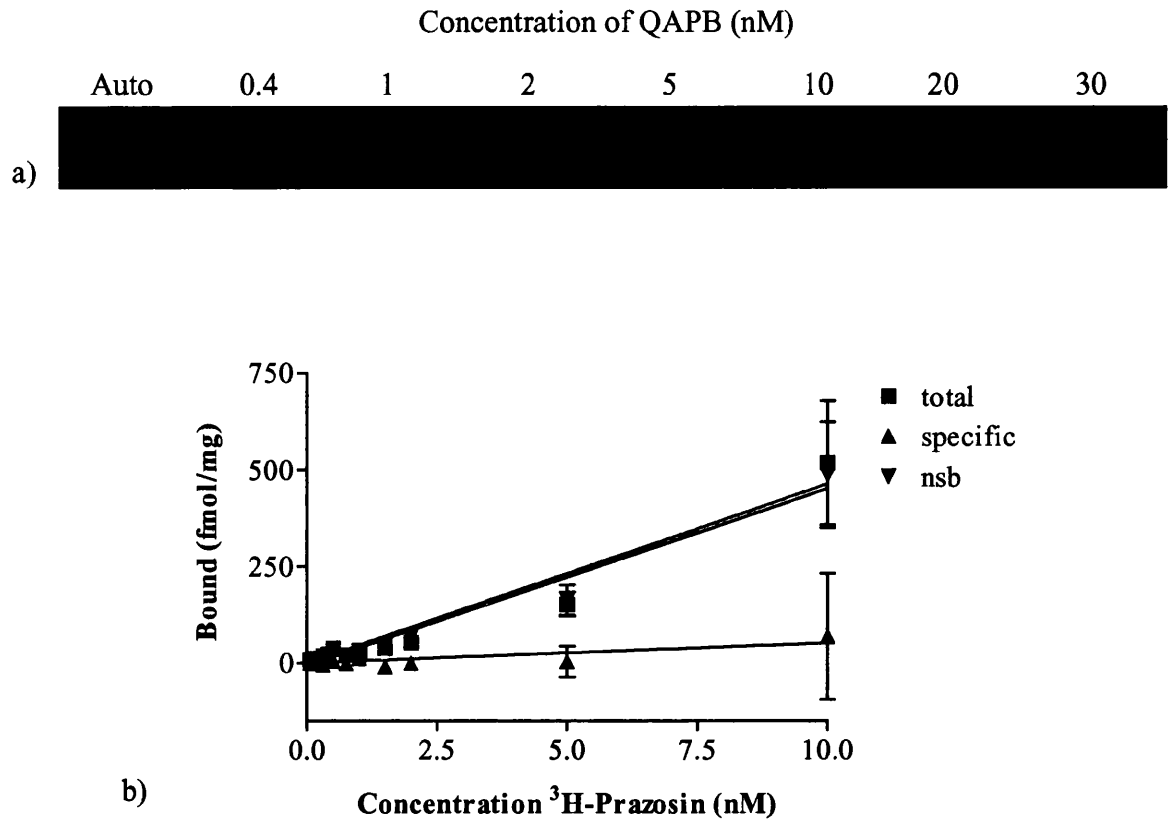
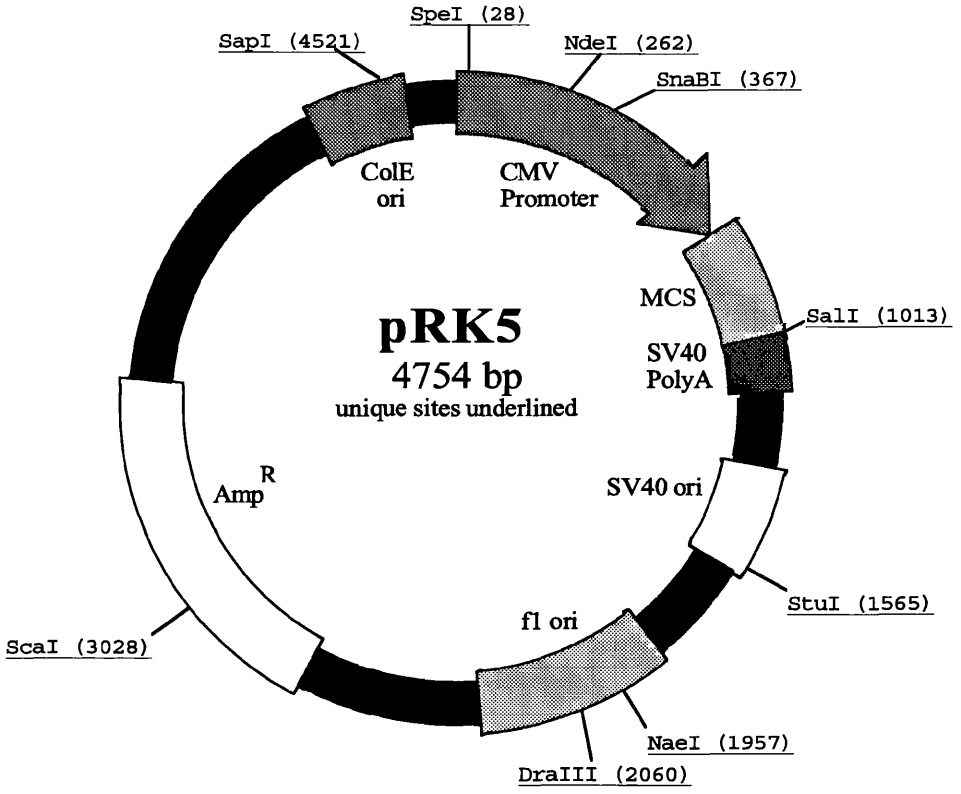
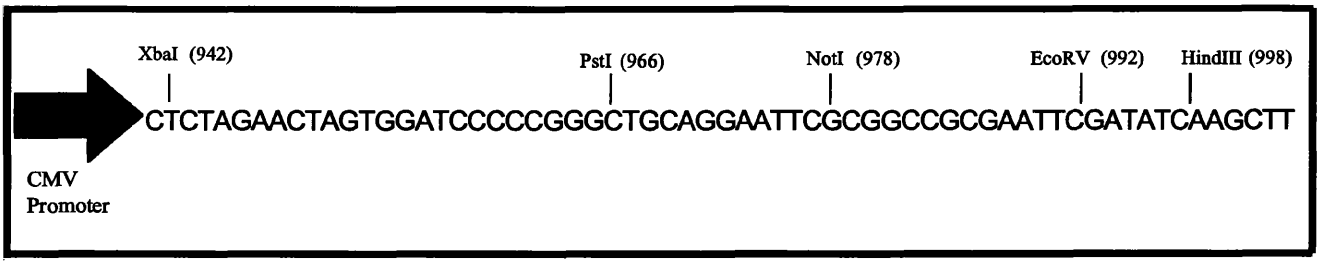
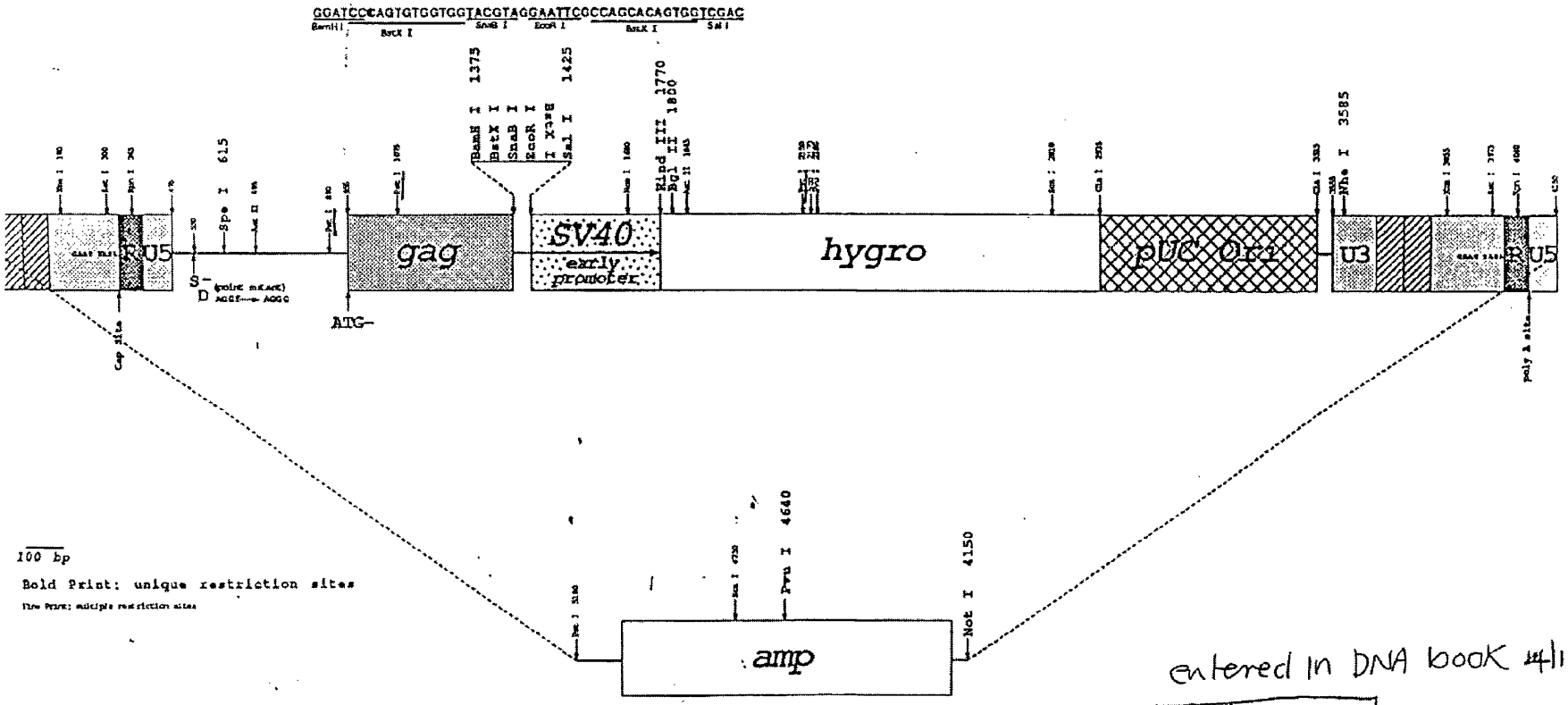


Figure 1.1 (a) QAPB binding on non-transfected NCB20 cells. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-30nM) were added cumulatively and images were collected at 1-minute intervals. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b) Saturation binding of ^{3}H-Prazosin to membranes from non-transfected NCB20 cells. Non-specific binding was determined in the presence of 10μM phentolamine. Results are the mean (±SEM) of at least three experiments performed in duplicate.


SpeI (28)
SapI (4521)
NdeI (262)
SnaBI (367)
ColE ori
CMV Promoter
MCS
SalI (1013)
pRK5
4754 bp
unique sites underlined
SV40 PolyA
Amp R
SV40 ori
StuI (1565)
f1 ori
ScaI (3028)
NaeI (1957)
DraIII (2060)


XbaI (942)
PstI (966)
NotI (978)
EcoRV (992)
HindIII (998)
CTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGCGGCCGCGAATTCGATATCAAGCTT
CMV Promoter

pBabe Hygro (5.2 kb)



entered in DNA book 4/11/91

DNA# 1994

Select for constructs derived from this vector in Ampicillin (100µg/ml) + Hygromycin (25 µg/ml)

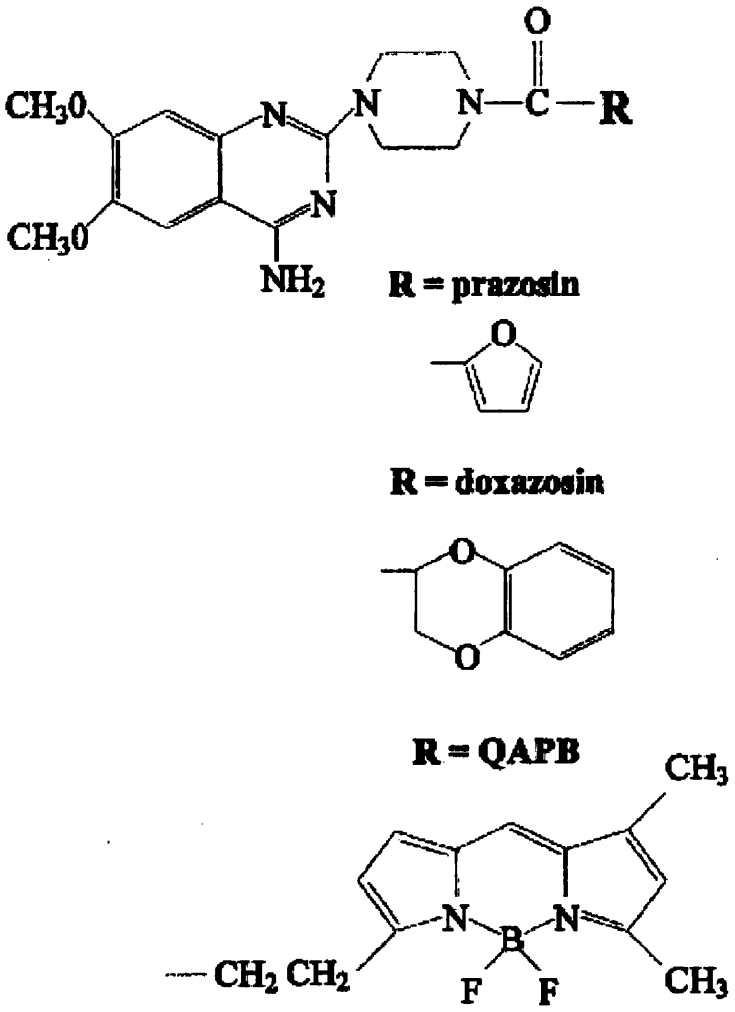
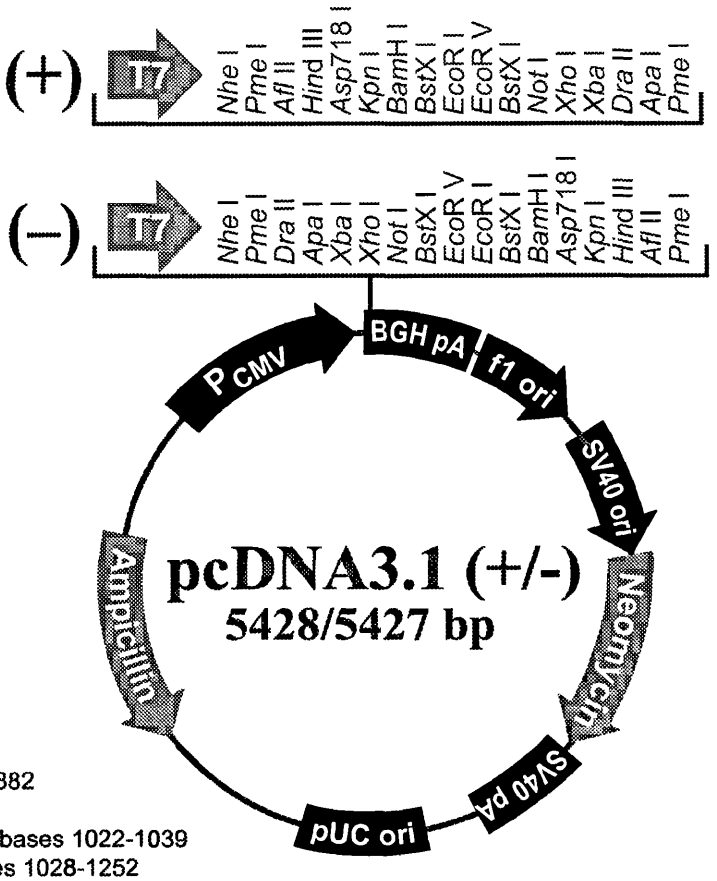


Figure 1.4 The structure of quinazolinyl piperazine (top) and the various substituents (R) that distinguish prazosin, doxazosin and QAPB. This particular form of BODIPY is excited at 488nm and emits above 515nm. The compound was obtained from Molecular Probes and is listed in their catalogue as "BODIPY FL-prazosin" but since it lacks the furan group which defines prazosin, as opposed to other compounds which share the quinazolinyl piperazine group, such as doxazosin, we refer to it by an acronym, "QAPB", derived from its chemical name (quinazolinyl piperazine borate-dipyrromethene).



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

Figure 1.5 QAPB binding on NCB20 cells stably transfected with the mouse α_{1b}-AR. Non-specific binding was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations of QAPB, as indicated, were added cumulatively and images were collected at 1-minute intervals. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. Images are representative of a number of similar experiments carried out using this cell line.

Figure 1.6 Diagram of vector pcDNA3.1 (+).

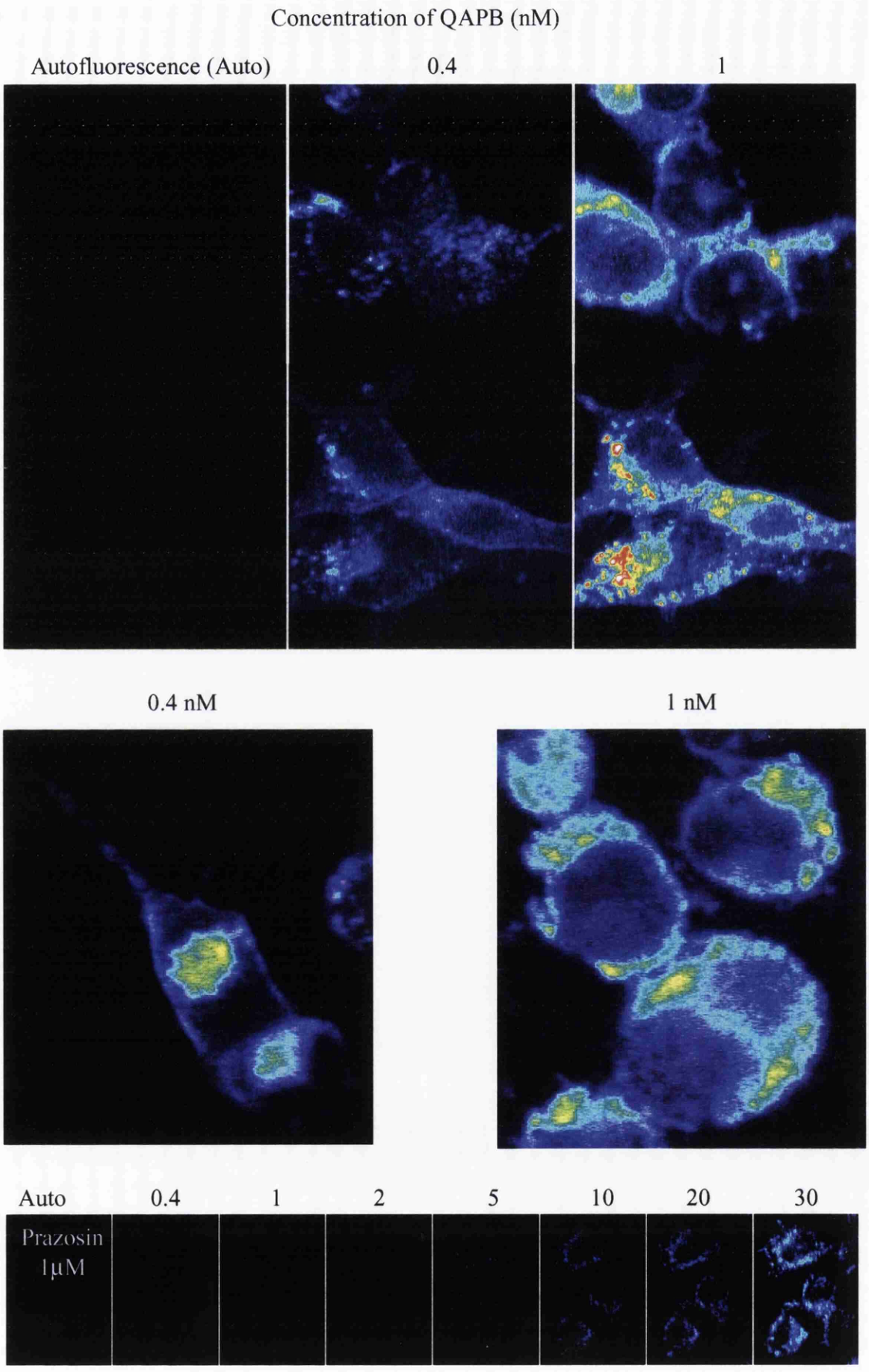
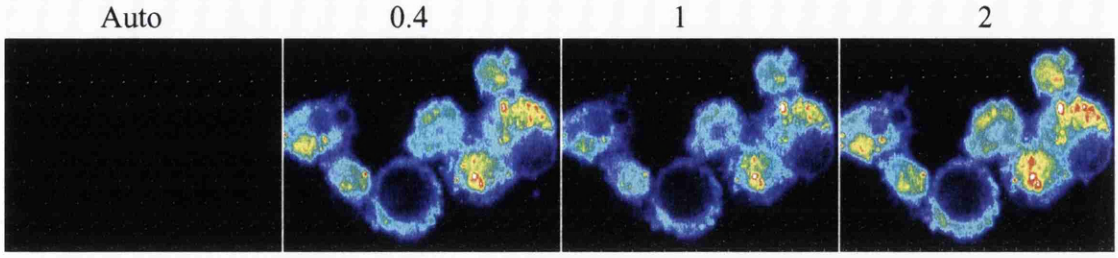
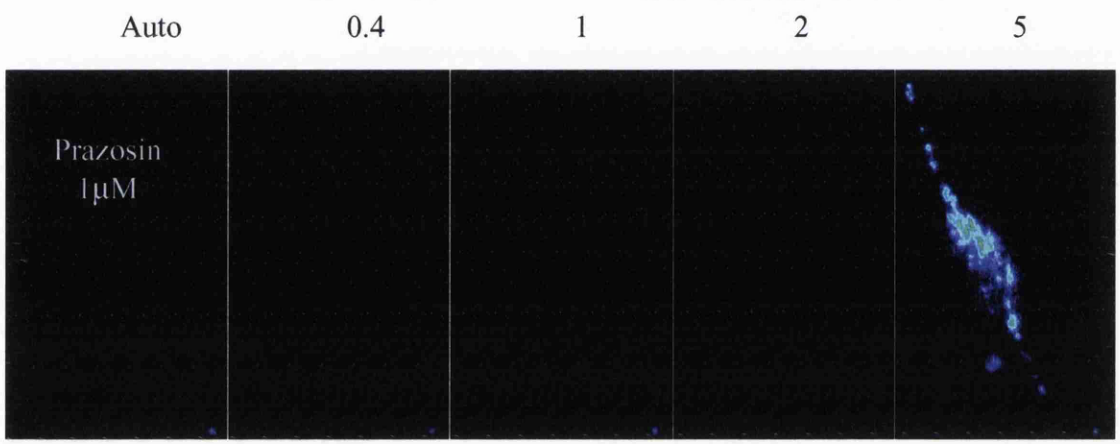

Concentration of QAPB (nM)
Autofluorescence (Auto)
0.4
1
0.4 nM
1 nM
Auto
0.4
1
2
5
10
20
30
Prazosin
1μM

Figure 1.7 QAPB binding on NCB20 cells stably transfected with the human α_{1b}-AR. Non-specific binding was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. (a) Increasing concentrations of QAPB, as indicated, were added cumulatively and images were collected at 1-minute intervals. (b) Equilibration of 1nM QAPB binding to receptive sites over a 5 minute time period. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. Images are representative of a number of similar experiments carried out using this cell line.

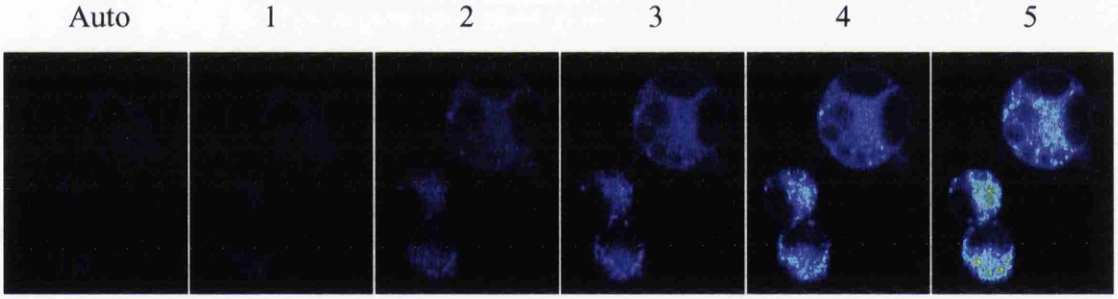
Concentration of QAPB (nM)





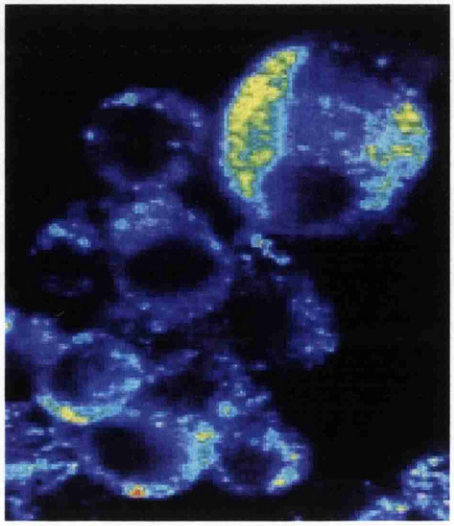
a)

Equilibration of 1nM QAPB over time (mins)



b)

0.4nM



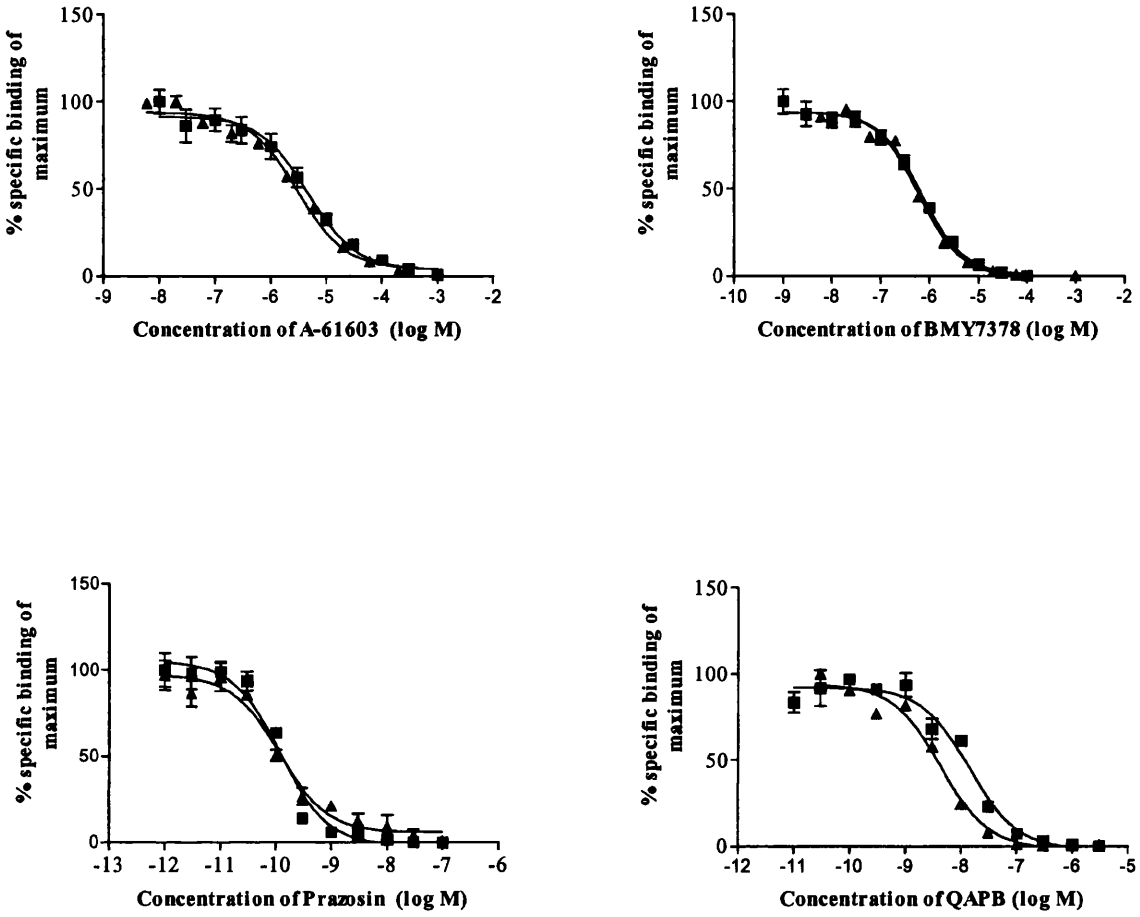


Figure 1.9 Displacement of 0.2nM ^{3}H-Prazosin binding to mouse (■) and human (▲) α_{1b}-AR subtype membranes by increasing concentrations of prazosin, QAPB, A-61603, and BMY-7378. Non-specific binding was determined in the presence of 10μM phentolamine. Values are the mean (±SEM) of at least three experiments performed in duplicate.

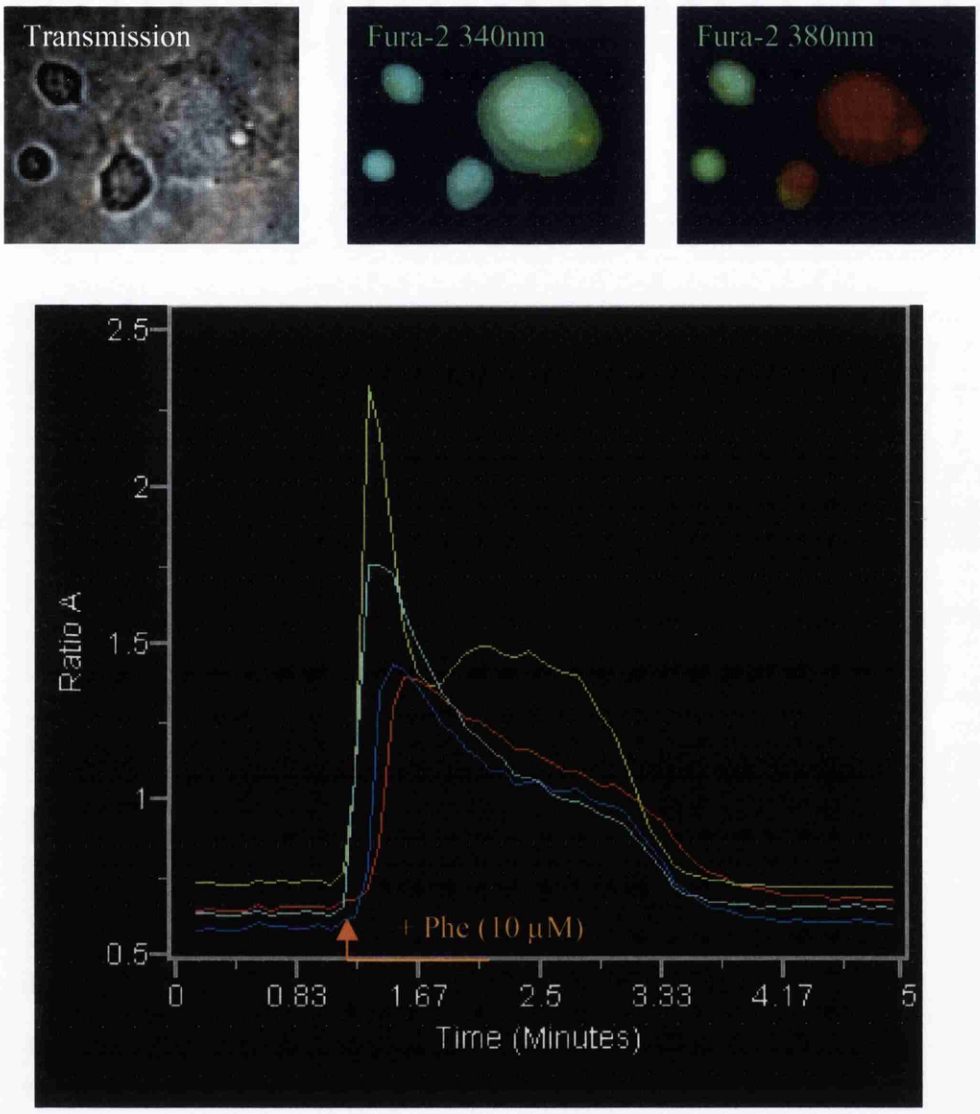


Figure 1.10 Intracellular calcium respone to 10µM phenylephrine in NCB20 cell stably expressing the mouse α_{1b}-AR. Cells were loaded with the fluorescent indicator dye Fura-2/AM (1.5µM) as described in the methods text. Time-dependent changes in $[Ca^{2+}]_i$ were calculated from the ratio of two background subtracted images. A transmission image is also shown.

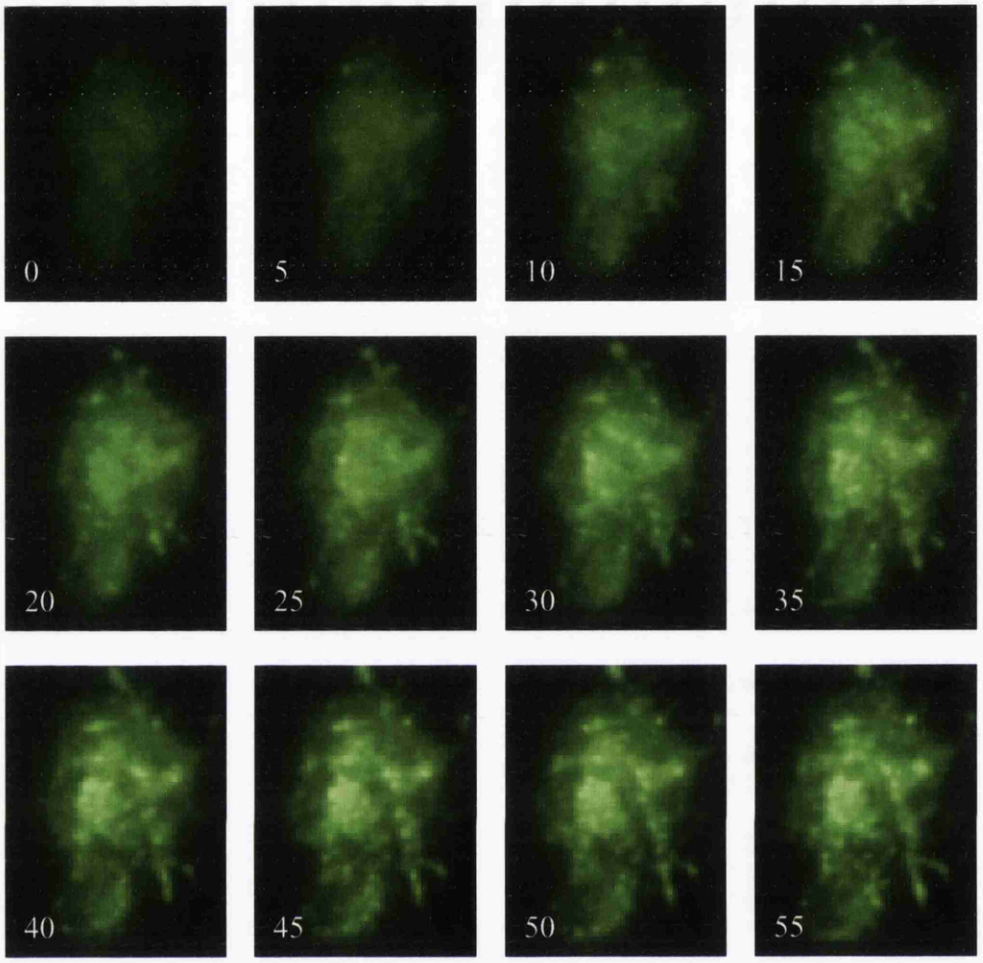


Figure 1.11a 5nM QAPB binding to mouse α_{1b}-ARs stably expressed in NCB20 cells. Association is over a 54min time period.

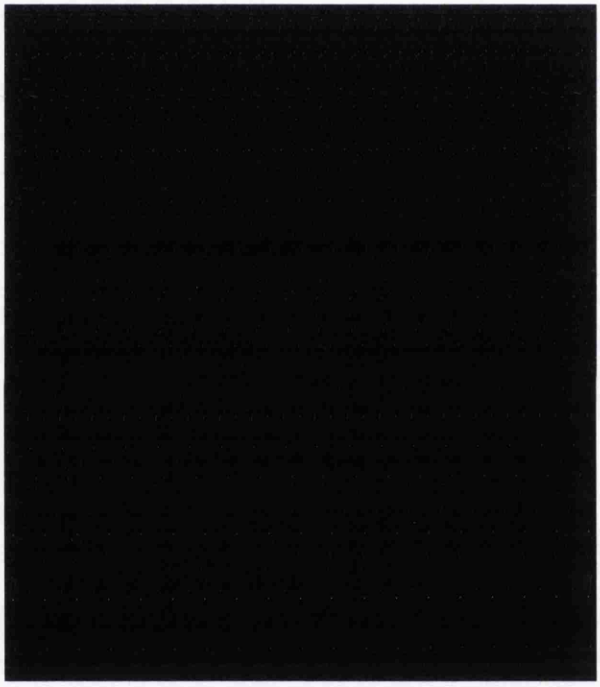


Figure 1.11b A movie of QAPB binding to mouse α_{1b}-ARs stably expressed in NCB20 cell. The movement of these receptors over a 54min time period.

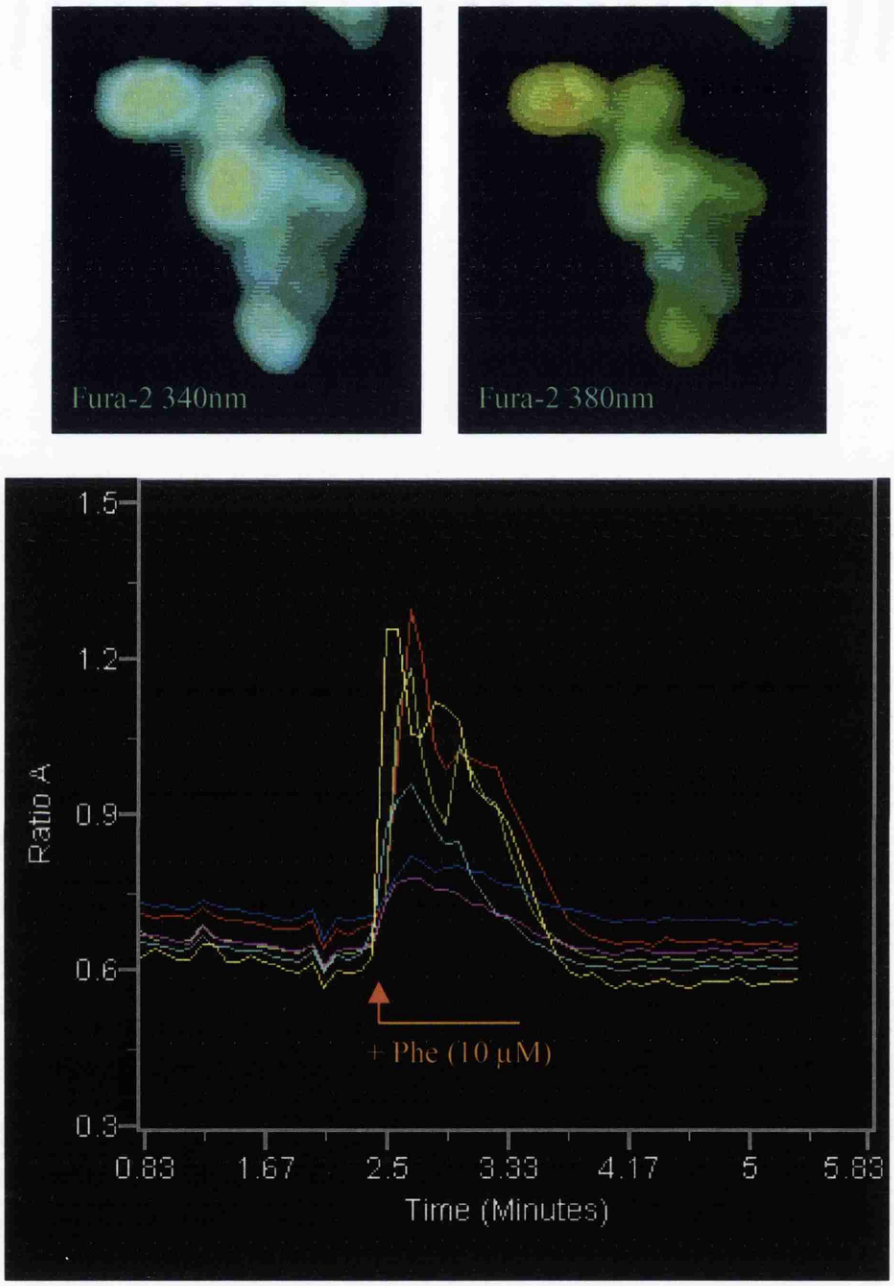
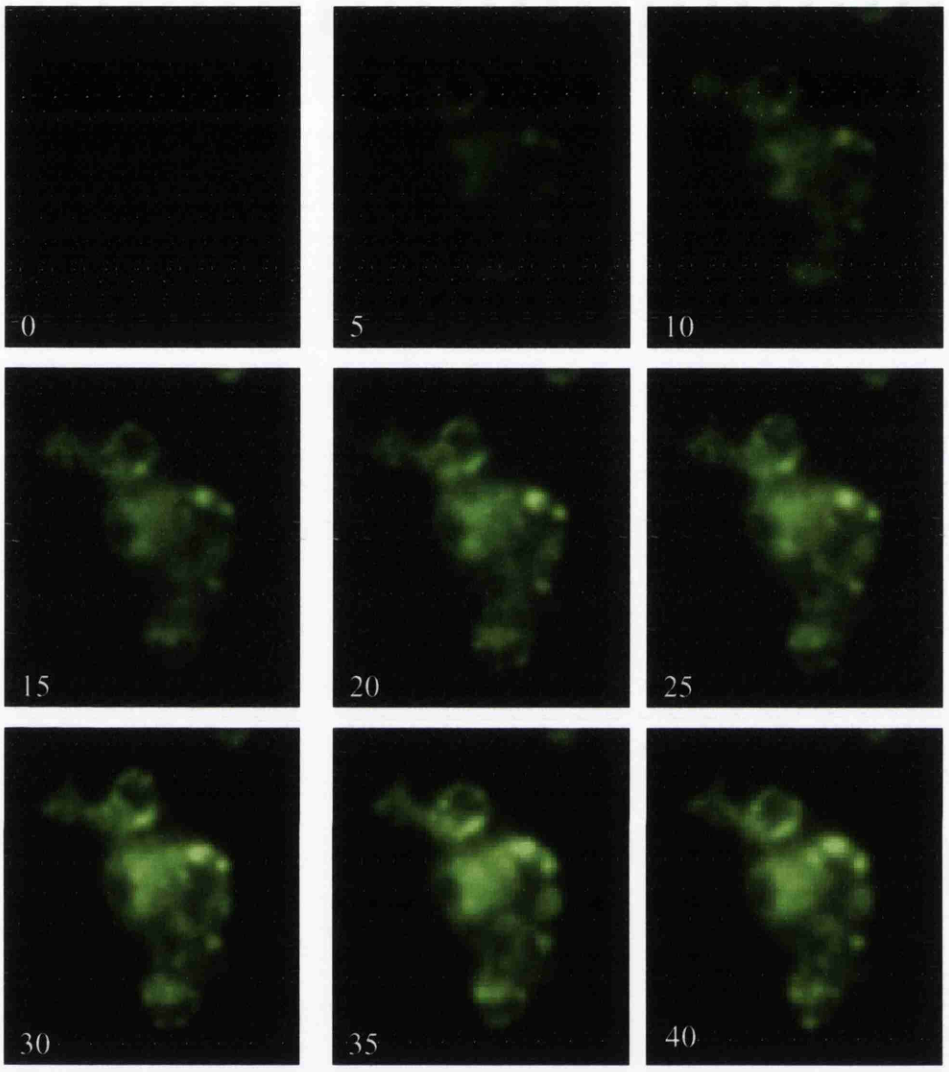
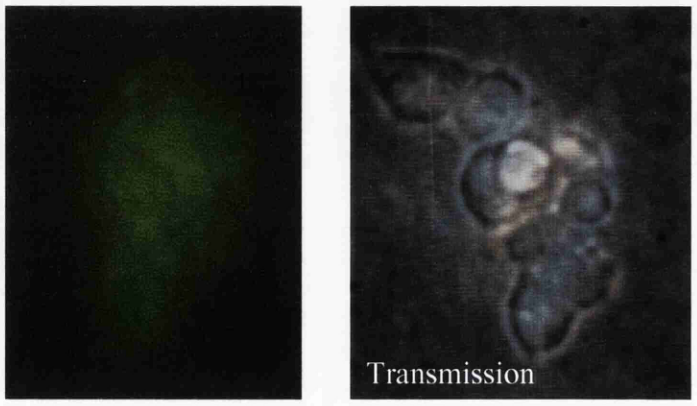


Figure 1.12 Intracellular calcium respone to 10µM phenylephrine in NCB20 cells stably expressing the human α_{1b}-AR. Cells were loaded with the fluorescent indicator dye Fura-2/AM (1.5µM) as described in the methods text. Time-dependent changes in $[Ca^{2+}]_i$ were calculated from the ratio of two background subtracted images.



a)



b)

Figure 1.13 (a) 5nM QAPB binding to human α_{1b}-ARs stably expressed in NCB20 cells. Association is over a 41min time period. (b) A movie of the intracellular movements of the bound receptor, alongside a transmission image.

```
   1 actccgagag ccccattaga ggtaagcatc cccaccctac cttttaactg aagcgtgaaa
  61 caggggggaaa aaaataatcc agcgggcccc tggggtgtat gaaccgggat gcccacaccc
 121 ggatcccctg ctctgctccc cgccccatcc ccgcagaggg agcggtgccg ggcgcggcag
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1981 tcggcgtcac ccagcccggg atacctgggt cgtggaacgc agccacccgt ggagctgtgc
2041 gccttccccg agtggaaacc cggagcgctg ctcagcctgc cagagcctcc tggccgccgc
2101 ggccgcctcg actccgggcc actcttcact ttcaagctcc tgggcgagcc tgagagcccg
2161 gggaccgaag gcgacgccag caatgggggc tgcgacacca caactgacct ggccaacggg
2221 cagccgggct tcaagagcaa catgcccctg gcgcccgggc acttttag
```

Figure 1.14 Nucleotide sequence of the full length mouse α_{1b}-AR

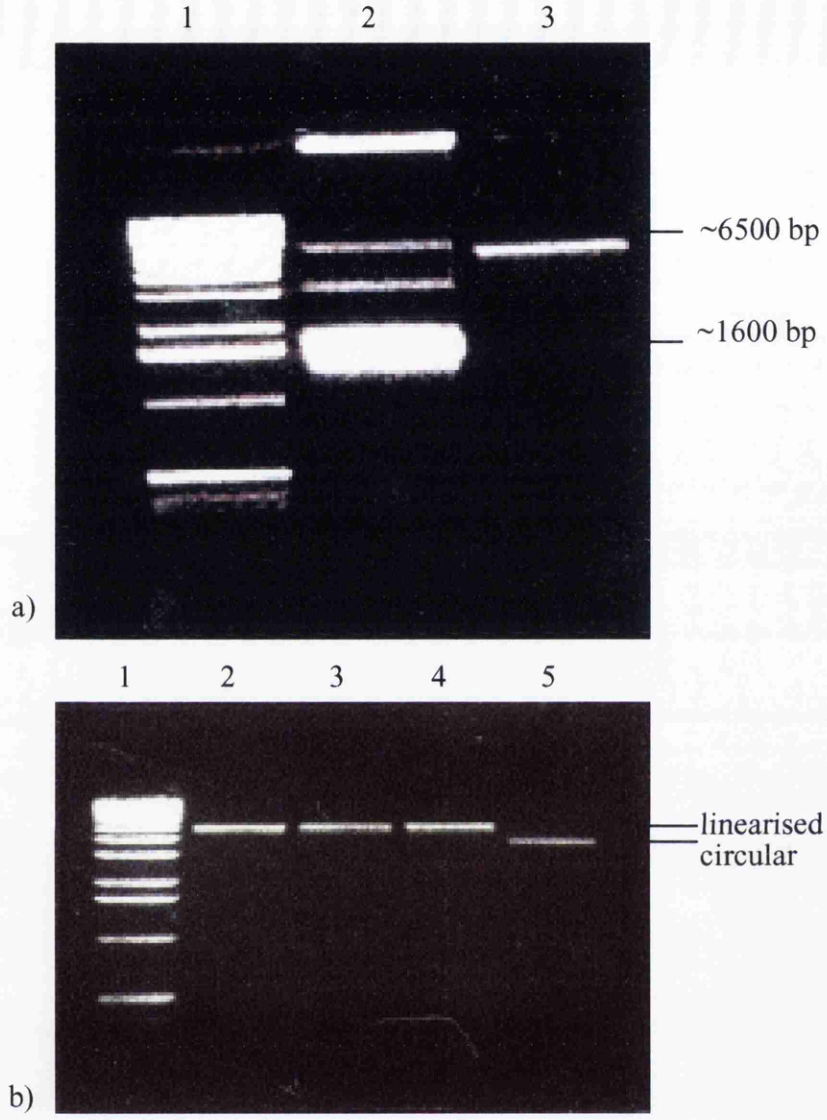


Figure 1.15 (a) PCR reaction products were analysed by agarose gel electrophoresis followed by ethidium bromide staining. Lane 1; separation of molecular weight markers (0.07-12.2kbp). Lane 2; separation of PCR products amplified with FLAG-primers. Lane3; mouse α_{1b}-AR cDNA subcloned into pRK-5 (b) Restriction analysis to check the activity of *NheI* and *XbaI* sites within the MCS of pcDNA3.1 (+). Lane 1; separation of molecular weight markers (0.07-12.2kbp). Lanes 2-5 represent *NheI / XbaI*, *XbaI*, *NheI* digested, and uncut, pcDNA3.1(+) respectively.

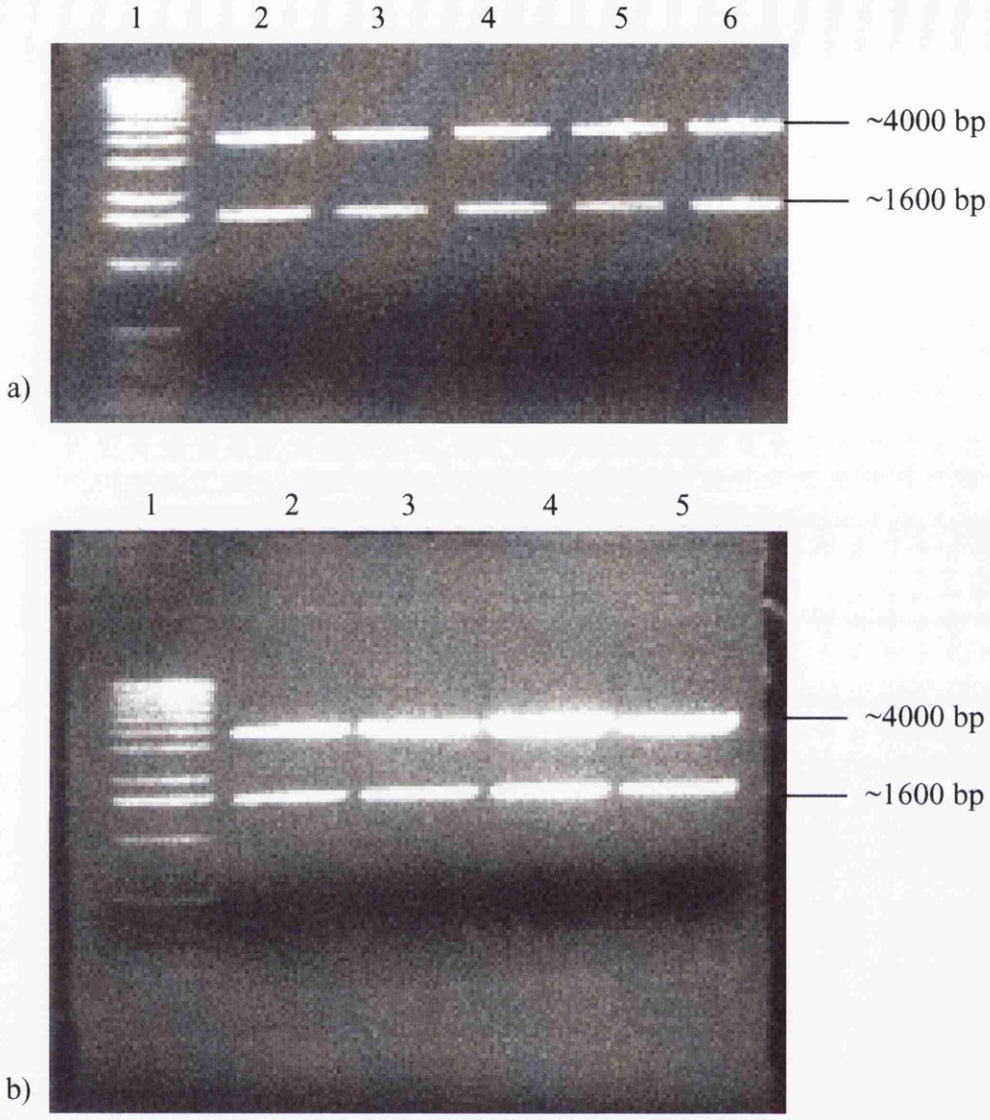
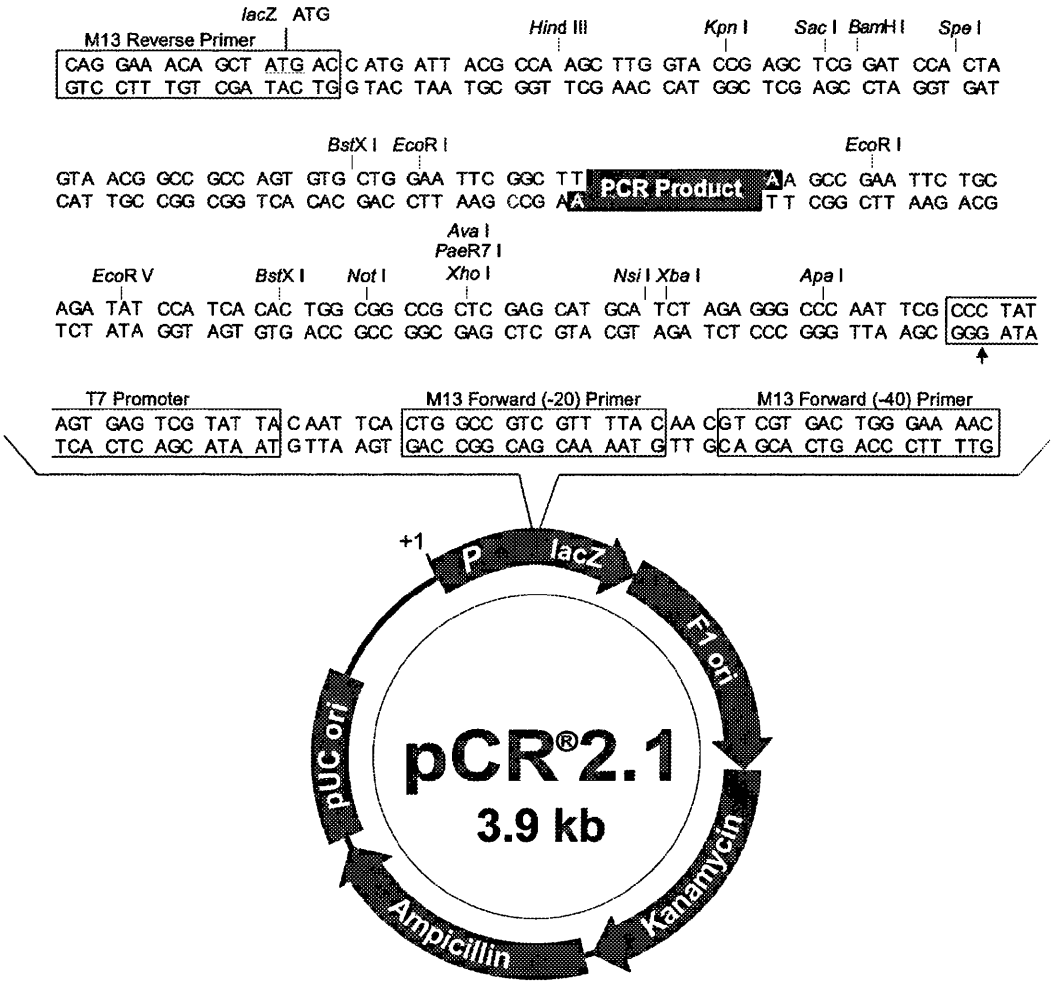


Figure 1.16 Positive colonies from the subcloning of the FLAG-tagged mouse α_{1b}-AR into the MCS of pCR®2.1. Digests were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. (a) Lane 1; separation of molecular weight markers (0.07-12.2kbp). Lanes 2-6; *EcoRI* digests of FLAG-tagged insert from the MCS of pCR®2.1. (b) Lane 1; separation of molecular weight markers (0.07-12.2kbp). Lanes 2-5; *NheI* / *XbaI* digest of FLAG-tagged insert from pCR®2.1.

Figure 1.17 Nucleotide sequence of N-terminally tagged mouse α_{1b}-AR. 81-86; *NheI* site, 87-92; KOZAC sequence, 93-95; start codon, 96-119; FLAG epitope, 120-980; partial sequence of mouse α_{1b}-AR.


lacZ ATG
M13 Reverse Primer
Hind III
Kpn I
Sac I
BamH I
Spe I
CAG GAA ACA GCT ATG AC C ATG ATT ACG CCA AGC TTG GTA CCG AGC TCG GAT CCA CTA
GTC CTT TGT CGA TAC TG G TAC TAA TGC GGT TCG AAC CAT GGC TCG AGC CTA GGT GAT
BstX I
EcoR I
EcoR I
GTA ACG GCC GCC AGT GTG CTG GAA TTC GGC TT
PCR Product
A A GCC GAA TTC TGC
CAT TGC CGG CGG TCA CAC GAC CTT AAG CCG AA
TT CGG CTT AAG ACG
Ava I
PaeR7 I
EcoR V
BstX I
Not I
Xho I
Nsi I
Xba I
Apa I
AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
TCT ATA GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
T7 Promoter
M13 Forward (-20) Primer
M13 Forward (-40) Primer
AGT GAG TCG TAT TA C AAT TCA CTG GCC GTC GTT TTA C AA C GT CGT GAC TGG GAA AAC
TCA CTC AGC ATA AT G TTA AGT GAC CGG CAG CAA AAT G TT GCA GCA CTG ACC CTT TTG
+1
P
lacZ
F1 ori
pUC ori
pCR®2.1
3.9 kb
Ampicillin
Kanamycin

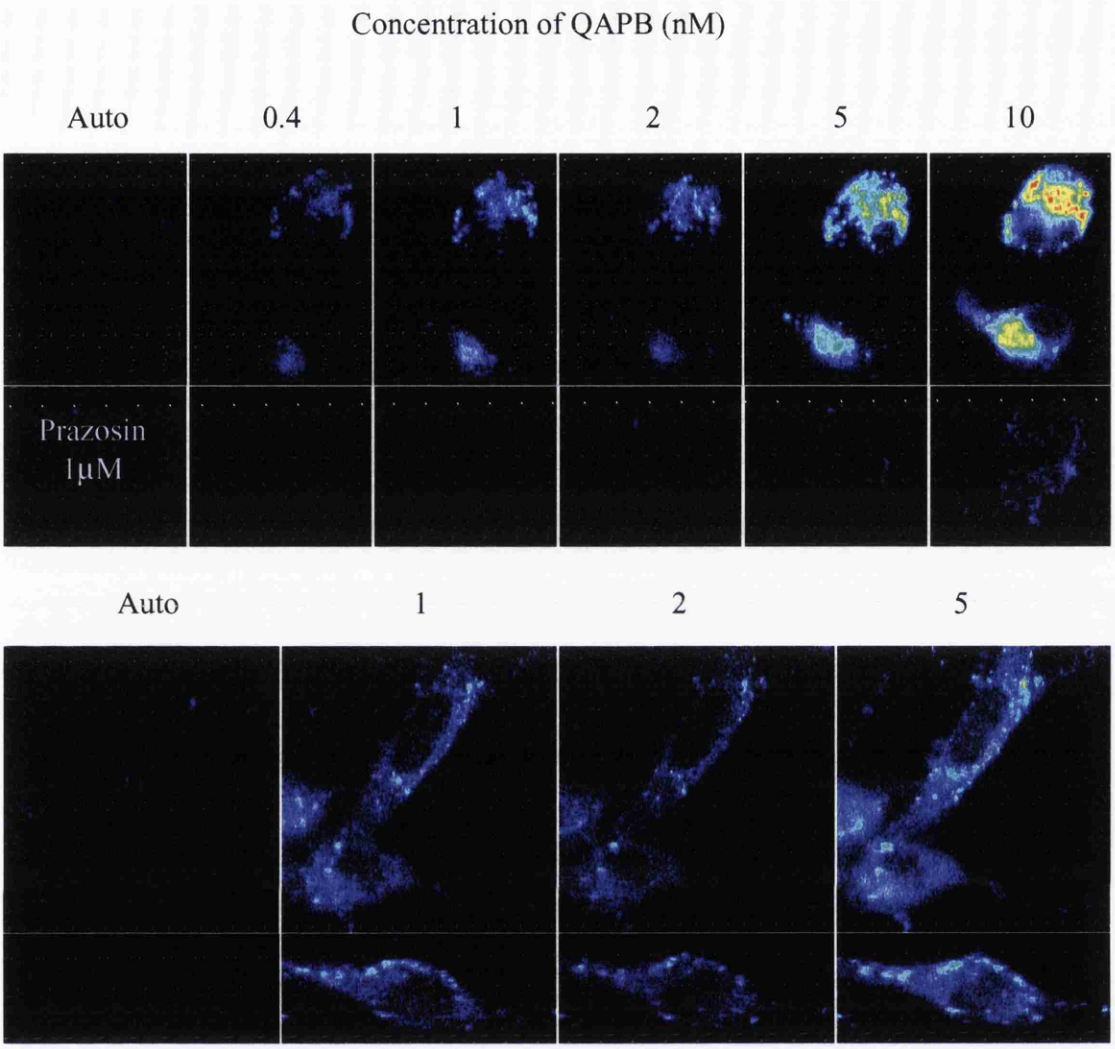


Figure 1.18 QAPB binding on NCB20 cells stably transfected with FLAG-tagged mouse α_{1b}-AR. Non-specific binding was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations of QAPB, as indicated, were added cumulatively and images were collected at 1-minute intervals. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore.

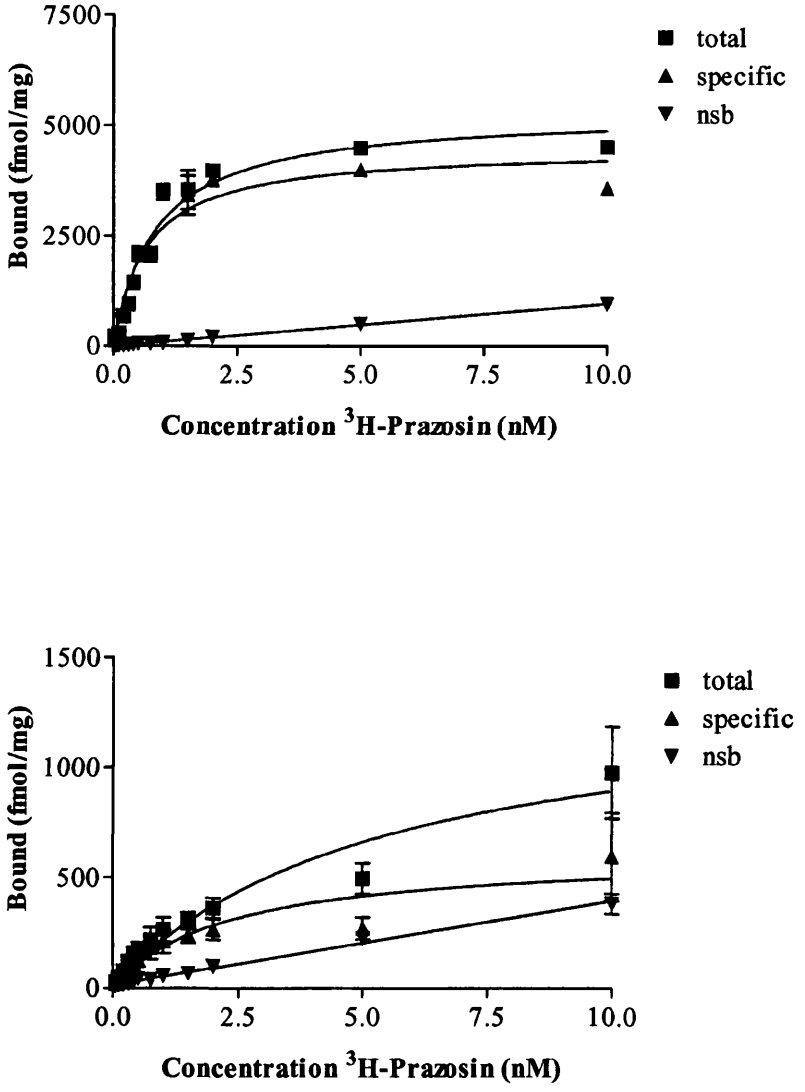
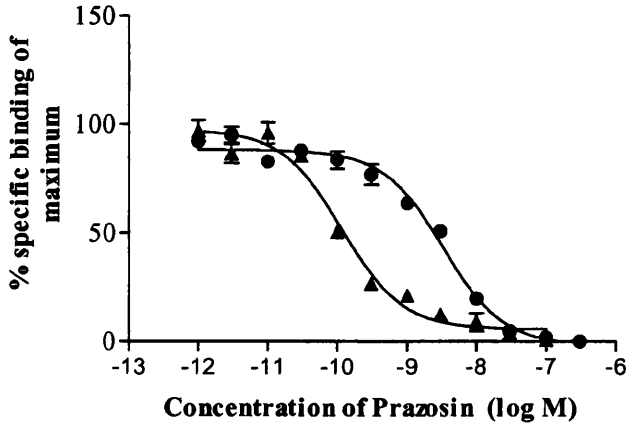


Figure 1.19 Specific saturation binding of ^{3}H-Prazosin to membranes from NCB20 cells, stably expressing mouse (top) and FLAG/mouse (bottom) α_{1b}-ARs. Non-specific binding was determined in the presence of 10μM phentolamine. Data are the mean (±SEM) of at least three experiments performed in duplicate.



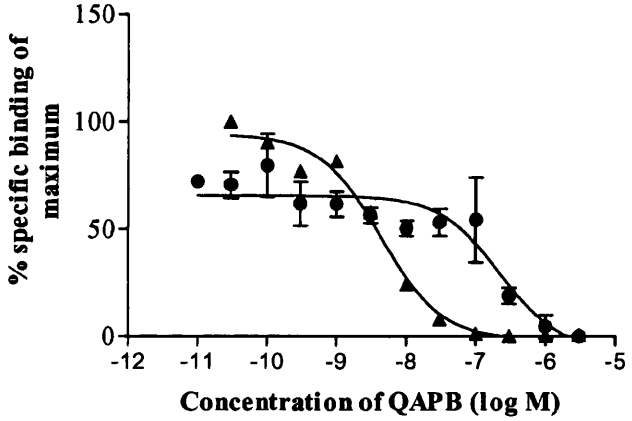


Figure 1.20 Displacement of 0.2/2nM 3H-Prazosin binding to mouse (▲) and FLAG-tagged (●) α_{1b}-AR subtype membranes, respectively, by increasing concentrations of prazosin and QAPB. Non-specific binding was determined in the presence of 10μM phentolamine. Values are the mean (±SEM) of at least three experiments performed in duplicate.

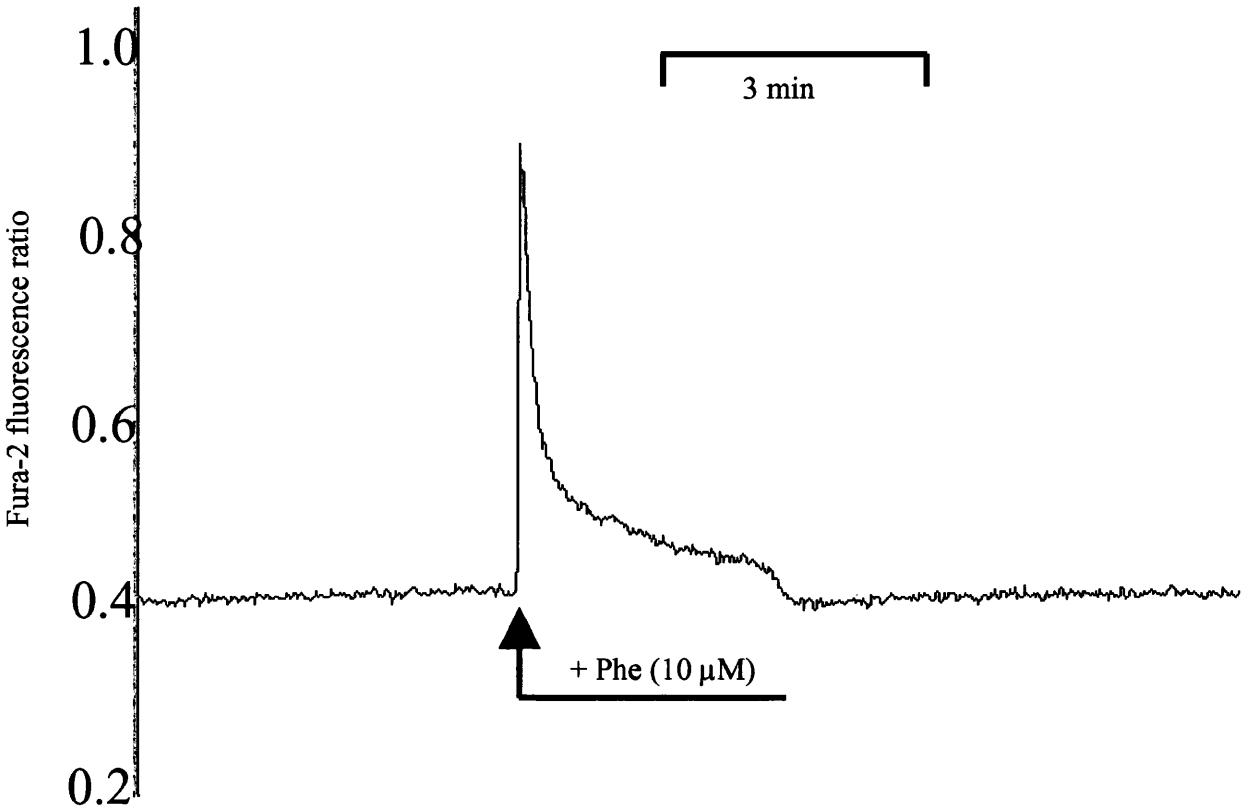
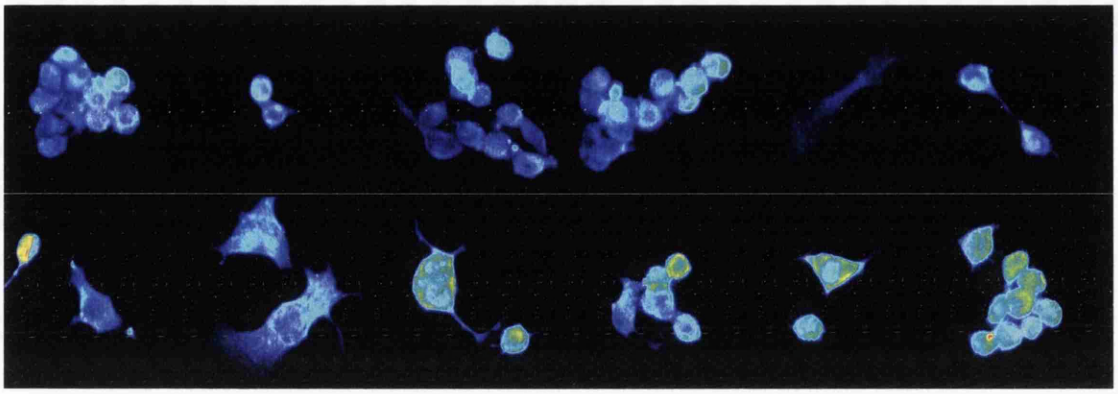


Figure 1.21 Intracellular calcium respone to 10μM phenylephrine in NCB20 cell stably expressing the FLAG-tagged mouse α_{1b}-AR. Cells were loaded with the fluorescent indicator dye Fura-2/AM (1μM) as described in the methods text. The peak calcium elevation was quantified by the dual wavelength ratio method following addition of the indicated concentration of agonist.

a)



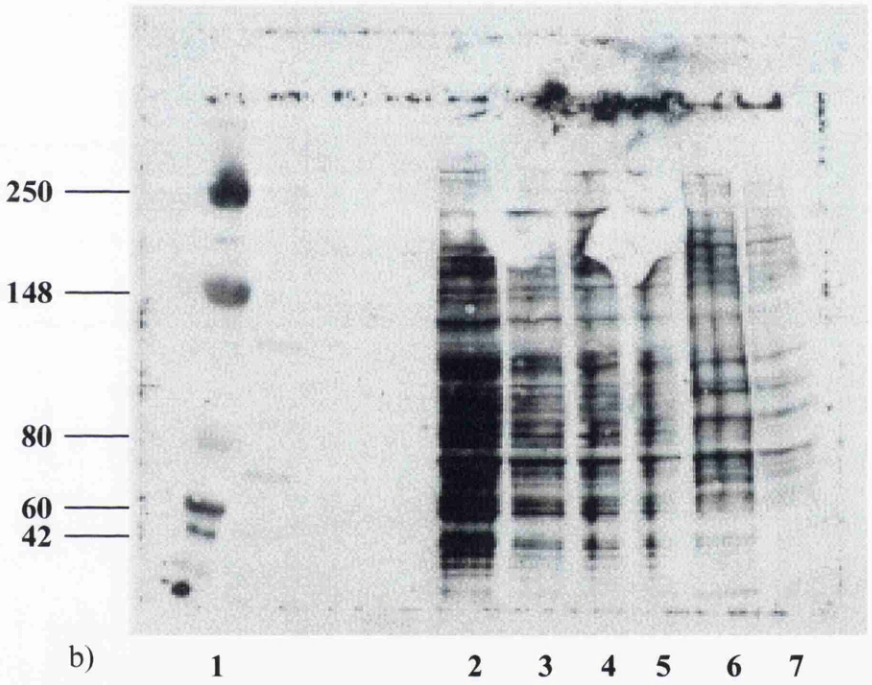


Figure 1.22 (a) Demonstration of the lack of immunostaining specificity of Anti-Flag ® M5 monoclonal antibody in NCB20 cells stably transfected with FLAG-tagged mouse α_{1b}-AR (top panel) and non-transfected cells (bottom panel). Immunostaining procedures were performed as described in Methods. (b) Western blot showing lack of specificty of Anti-Flag ® M5 monoclonal antibody to the FLAG-tagged mouse α_{1b}-AR. (Lanes 2, 3) FLAG-tagged plasma membranes; (Lanes 4, 5) contain non-transfected NCB20 plasma membranes; (Lanes 6, 7) contain non-transfected NCB20 cells. The dilution of both Anti-Flag ® M5 monoclonal antibody and secondary antibody (Goat anti-mouse IgG-HRP) was 1:2000. Molecular mass (kDa) is given at the left.

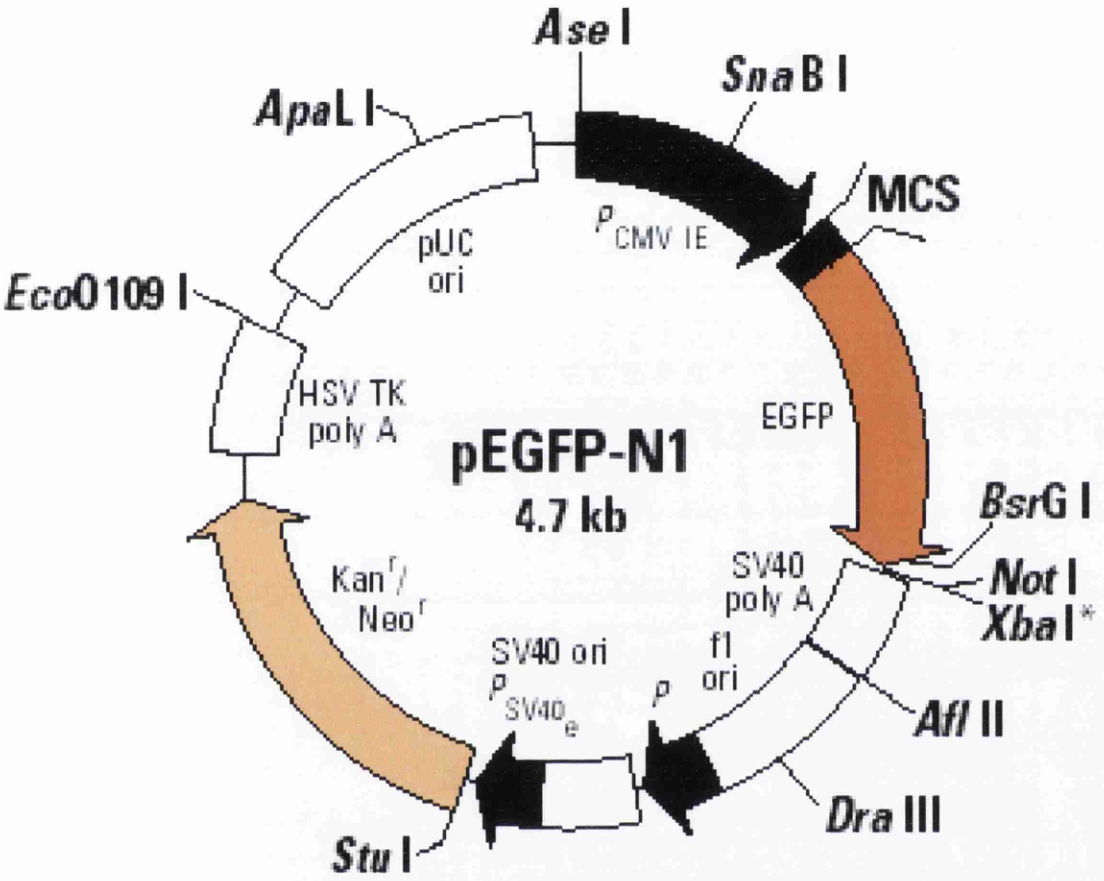
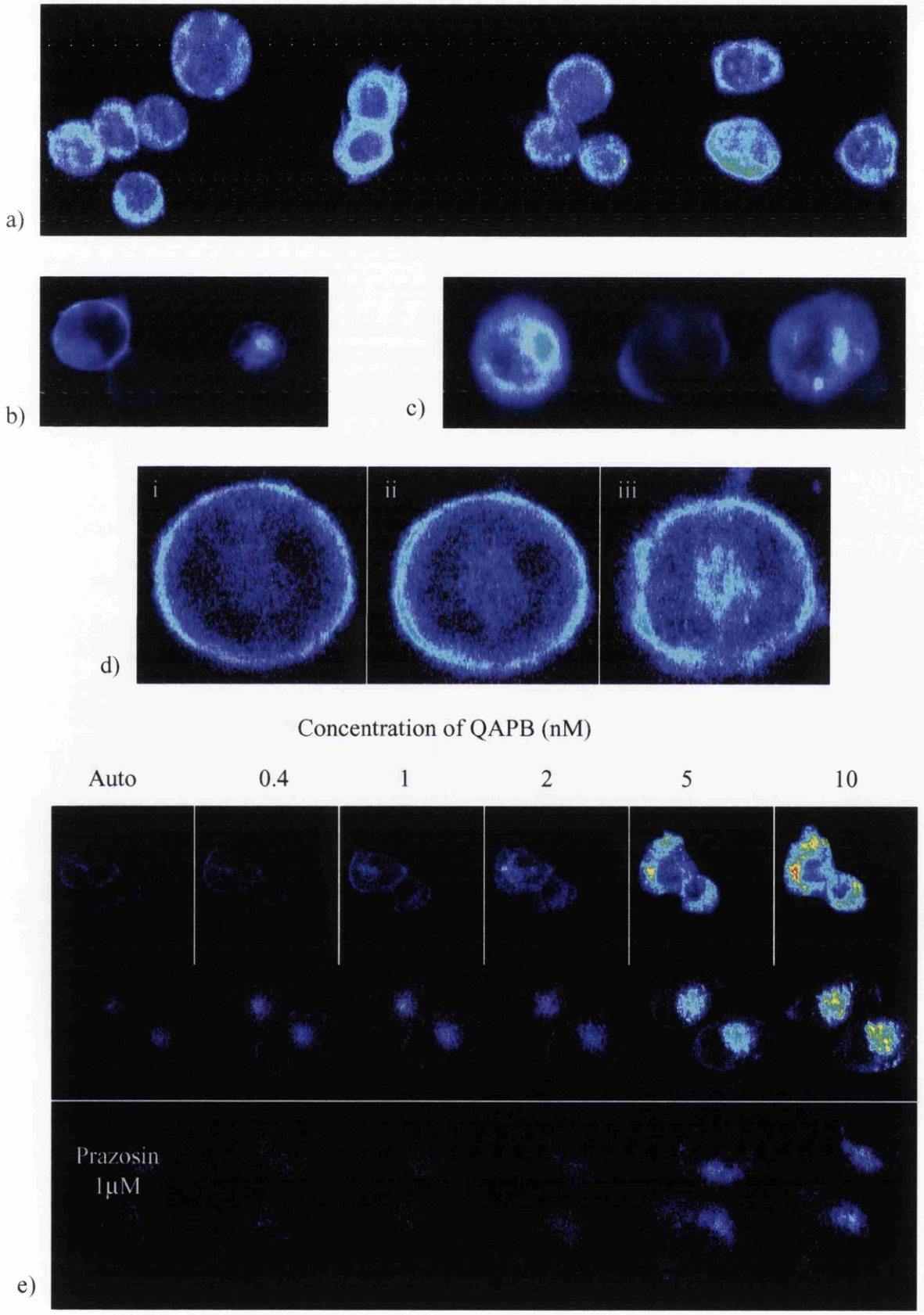


Figure 1.23 Diagram of vector pEGFP-N1

Figure 1.24 NCB20 cells were transfected with GFP-tagged human α_{1b}-AR constructs. (a, d) GFP-associated fluorescence represents the stable expression of pEGFP/human α_{1b}-AR in NCB20 cells. (di) GFP-associated fluorescence alone, (d ii, iii) 2nM QAPB binding to the same cell. (b) GFP-associated fluorescence represents the transient expression of pEGFP/human α_{1b}-AR. (c) GFP-associated fluorescence represents the transient expression of pmouseα_{1B}-AR /human α_{1b}-AR. (e) QAPB binding to NCB20 cells stably expressing low levels of pEGFP/human α_{1b}-AR. Non-specific binding was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-10nM) were added cumulatively and images were collected at 1-minute intervals. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore.


a)
b)
c)
i
ii
iii
d)
Concentration of QAPB (nM)
Auto
0.4
1
2
5
10
Prazosin
1μM
e)

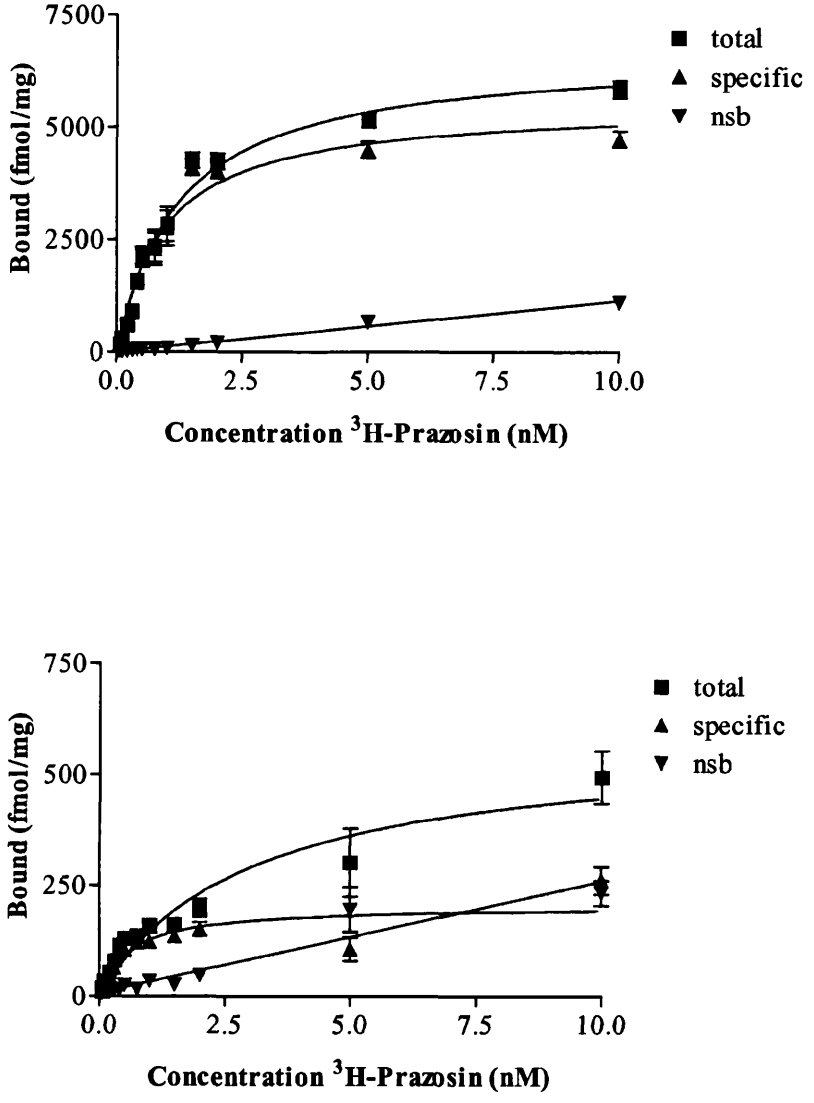


Figure 1.25 Specific saturation binding of ^{3}H-Prazosin to membranes from NCB20 cells, stably expressing human (top) and GFP-tagged (bottom) α_{1b}-ARs. Non-specific binding was determined in the presence of 10μM phentolamine. Data are the mean (±SEM) of at least three experiments performed in duplicate.

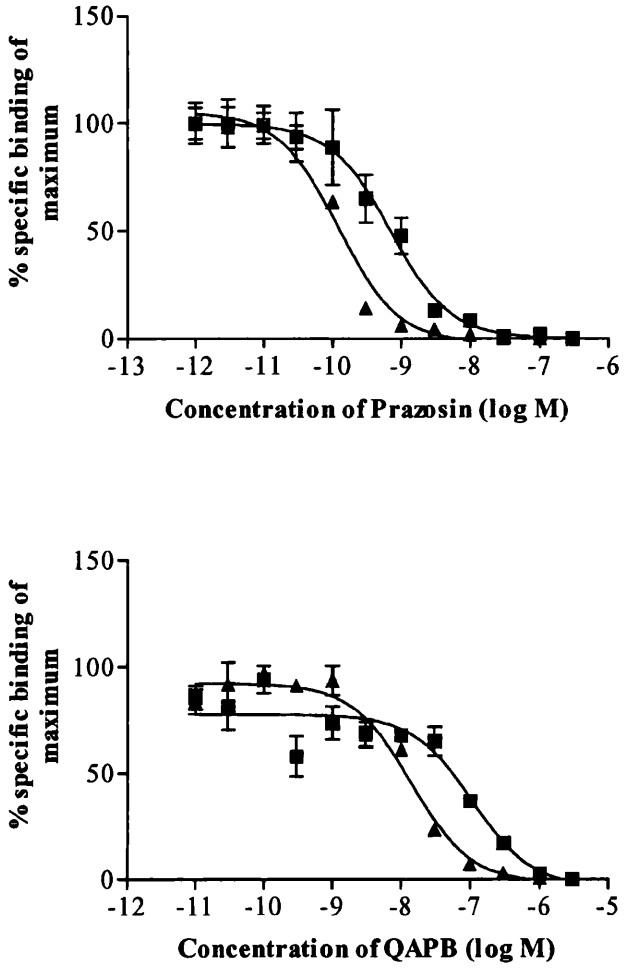


Figure 1.26 Displacement of 0.2nM 3H-Prazosin binding to human (▲) and GFP-tagged (■) α_{1b}-AR subtype membranes, respectively, by increasing concentrations of prazosin and QAPB. Non-specific binding was determined in the presence of 10μM phentolamine. Values are the mean (±SEM) of at least three experiments performed in duplicate.

	(i)		(ii)			
α_{1b}-AR construct	K_D	B_{max}	K_i (nM)			
	(nM)	(fmol/mg)	prazosin	QAPB	A-61603	BMY7378
mouse α_{1b}-AR	0.7±0.16	4467±363.4	0.09±0.1	3.4 ±0.09	3651±0.07	532 ±0.05
human α_{1b}-AR	0.9±1.67 0.9±0.17	7781±566.5 5501±346.7	0.10 ±0.1	11 ±0.1	2458 ±0.09	499 ±0.07
FLAG/mouse α_{1b}-AR	2.3±0.83	608.9±94.74	1.83 ±0.08	119 ±0.2	-	-
pEGFP/human α_{1b}-AR	0.6±0.26	203.7±28.19	0.44 ±0.06	75 ±0.2	-	-

Table 1 (i) ^{3}H-Prazosin saturation binding parameters in membranes from NCB20 cells stably transfected with α_{1b}-AR constructs. (ii) Comparison of affinity estimates from NCB20 cells stably transfected with α_{1b}-AR constructs. Data are means ±SEM of a minimum of three experiments.

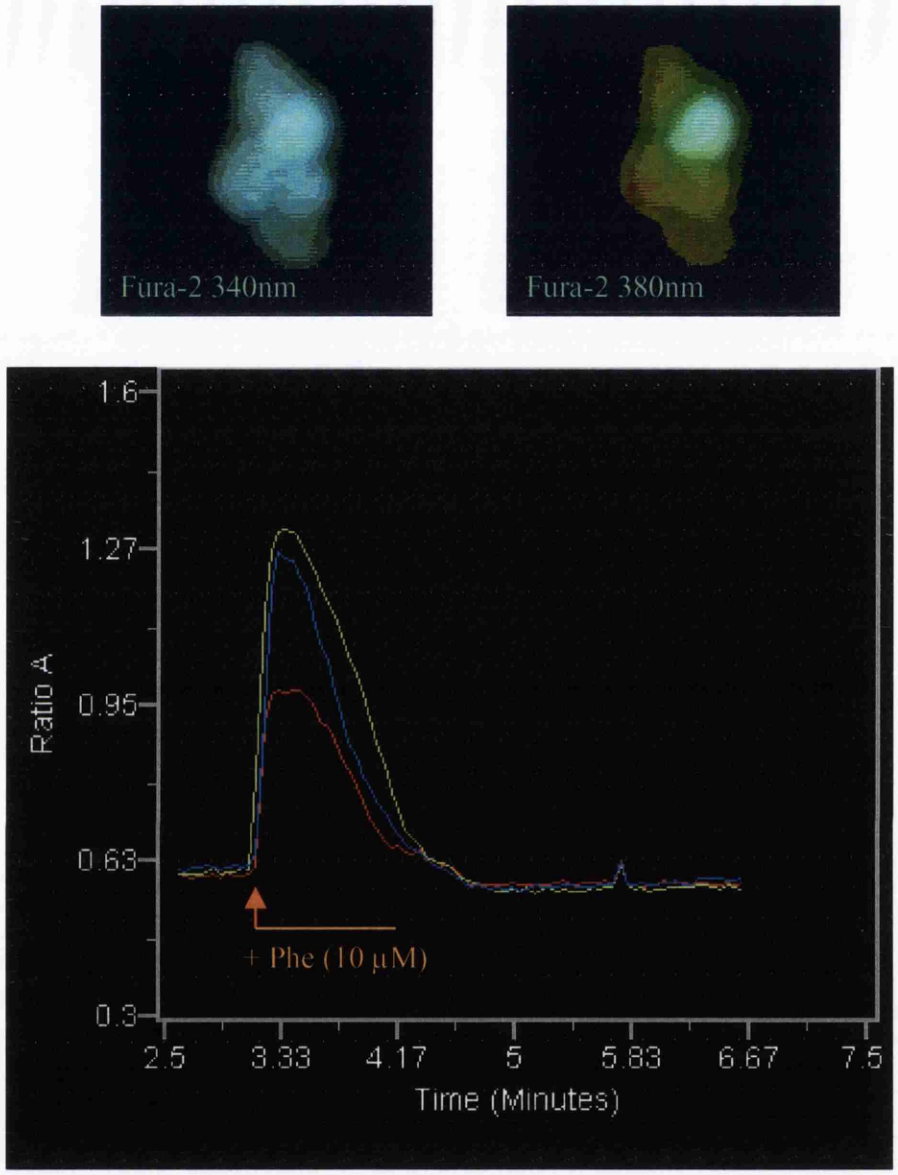


Figure 1.27 Intracellular calcium respone to 10µM phenylephrine in NCB20 cell stably expressing GFP-tagged human α_{1b}-ARs. Cells were loaded with the fluorescent indicator dye Fura-2/AM (1.5µM) as described in the methods text. Time-dependent changes in $[Ca^{2+}]_i$ were calculated from the ratio of two background subtracted images.

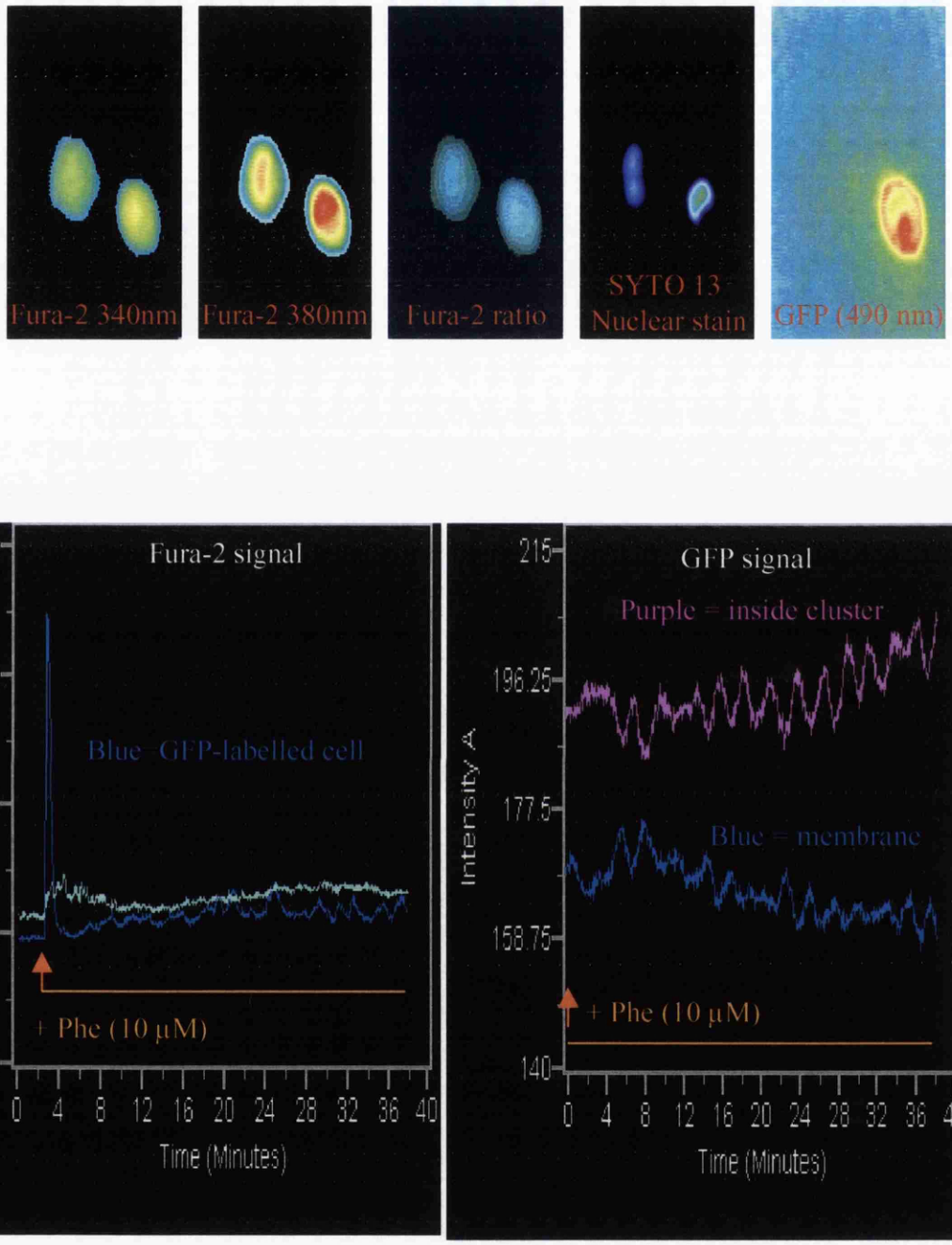


Figure 1.28a Changes in $[Ca^{2+}]_i$ and GFP intensity in response to phenylephrine (10µM) in NCB20 cells stably expressing GFP-tagged human α_{1b}-ARs. Cells were loaded with the fluorescent indicator dye Fura-2/AM (1.5µM) as described in the methods text. Time-dependent changes in $[Ca^{2+}]_i$ were calculated from the ratio of two background subtracted images. Changes in GFP intensity in the membrane and intracellular region were also recorded over time. The nucleus was labelled with SYTO 13.

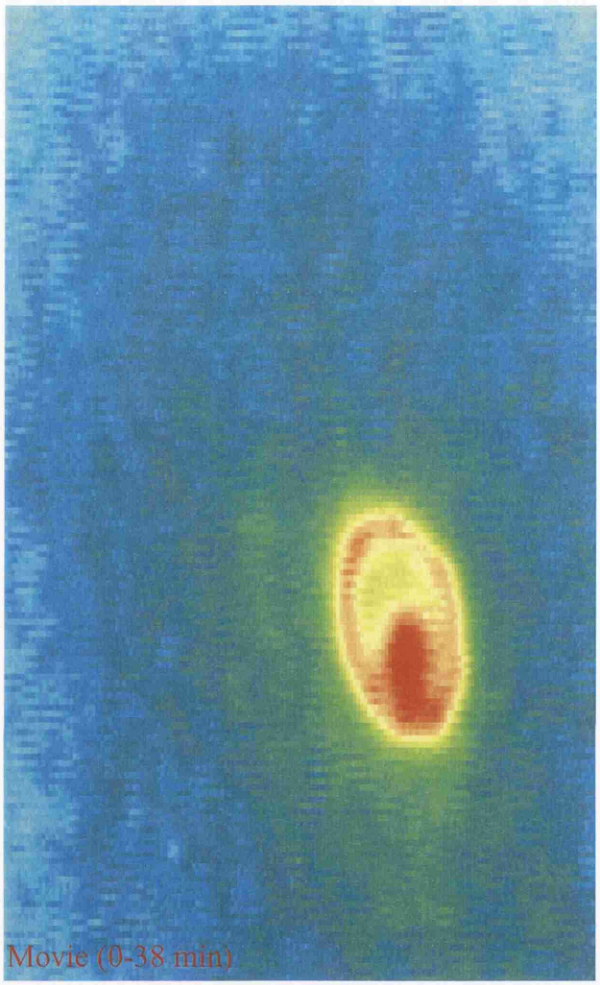


Figure 1.28b Movie showing movement of GFP-tagged human α_{1b}-ARs stably expressed in NCB20 cells in response to 10μM phenylephrine

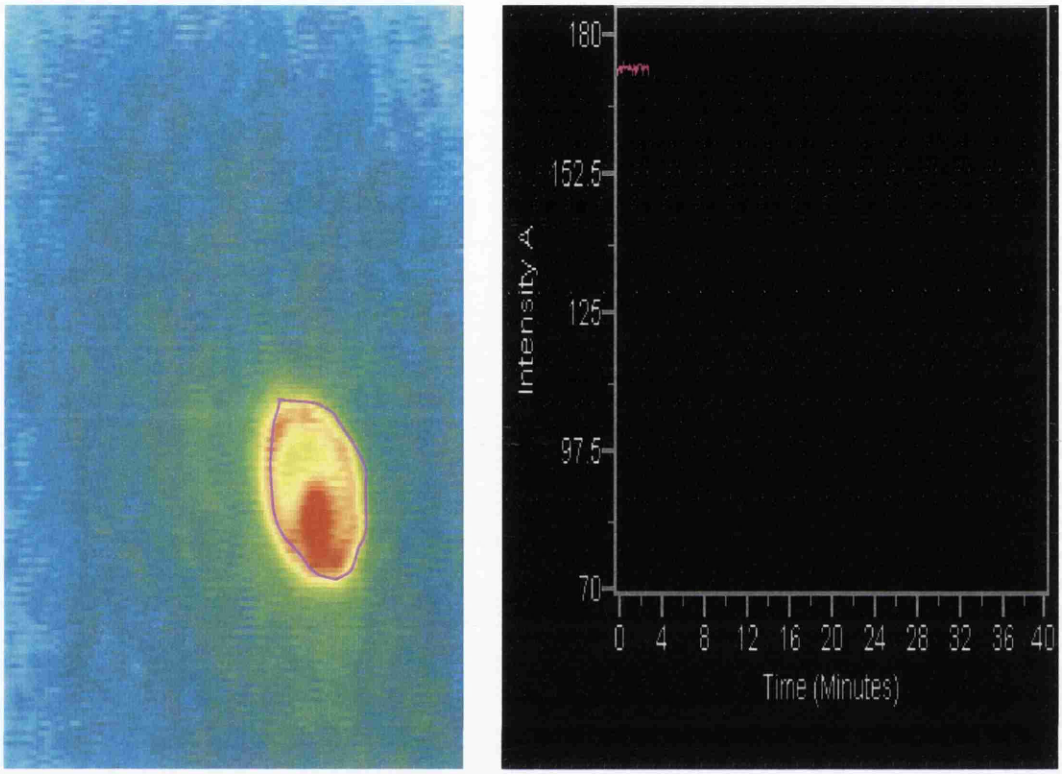


Figure 1.28c Overall GFP-intensity in the cell prior to agonist stimulation.

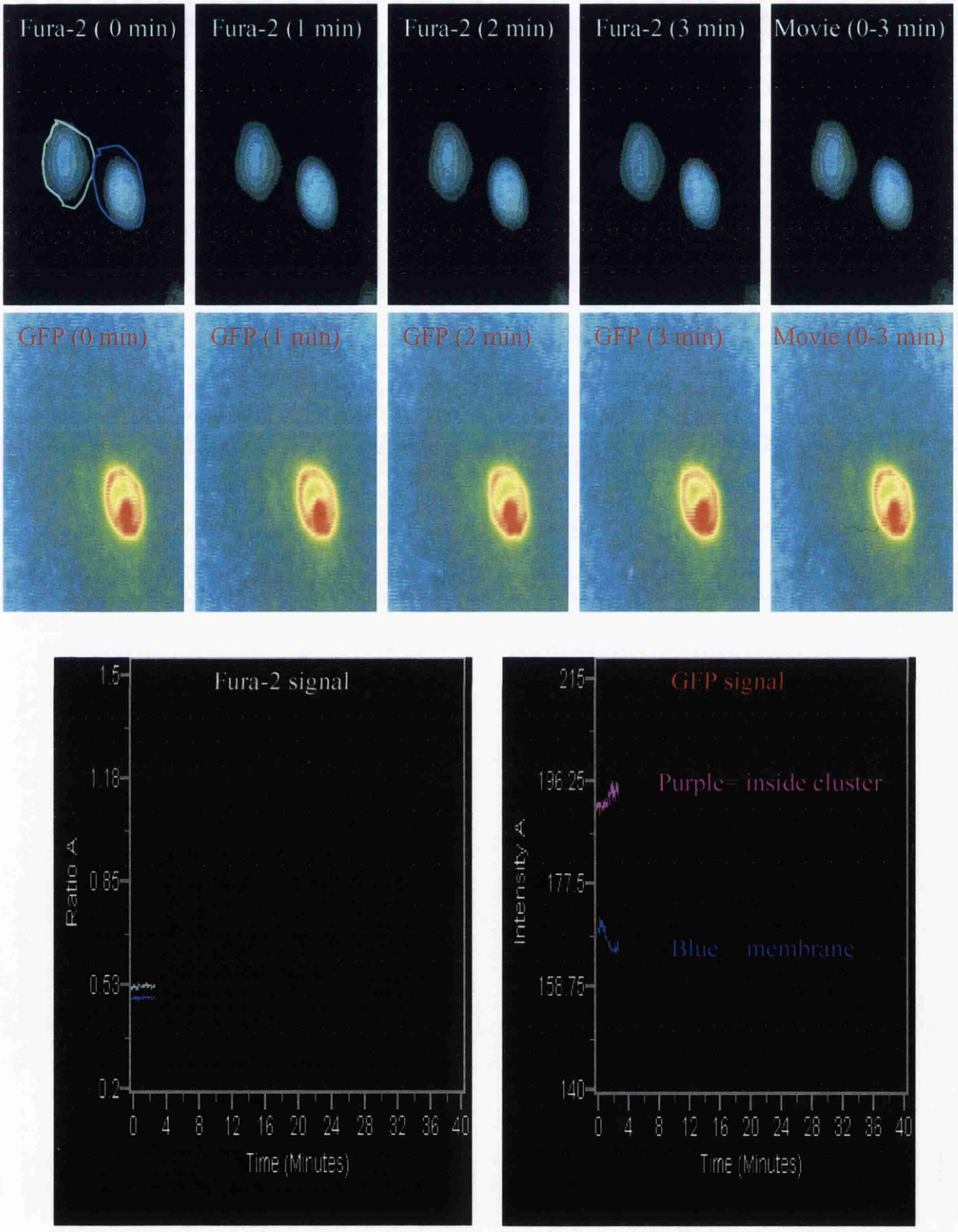


Figure 1.28d Measurement of Fura-2 and GFP signals in unstimulated NCB20 cells stably expressing GFP-tagged humanα_{1b}-ARs (0-3 mins).

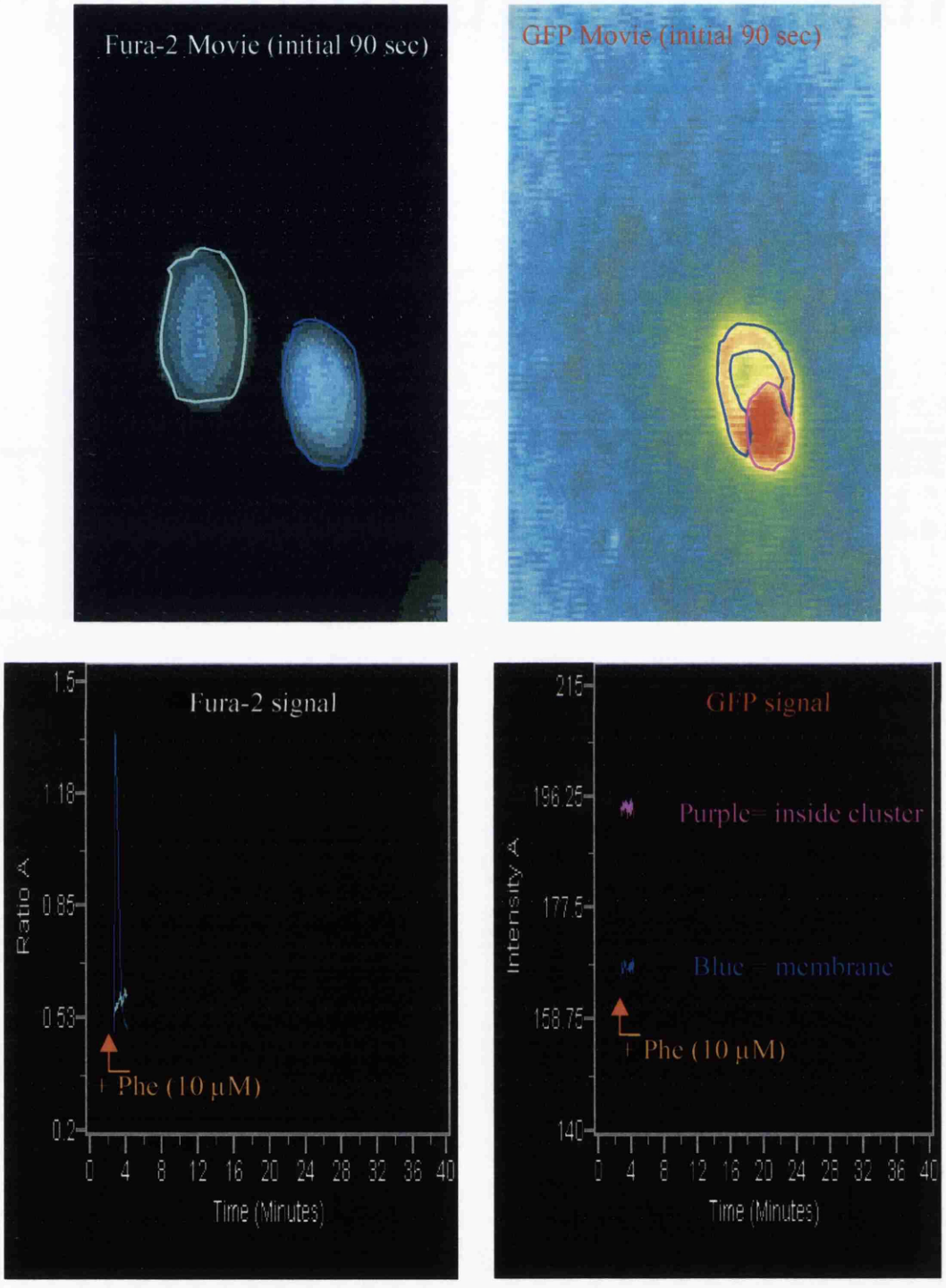


Figure 1.28e Combined effects produced during initial 90sec exposure to agonist (10µM Phe).

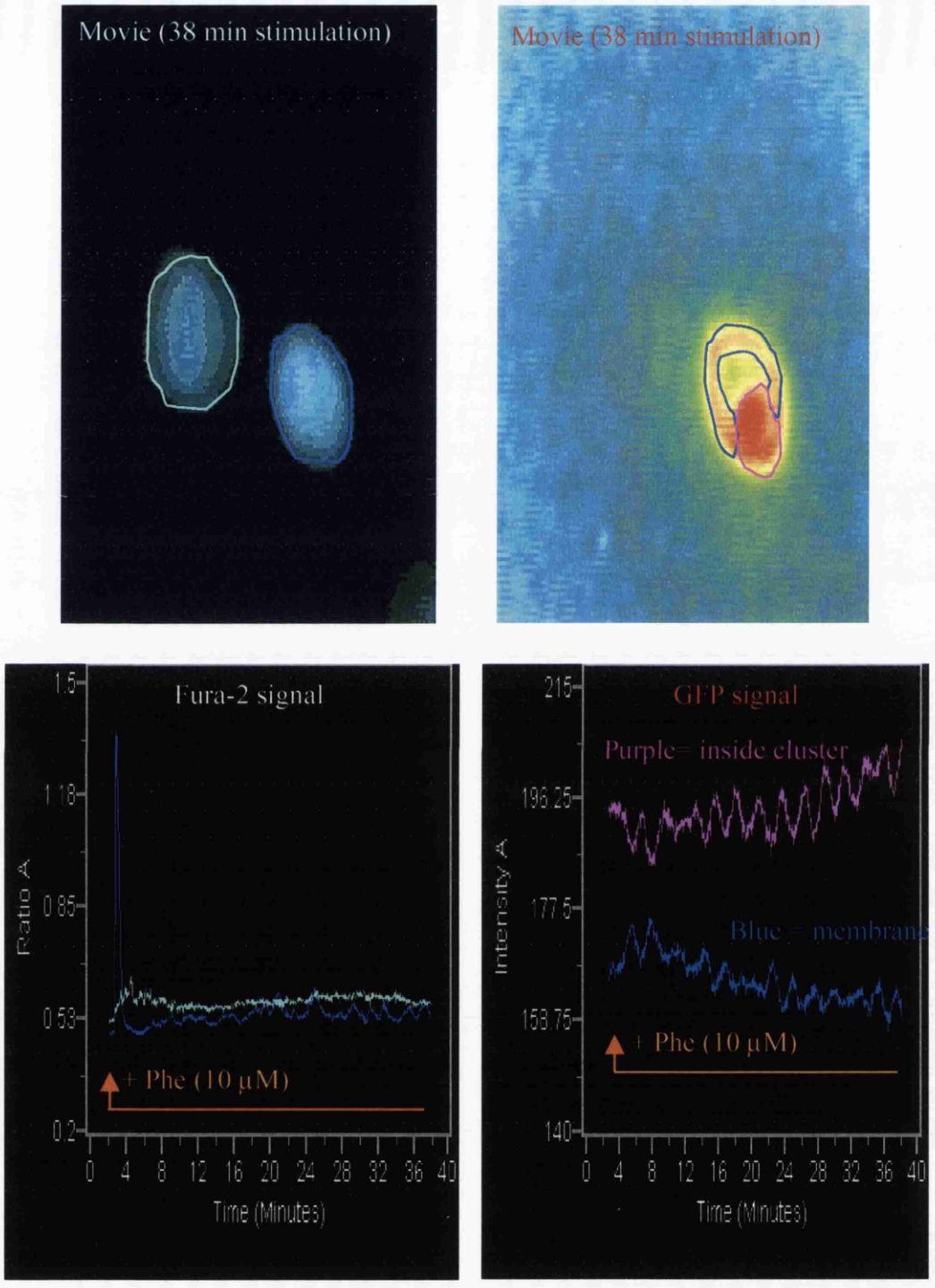


Figure 1.28f Combined effects produced during prolonged (35 min) agonist exposure (10µM Phe).

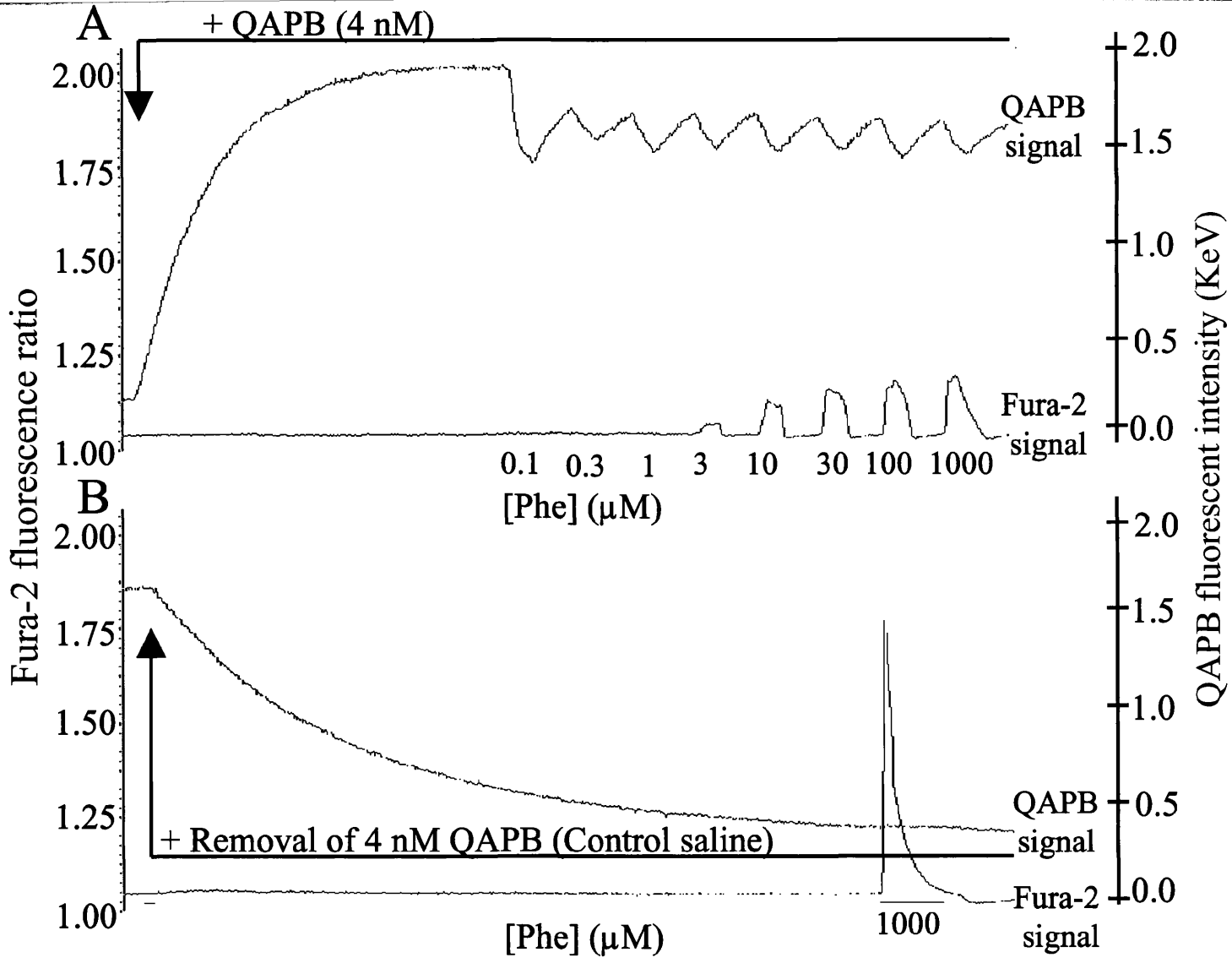


Figure 1.29 QAPB binding to α_1-ARs does not elicit an intracellular Ca^{++} response. Cells were loaded with the fluorescent indicator dye Fura-2/AM (1μM) as described in the methods text.

Chapter 2.

Subcellular distribution, characterisation and modulation of α_1-ARs in cultured hepatocytes.

Chapter 2

Introduction

The previous chapter dealt with the use of fluorescence based methods to enable the analysis of the subcellular distribution of recombinant α_{1b}-ARs. Initially, a fluorescent ligand method was employed to locate α_{1b}-ARs stably expressed in live, NCB20 cells. Localisation of receptive sites was validated with the use of GFP-tagged α_{1b}-ARs. Coupled with calcium imaging, this allowed the study of distribution and active properties of these molecules. The methods developed in the previous chapter were then used to examine the properties of native receptors on isolated hepatocytes.

The rationale behind using hepatocytes was based on their ease of isolation, and reports that α_{1B}-ARs are the main α_1-AR subtype present in murine liver (although the possibility that other α_1-AR subtypes could be present cannot be ruled out) (García-Sáinz et al., 1994). Since colonies of $\alpha_{1B}^{-/-}$ mice (Cavalli et al., 1997) were available, the liver was therefore of great interest to us from a pharmacological/physiological point of view. Our main interest was to assess the subcellular distribution of native α_1-ARs in isolated hepatocytes and to hypothesise as to their functional location.

The possibility of up regulation of another α_1-AR subtype to compensate for the loss of the α_{1B}-AR in the knockout model would indicate a lack of subtype-specific function of α_1-ARs in the liver. In fact, although it has been shown that the genes for the three subtypes of α_1-AR are expressed in discrete, tissue-specific patterns in the same organism (e.g., Price et al., 1994a), evidence is available suggesting that the liver may express distinct α_1-AR subtypes in different species (see general

introduction). The reason for such variability in the liver is unknown, and reports concerning the regulation of the expression of these receptors are limited. However, it is clear from such data that there is no 'tissue-specific' pattern of expression and that data cannot be extrapolated without verification from one species to another. Therefore, the characterisation of hepatic adrenoceptors expressed in different species is considered of great interest since it may give insight into the physiological and evolutionary significance of such variation. Characterisation of the α_1-AR subtype(s) expressed in the α_{1B}-AR knockout liver will also be of interest, as it raises the possibility that α_1-AR subtypes may be interchangeable within one species. In fact, a recent study by González-Espinosa et al. (1999) has shown that cell isolation alters α_1-AR mRNA expression. A transition from α_{1A}-AR to α_{1D}-AR occurs during the isolation procedure of guinea pig hepatocytes (González -Espinosa et al., 1999).

Since α_1-AR subtype modulation/transition can occur with age (Gurdal et al., 1995a,b; Xu et al., 1997; Ibarra et al., 1997; Rudner et al., 1999; Yamamoto et al., 2001), it seems plausible that a similar situation could occur in the liver.

Although there have been no reports of the ontogenic transition between α_1-AR subtypes in the liver, studies beginning in the early 80's, have reported ontogenic modulation (McMillan et al., 1983) as discussed in the general introduction.

Another interesting feature regarding hepatic α_1-ARs is the existence of sites with low affinity for prazosin. The human liver has been shown to predominantly contain mRNA for α_{1A}-AR, although that for the α_{1B}-AR subtype has been detected (Weinburg et al., 1994; Price et al., 1994b). With reference to the splice variant α_{1A-1}, found in human liver (Chang et al., 1998), it is interesting to note that when these

isoforms are expressed in cell lines and IP_3 accumulation measured, they display α_{1L}-AR pharmacology (Ford et al., 1997; Chang et al., 1998).

Shibita et al. (1995) have detected low and high affinity sites for the α_{1A}-AR using selective ligands KMD-3213 and 5-MU in human liver membranes. This low affinity population of α_1-ARs corresponds to the α_{1L}-AR, with an abundance of 40-46% of the total α_1-AR population.

Another interesting observation made by Ohmura and Muramatsu, is the existence of 2 distinct α_1-AR subtypes in rabbit liver membranes. Using rat liver as a control, because exclusive expression of α_{1B}-AR subtype was demonstrated, they found that [^{3}H]-prazosin bound to two distinct affinity sites. Competition experiments with a series of antagonists indicated pharmacological characteristics of high and low prazosin sites suggesting their identity as α_{1A} and α_{1L}-subtypes respectively (Ohmura and Muramatsu, 1995).

Most mRNA distribution studies in the rat have detected only α_{1B}-AR mRNA in the liver, with no detection of an α_{1A}-AR signal (Lomasney et al., 1991; Price et al., 1994a; Rokosh et al., 1994). However, Scotfield et al. (1995), using RT-PCR, detected a minor component of the α_{1A}-AR in rat liver. Other pharmacological studies of liver plasma membranes from rodents, namely rat and hamster have suggested an essentially homogeneous population of α_1-ARs of the α_{1B}-AR subtype (García-Sáinz et al., 1994). However, an exception to this was observed in competition binding curves with prazosin using mice liver membranes. Analysis of these data indicated that the competition curve with prazosin in mice liver membranes was best fitted to a two-site model (García-Sáinz et al., 1994).

Reports thus far have indicated that although one subtype predominates in the livers of various species, it is apparent that sites exist that have a low affinity for prazosin.

There is however no indication as to a functional role for these receptors or 'affinity state' as yet.

To identify and characterise α_1-ARs present in liver of diverse species, several approaches have been employed; mRNA analysis, radioligand binding in plasma membranes and functional studies using whole cells. However, these approaches do not complement each other as detection of mRNA for an α_1-AR subtype, does not necessarily signify the expression of receptor protein, nor that it mediates a specific action. An example of this is seen in the carcinoma cell line, HepG2. Northern analysis indicated the presence of both α_{1D}-AR and α_{1B}-AR mRNA, yet this cell line lacked α_1-ARs in radioligand binding assays (Kost et al., 1992). Furthermore, the α_1-AR subtype expressed in the guinea pig liver has been especially difficult to characterise. Pharmacological and radioligand binding analysis of guinea pig hepatocytes indicated the presence of α_{1A}-ARs (García-Sáinz et al., 1992a,b, 1995a). However, Northern analysis revealed a hybridisation signal with the α_{1D} but not with the α_{1A}-AR probe (García-Sáinz et al., 1992b). It has since been observed that during isolation of guinea pig hepatocytes with collagenase, degradation of α_{1A}-AR mRNA occurs with a concomitant increase in the transcription of the α_{1D}-AR gene (González -Espinosa et al., 1999).

It is well documented that adrenoceptor mechanisms in the liver display a unique form of plasticity. In the male adult rat liver, the best studied hepatic model, adrenoceptors of the α_1 and β_2 subtypes are present, but functionally α_1-ARs mediate the adrenergic effects almost exclusively (Exton et al., 1980). The same effect, however, is mediated by a predominantly β-adrenergic mechanism in the liver of the foetus (Sherline et al., 1974). This transition is a logical adaption to the development of sympathetic innervation, as there is an increased switch from

circulating AD from the adrenal medulla to NA derived from sympathetic nerves (McMillian et al., 1983). This changeover is metabolically appropriate to the substitution of milk for a mature diet that is carbohydrate rich. The consequent climb in glycogen stores requires the substitution of gluconeogenesis (closely linked to β-adrenergic function) for glycogenolysis (α_1-AR dependent) as the source for mobilization of glucose (McMillian et al., 1983; Huff et al., 1991; Morgan et al., 1983). This maturational change in adrenergic-mediated glycogen phosphorylase activation from β_2- to predominantly α_1-adrenergic control is related to concomitant changes in the respective receptor densities (Bendeck and Noguchi, 1985).

The α_{1B}-AR mediates the acute metabolic effects of catecholamines in the liver and is also involved as a comitogen in the regenerative response after loss or injury of liver tissue (Cruise et al., 1985 and 1987; Kunos et al., 1987 and 1995).

Expression of the α_{1B}-AR gene in the rat liver is controlled by hormonal and developmental factors in a tissue-specific manner as exemplified in hypothyroidism, which increases the level of α_{1B}-AR mRNA in the rat heart but decreases it in the rat liver (Lazar-Wesley et al., 1991). In primary cultures of rat hepatocytes, high cell density prevents the decline in α_{1B}-AR expression observed at low cell densities (Kajiyama and Ui, 1994) whereas in primary cultures of myocardiocytes, increasing cell density decreases α_{1B}-AR expression (Bishopric et al., 1991).

Several other reports exist in the literature showing changes in α_{1B}-AR expression due to conditions that are associated with hepatocyte dedifferentiation (Bevilacqua et al., 1991; Kost et al., 1992; Kunos et al., 1987, 1995; Rossby et al., 1991). Such regulation has been shown to occur at the transcriptional level under many of these

conditions, such as after partial hepatectomy (Kunos et al., 1995), during primary culturing of hepatocytes (Ishac et al., 1992), and in response to glucocorticoids (Sakaue et al., 1991), cyclohexamide (Hu et al., 1993), phorbol esters (Hu et al., 1993), thyrotropin, and cAMP (Kanasaki et al., 1994).

As mentioned above, *in vitro* incubation of hepatocytes acutely isolated from adult male rats leads to a rapid conversion of the adrenergic activation of glycogenoylsis from an α_1-AR to a β_2-AR mediated response within 4 hours. Incubation of hepatocytes for 4hours results in a decrease in phosphorylase activation and IP_3 accumualtion in response to phenylephrine, a 40% decrease in α_1-AR density, and a 70% decrease in α_{1B}-AR mRNA levels. This is accompanied by an emergence of a phosphorylase response to isoproterenol, no significant change in β_2-AR density, and a twofold increase in β_2-AR mRNA levels (Ishac et al., 1992). Evidence suggests that this conversion is related to changes in the expression of the respective genes (Lazar-Wesley et al., 1991; Ishac et al., 1992), although it is clear that additional mechanisms, such as selective changes in the coupling of these receptors to their respective G-proteins (Kunos et al., 1987; Itoh et al., 1984), or changes in G-protein expression (Rapeijko et al., 1989), are also involved. It is interesting that this switch is prevented when cell-cell contacts through plasma membranes are favoured during culture by increasing the cell density (Kajiyama and Ui, 1994). Such cell-cell interactions were reported to be an important factor in inhibition of cell growth, in favour of cell differentiation or expression of liver-specific characteristics (Nakamura et al., 1983, 1984).

The inverse regulation of α_{1B}-AR and β_2-AR has been observed in a number of different physiological and pathological conditions (Aggerbeck et al., 1983; Chan et al., 1979; Preiksaitis et al., 1982; García-Sáinz et al., 1986; Christoffersen and

Berg, 1975; Blair et al., 1979; Itoh et al., 1984; Kunos et al., 1984; Nakamura et al., 1984; Schwartz et al., 1985; Sandes et al., 1986). Reports have shown remarkable similarity of the altered response pattern under these various conditions, suggesting that it represents a unique form of receptor regulation. Furthermore, there is a close parallel between the conversion of the AR response and a shift from liver-specific to growth-related functions suggesting a role in the process of liver cell growth and differentiation. Whereas the activation of glycogenolysis by α_{1B}-AR or β_2-AR is a unidirectional response, this is not the case for effects on liver cell proliferation. α_{1B}-ARs are mitogenic, increase DNA synthesis, and their inhibition can prevent liver regeneration after partial hepatectomy (Michalopoulos, 1990). In contrast, in proliferating liver cells stimulation of β_2-ARs strongly inhibits the G1-S phase transition (Refsnes et al., 1992), and thus may be involved in the termination of the proliferative process. This, and the reported shift from α_1-AR to β_2-AR in human hepatocellular carcinoma (Bevilacqua et al., 1991), could suggest that this form of regulation is important in terminating hepatocyte proliferation in order to prevent potential progression into malignant transformation (Kunos et al., 1995).

The work in this chapter shows preliminary studies of the subcellular distribution, characterisation, and modulation of α_1-ARs during culture of hepatocytes isolated from wildtype and α_{1B}-AR knockout liver. Firstly, basic binding properties and general distribution of α_1-ARs was assessed using fluorescent ligand binding. Since reports in the literature state that ontogenic modulation of α_1-ARs occur, studies were carried out in both strains at a specified age. Further caution was taken when interpreting this data as reports maintain that rapid changes in α_1-AR density occur during culture of acutely isolated cells (Ishac et al., 1992). Animals were initially sacrificed at 4months then due to availability and time constraints; work began on

3month-old animals. Comparisons of specific QAPB binding in hepatocytes from both strains at both ages were subsequently carried out.

Since culturing of hepatocytes has been reported to modulate α_1-ARs, I therefore began some preliminary work looking at changes in α_1-AR density after 4, 24 and 48 hours in culture.

Confirmation that these receptors correspond to the binding sites for QAPB and display α_1-AR pharmacology was established. Thereafter characterisation of α_1-AR subtype(s) expressed was assessed by quantitative competition versus the development of fluorescence employing the discriminatory antagonists BMY-7378 and RS100329. Such experiments allowed me to determine whether a compensatory up-regulation of one of the other α_1-AR subtypes had been established in the knockout. The possibility of re-introducing the α_{1b}-AR into knockout cells and restoring 'normal' pharmacological function was also considered.

Little is known about the functional location of α_1-AR subtypes seen in native systems, especially in hepatocytes. Our main aim was therefore to uncover the functional location of native α_1-ARs in hepatocytes. Although the specificity of QAPB binding to recombinant α_{1b}-ARs was confirmed in chapter 1, it was also necessary to validate the location of the fluorescent ligand with that of the native receptor. A GFP-tagged α_{1b}-AR construct designed to mimic the expression and localisation of the native receptor would therefore prove invaluable. Coincidentally, at this time, cloning of the promoter from the mouse α_{1B}-AR gene came to fruition in Dianne Perez's laboratory, and confirmation of its fidelity and tissue-specific expression (Zuscik et al., 1999) paved the way forward using GFP as a valuable reporter tool. A promoter-reporter construct was designed (Perez) and thereafter

employed to locate the α_{1b}-AR in its native environment. The competency of any given cell/tissue to activate transcription of the α_{1b}-AR gene and hence express the GFP-tagged receptor allowed us scope to visualise expression and location at a subcellular level. It was anticipated that such studies would indicate a possible cell-specific functional location of the α_{1B}-AR.

A recent finding has indicated a role for the multifunctional protein, gC1q-R, in the regulation of subcellular distribution and expression of α_{1B}-ARs (Xu et al., 1999; Hirasawa et al., 2001). gC1q-R was found to bind with the carboxyl-terminal cytoplasmic domain of the α_{1B}-AR in a yeast two-hybrid screen of a cDNA library prepared from rat liver. The interaction was confirmed by specific co-immunoprecipitation of gC1q-R with full-length α_{1b}-ARs expressed in transfected COS-7 cells. Using tagged-constructs and fluorescence confocal microscopy co-localisation of these proteins in intact cells was also visualised. Interestingly, the α_{1b}-ARs were exclusively localised to the region of the plasma membrane in COS-7 cells that expressed the α_{1b}-AR alone, whereas gC1q-R was localised in the cytoplasm in COS-7 cells that expressed gC1q-R alone; however, in cells that co-expressed α_{1b}-ARs and gC1q-R, most of the α_{1b}-ARs were co-localised with gC1q-R in the intracellular region, and a remarkable down-regulation of receptor expression was observed (Xu et al., 1999). Further work by this group has revealed that this protein appears to regulate the expression level and cellular localisation of the α_{1B}-AR through its carboxyl terminal. These data strongly suggest that gC1q-R plays a regulatory role in the function of the α_{1B}-AR. However, since species heterogeneity exists in hepatic α_1-AR expression, the specificity of gC1-qR must be questioned.

Chapter 2

Methods

Hepatocyte isolation and culture

The time between sacrifice of the animal and isolation of hepatocytes did not exceed 1 hour, during which the liver was maintained at 37°C under sterile conditions. Mouse hepatocytes were isolated by a non-perfusion procedure performed without vascular access by multiple injections into the hepatic parenchyma (Adapted from David et al., 1998). The liver portion was placed in a sterile universal containing Hepatocyte Perfusion Media (Life technologies, Inc) and incubated for ~1hour at 37°C in a water bath. This same buffer was then injected using a 10ml syringe via an 18G needle into all areas of the piece of liver until the tissue softened and a cell suspension was released. The flow rate was approximately 10ml/min. The solution was not re-circulated. The cell suspension was centrifuged at 50g for 1 minute at room temperature. The cell pellet was then washed twice with Hepatocyte Attachment Media (Life technologies, Inc) at 50g for 1 minute to isolate hepatocytes from other cell types. Cell viabilty was determined by trypan blue exclusion (0.04% final concentration in protein-free medium). The cell pellet was finally resuspended in a minimal volume of Attachment Media and cells were allowed to attach to glass cover slips. After this attachment period, the medium was removed and replaced with HepatoZYME-SFM (Life technologies, Inc). Cultures were maintained at 37°C in a humidified atmosphere (95% air, 5% CO_2). Cells were analysed at 4, 24, and 48 hours post isolation.

Transient transfection of primary hepatocytes

Hepatocytes were isolated, plated and cultured overnight prior to transfection. 5μg of plasmid DNA per well was complexed with 15μg DOTAP transfection reagent (Boehringer, Mannheim), diluted to a volume of 250μl with culture medium, and allowed to sit for 15 minutes at room temperature. A further 2mls of culture medium was added to the complex, gently mixed, added drop-wise to the cells in culture which were then incubated for 36 hours from the start of the transfection to allow for expression of the plasmid.

Confocal Microscopy

Identical parameters (brightness, contrast, laser intensity, excitation and emission wavelengths, slit size and frame averaging) were set in every experiment, unless stated otherwise. The objective used throughout this chapter was a Nikon x40, oil.

Whole cell image analysis

Images were collected and analysed using Universal Imaging's 'Metamorph' software. Cells were grown on cover slips for 4, 24, and 48 hours prior to use, as indicated. Cover slips were mounted in a flow chamber (WPI) and placed on the stage of an invert (Nikon Diaphot) microscope fitted with a Noran Odyssey Laser Scanning Confocal Module. Fluorophores were excited using a 488 nm argon laser and detected with a 515 nm band pass filter. In all experiments, a 15μm slit was used and all other parameters were kept constant.

QAPB

Using cell autofluorescence as an indication, a suitable group of cells was selected and the focal plane fixed by locking the focus motor. The system was then set to

acquire images (64 frame averages; 2.56-s exposure) at 1-min intervals, unless stated otherwise) at 1 minute intervals. After a baseline was established, typically 5-6 minutes, the first concentration of fluorescent ligand was added and allowed to equilibrate (i.e., until no further increase in fluorescence) for at least 5minutes. After equilibration the next concentration of fluorescent ligand was added, without washing, and given time to equilibrate as before. Once saturation has been reached the individual cells were outlined using Metamorph's define-region tool and the fluorescent intensity values representative of each concentration at equilibrium were recorded. Non-specific binding was defined as fluorescent binding in the presence of 10μM prazosin. The composition of the Hepes buffer used in all single cell work was as follows: NaCl 130mM, KCl 5mM, HEPES 20mM, Glucose 10mM, MgCl 1mM, CaCl 1mM.

Inhibition of QAPB-associated fluorescence binding

Cells were mounted on a flow chamber bath and experiments carried out in a similar manner to that described above. Hepatocytes were pre-incubated for 30 minutes with inhibitors, BMY-7378 or RS100329 (Dr. Michelson, Roche Bioscience, Palo Alto, CA) prior to addition of QAPB. Characterisation of α_1-AR subtypes expressed was estimated as a measure of the ability of either drug to inhibit the development of QAPB-associated fluorescence.

GFP detection

Using GFP-associated fluorescence, suitable cells were chosen and images acquired. Non-transfected cells were cultured alongside as a comparison.

ArrayScan analysis

Hepatocytes were isolated from 3 and 4month-old wildtype and knockout mice, plated onto Grenier 96 well clear view plates and cultured overnight at 37°C. Approximately 24hours post-isolation, cells were incubated with the appropriate inhibitory antagonist for 30minutes prior to loading with 5nM QAPB and 10μg/ml Hoechst 33342 (bisBenzamide).

After a further 1hour at 37°C, images of cells were acquired using the ArrayScan™ HCS system. Images were converted to the appropriate format and thereafter; average fluorescent intensity values were measured using Metamorph Imaging software.

Chapter 2

Introduction to results

My initial objective was to transfect labelled α_{1b}-ARs into native cells in order to visualise the natural population enabling study of their distribution, co-localisation with QAPB and transposition in response to stimuli. It was also anticipated that comparisons would be made between the distributions of the α_{1b}-AR populations under the control of a viral versus mouse α_{1B}-AR-specific promoter. The liver was chosen because it is reported to express only α_{1B}-ARs, for which much knowledge is available at a molecular level. The objective in using α_{1B}-AR knockout mice was to produce a situation where only the labelled receptors were present.

This primary objective was frustrated for the unforeseen technical reason that primary cultures of hepatocytes acquire a strong green autofluorescence when cultured for the several days required for transfection studies.

An alternative study was to focus on existing populations of native α_1-ARs in wildtype and knockout cells, and attempt to identify and characterise them using a combination of QAPB and subtype-selective antagonists.

Prior to this work, a colleague had carried out ^{3}H-prazosin binding studies on mouse liver plasma membranes, to confirm previous findings regarding the expression of the α_{1B}-AR in both wildtype and knockout tissue (Cavalli et al., 1997; Yang et al., 1998). From previous reports, it was anticipated that the knockout liver would not possess an α_1-AR population. Cavalli et al. (1997) reported a 98% decrease in α_1-AR density in the knockout liver when compared to the wildtype. In contrast, my colleague discovered only a 40% decrease in α_1-AR density in knockout liver tissue.

The population of α_1-ARs found in the wildtype liver was 50fmol/mg, identical to that of Yang et al. (1998). However, Cavalli et al. (1997) found only 20 fmol/mg of

α_1-AR protein in the wildtype liver. This discrepancy may explain the lack of α_1-ARs seen in the knockout by this group. Our data revealed that the α_1-AR density remained as high as 30fmol/mg in the knockout. The calculated K_D values in both strains were similar, and comparable to those of Cavalli et al. (1997) and Yang et al. (1998). Basing our expectations on the literature, to find a population of α_1-ARs, replacing the α_{1B}-AR subtype, was exciting.

The binding data from wildtype and knockout liver revealed a pure population of α_{1B}-ARs in the wildtype, as expected, and a pure population of α_{1A}-ARs in the knockout (C.Deighan, PhD thesis). All radioligand binding studies up to this point were carried out using 4month-old mice. Surprisingly, preliminary experiments carried out in 3month-old animals revealed only 8fmol/mg of α_1-AR protein in the knockout, yet 76fmol/mg in the wildtype. Although the K_D values remained unaltered in the wildtype, a 10-fold difference was noted in the knockout, with the affinity increasing from 1.0 (3months) to 0.1nM (4months). These results suggested to us that the compensatory up-regulation of the α_{1A}-AR must occur at some stage between 3 and 4months in the development of the mouse. The possibility of an age-dependent pattern of subtype expression was likely.

These outcomes thereafter dictated new objectives that had to be set at the single cell level. Initially, analysis of QAPB binding to α_1-ARs at a cellular level was assessed using isolated hepatocytes. The next objective was to assess whether subtypes could be identified at this level and whether the following questions could be answered. Do the α_{1B}-ARs (wildtype) and α_{1A}-ARs (knockout), as indicated by the ^{3}H-prazosin binding, have the same or different subcellular distribution?

The next stage was to consider the above issues at different ages, given the indication from the ^{3}H-prazosin binding that the knockout liver differs at 3 and 4months.

In addition, several factors had to be considered arising from the practicability of performing fluorescent binding on primary hepatocytes.

One of the issues that had to be considered was the time of pre-culture before carrying out the QAPB binding studies. A period of at least a few hours was necessary for the cells to 'stick down' and equilibrate in the their new environment. It is well documented in the literature that culturing of hepatocytes changes the expression and functionality of α_1-ARs. Therefore, since our objective was to understand the natural disposition of these receptors, prolonged incubation might lead to de-differentiation with consequent changes in distribution, function and eventually, changes in expression of receptor subtypes. A recent finding has shown that α_1-AR subtype transition occurs during the isolation of guinea pig hepatocytes (González -Espinosa et al., 1999). This opens up another possibility for alterations in subtype expression with this procedure. These issues were considered.

Other factors, not considered to represent a natural environment for hepatocytes, based on studies in the literature, were the ingredients of the culture media (see chapter 2 discussion). For example, the presence of nicotinamide, which is converted to endogenous fluorophores: NAD/NADP would probably contribute to the autofluorescence observed after several days in culture. However, all of these factors remained constant throughout so that direct comparisons could be made where applicable.

As for the functionality of α_1-ARs in cultured hepatocytes, it is well documented that within 4hours post-isolation, there is a switch from an α_1- to β-AR response and that reported changes in functionality do not necessarily reflect a corresponding change in receptor density (Kunos et al., 1995). Attempts were made to assess the functionality of α_1-ARs in hepatocytes. Unfortunately due to technical limitations with this cell

type, in their ability to withstand the flow system and remain attached to the cover slip during the experiment, this was not possible.

Another important experimental issue was how to identify α_1-AR subtypes on/within these cells. Several factors made the 'before and after' effect of antagonists on QAPB binding impractical. Due to photolytic 'fade' and prolonged protocols including a long wash phase, it was deemed impractical to carry out more than a single binding curve on each cell. In practice, this meant identifying suitably healthy cells by a combination of low autofluorescence and their bright field appearance, then applying sequentially increasing concentrations of ligand, capturing images at one minute intervals, and acquiring equilibrium data for each concentration of ligand applied. The difficulty that presented itself was the variability in the amount of fluorescence produced in each cell at the highest concentration of ligand used. This made it impossible to normalise the data to any 'control' level or to make a quantitative assessment of 'specific' binding. Essentially, a judgement had to be made for each cell: The 'maximum' fluorescence of the experimental cell at a specified concentration, relative to other cells under identical conditions. Also the resulting 'range' of binding levels under these conditions had to be compared with other data sets, the objective being to assess the 'amount of receptors'. Saturation binding curves of individual cells were constructed where applicable, and the fluorescence dissociation constant, FK_D was used as a general indicator of whether QAPB binding was to α_1-ARs. A criterion had to be applied in order to accept or reject binding according to whether it fell into the first line definition of α_1-ARs (that they had a high affinity for prazosin, or, in this case, a high affinity for QAPB). However, it

became evident that QAPB also bound to prazosin-sensitive sites, at concentrations higher than normally associated with 'typical' α_1-AR pharmacology.

Lastly, a judgement had to be made for each cell in the presence of subtype-selective antagonists in an attempt to identify the receptor subtypes. The variation in α_1-AR expression between individual cells was so high, difficulties arose when trying to establish inhibition of QAPB-associated fluorescence in the presence of antagonists.

Taken together, the data produced two general types of complication. Variability in the 'amount' of maximum binding within a given situation, and secondly, variability in the estimate of affinity by a larger degree than expected. QAPB appeared to bind to 'high' and 'low' affinity populations in this cell type. In some cases, the rise and fall of fluorescence intensity levels during increasing concentrations of QAPB gave rise to saturating and non-saturating sites within the concentration range used, suggesting that the 'low' affinity sites might have been considered 'non-specific'. However they were in fact sensitive to cold prazosin and thus 'specific' in this sense.

Results

Fluorescent ligand binding on hepatocytes isolated from livers of 3month-old knockout mice, cultured for ~4hours.

QAPB binding to hepatocytes isolated from 3month-old knockout mice showed that cells could express either high or low affinity populations of α_1-AR. Some cells expressed low, some high and others both populations (figure 2.1a). QAPB-associated fluorescence binding to high affinity (FK_D 1.00nM-cell 1, 0.10nM-cell 2), low density (0.12 and 0.03 (fluorescence intensity), respectively) populations was saturable (figure 2.1b) and completely abolished in the presence of 1μM prazosin (figure 2.1a, bottom panel). Binding to low affinity sites was non-saturable within the concentration range (0.4-20nM QAPB). However, the programme used to quantify these data, GraphPad Prism, gave estimated values where applicable. FK_D values of 21.80nM (cell 5) and 29.22nM (cell 6) were estimated for these populations of receptors. In the presence of prazosin, the residual fluorescence seen at 20nM QAPB represented non-specific binding, but only in the cell in question. It did not prove practical to carry out two binding curves on one cell making it impossible to define the non-specific component in individual cells. Since the α_1-AR complement of hepatocytes was so varied, it was not possible to quantify non-specific binding per se. However, since so little fluorescence was found in cells incubated with QAPB in the presence of prazosin (1μM) (figure 2.1e), it was assumed that at this age, under these culture conditions, QAPB binding was specific below 20nM. The overall density of α_1-ARs between individual cells varied considerably, emphasising the quantitative heterogeneity of α_1-AR expression in this cell type.

The distribution of QAPB binding was diffuse throughout the cytoplasm of all cells, although 'holes' were present suggesting organelles that did not contain receptors or were impermeant to the ligand. In fact, a report in the literature describes the appearance of vacuoles that arise from cellular reorganisation as hepatocytes become active in lipid glyconeogenesis (May et al., 1982).

Comparison of fluorescent ligand binding on hepatocytes isolated from livers of 3month-old wildtype and knockout mice, cultured for ~4hours.

QAPB bound to different cells in a manner indicating either high or low affinity sites both in wildtype and knockout hepatocytes isolated from 3month-old mice. The overall density of α_1-ARs within these populations was much greater in cells isolated from the wildtype animal. It was observed in both wildtype and knockout cells, at 3months, that the α_1-AR populations with low affinity for QAPB did not saturate at concentrations up to 20nM (figure 2.2).

Comparison between hepatocytes isolated from livers of 3 or 4month-old knockout mice, cultured for ~4hours.

The pattern of QAPB binding in the knockout at 4months differed from that seen at 3 months, in that the population of non-saturating, low affinity sites seen in younger animals became more apparent allowing a more straightforward quantification of these sites (figure 2.3). Estimations of ligand affinities and receptor densities were made, as indicated in the table (figure 2.3d).

Comparison of hepatocytes from wildtype mice at 3 and 4 months (cultured for ~4hours).

The fluorescent binding to individual hepatocytes isolated from 4month-old wildtype liver was very variable and created difficulty when trying to draw comparisons with cells from the 3month-old animal (figure 2.4). However, quantification revealed that specific binding of QAPB at low concentrations was higher in cells from the 4month-old animal. The appearance of QAPB binding to high and low affinity populations was evident at both ages, as also seen in the knockout.

Comparison of hepatocytes from 4month-old wildtype and knockout mice (cultured for 4 hours or 24 hours).

QAPB binding on hepatocytes isolated from 4month-old wildtype and knockout mice was variable for reasons mentioned above, although no obvious differences were apparent between the strains (figure 2.9). After 24 hours in culture, the overall increase in receptor density allowed the visualisation of two separate populations of α_1-ARs more readily (figure 2.9). Quantification of the density and affinity of high or low affinity α_1-AR populations seen in individual cells was possible in some cases, but not all. Therefore, to draw conclusions from comparisons made between the 2 strains at this age was not feasible. General conclusions about the nature of QAPB binding to either wildtype or knockout hepatocytes at 4months could not be made until a more substantial study was carried out to account for the extensive heterogeneity seen in this native cell type.

Effect of varying the duration of pre-culture for ~4, 24 or 48 hours on hepatocytes from 4month-old wildtype mice

Hepatocytes demonstrating high affinity binding showed a greater amount of specific binding of QAPB when cultured for 24 compared with 4hours (2.20 and 0.24 (fluorescence intensity) respectively). The fluorescent affinity for QAPB binding to high affinity α_1-ARs did not alter significantly during this time period (0.37and 0.31nM respectively). The density of low affinity populations of α_1-ARs also increased over time in culture. However this was likely to be accompanied by a proportional increase in non-specific binding at higher concentrations of QAPB. The pattern of QAPB binding to low affinity sites, after each time point, was very similar, suggesting that there is no change in the affinity of QAPB binding to α_1-ARs after culturing of hepatocytes.

Effects on fluorescent ligand binding of pre-culturing hepatocytes from 3month-old knockout mice for different times.

Cells cultured for 24 or 48hours, and showing a high affinity for QAPB, showed an increase in the amount of specific binding compared with that seen at 4hours (2.79, 7.58, and 0.12/0.03 (fluorescence intensity), respectively). The fluorescence binding affinity for QAPB on cells cultured for different time periods did not alter significantly (figure 2.6b).

A similar situation was observed in a population of cells expressing low affinity sites (figure 2.6c): the density of α_1-ARs also increased over time in culture, but this was difficult to quantify since a high degree of binding in cells pre-incubated with prazosin, suggested a proportional increase in non-specific binding of QAPB at the high concentration range necessary. At this age, and at each culture time, these low affinity sites remained non-saturable within the concentration range.

The pattern of QAPB binding to both populations of α_1-AR after each time point was very similar, showing an overall increase in α_1-AR density, with no obvious changes in affinity. Again, quantification of the non-specific component in each experimental cell was not possible, and estimates were made.

Effects on fluorescent ligand binding of pre-culturing hepatocytes from 4month-old knockout mice for different times.

The effects of extending the pre-incubation period on the pattern of QAPB binding to hepatocytes isolated from 4 month-old mice, were similar to those at 3 months. Overall, culturing hepatocytes for between 4 and 24 hours caused an increase in the density of α_1-ARs (figure 2.8b), although direct comparisons could not be made in individual cells. Estimates of ligand affinity values were also variable at each time point.

Culturing for 24 hours did however have the advantage of emphasising the difference in QAPB binding to α_1-AR populations at 3 and 4months (figure 2.7). In 3month-old animals, after 24hours in culture, it was not possible to quantify the low affinity sites within the concentration range used. A population of 'lower' affinity sites did however appear in cells from the older animals, which were easily quantified. This observation was made after both 4 (figure 2.3d) and 24 (figure 2.7b) hours in culture.

Identification of native α_1-AR subtype(s) on cultured hepatocytes isolated from 3 and 4month-old knockout mice by inhibition of QAPB-associated fluorescence.

Hepatocytes isolated from 3 and 4month-old knockout mice bound QAPB in a concentration dependent manner (figures 2.1 and 2.3). Cultured (~4hours) hepatocytes (3month-old) were pre-incubated with 1nM RS100329 or BMY-7378.

On addition of cumulative concentrations of QAPB, the appearance of fluorescence was significantly inhibited in the presence of RS100329 yet unaffected by BMY-7378 (figure 2.11). Similarly, in cells from a 4month-old knockout mouse, pre-incubation with 1nM RS100329 resulted in complete blockade of QAPB-associated fluorescence up to 5nM QAPB but was unaffected by BMY-7378 (figure 2.13c). These data suggest that in hepatocytes isolated from the α_{1B}-AR knockout mouse, the α_1-AR subtype expressed is predominantly α_{1A}-AR. The presence of α_{1D}-ARs could not be completely ruled out since they may have been masked by the fluorescence from α_{1A}-AR, but there was no evidence to support its presence.

Identification of native α_1-AR subtype(s) on cultured hepatocytes from 4month-old wildtype mice by inhibition of QAPB-associated fluorescence.

Hepatocytes isolated from 4month-old wildtype mice bound QAPB in a concentration-dependent manner. Cultured (4 or 24hours) hepatocytes were pre-incubated for 30minutes with 1nM RS100329 or BMY-7378. On addition of cumulative concentrations of QAPB, either RS100329 or BMY-7378 reduced, but did not completely block the appearance of fluorescence (figure 2.12c). This was analysed at 5nM QAPB. Comparison of wildtype and knockout hepatocytes (figure 2.13c) demonstrated that BMY-7378 had minimal effect and that RS100329 reduced specific binding of QAPB to wildtype hepatocytes, but abolished it in the knockout. Collectively, these results suggest that the α_1-AR subtype expressed in wildtype hepatocytes has an α_{1B}-AR component (sensitive to prazosin, resistant to RS100329 and BMY-7378) while the RS100329-sensitive component suggests that some cells express the α_{1A}-AR subtype. In the knockout mouse at 4months-old, the binding of QAPB (abolished by RS100329) is exclusively to the α_{1A}-AR. Pre-culturing cells for

a longer period increases the amount of receptors expressed but no obvious changes in subtype expression were observed.

ArrayScan analysis to provide quantitative data.

Hepatocytes were isolated from 3 and 4month-old wildtype and knockout mice and cultured overnight prior to analysis. The following day, cells were incubated for 30minutes with the appropriate subtype-selective antagonist prior to α_1-AR detection using 5nM QAPB. After a further 1hour QAPB equilibration period, images were captured of control cells (5nM QAPB only) and those in the presence of either BMY-7378 or RS100329 (1nM). After images were collected, they were converted to the appropriate format and average fluorescence intensity values were measured using Universal Imaging's 'Metamorph' software.

After 24hours in culture, α_1-AR density was at a level suitable to carry out comparative inhibition experiments. In the presence of either RS100329 or BMY-7378, a statistically significant decrease in the average fluorescence intensity was observed in both wildtype or knockout cells at both ages (figures 2.14b, and c). In addition, a statistically significant increase in α_1-AR density was observed in knockout cells between 3 and 4months, the opposite of which was observed in wildtype cells (figure 2.14a). Interestingly, the lower α_1-AR density seen in the 3month knockout compared to the wildtype at the same age, appeared to balance out by the time the animal reached 4months, as the average fluorescence intensity values in both strains were similar (figure 2.14a).

Transient transfection of knockout hepatocytes with GFP-tagged α_{1b}-AR constructs.

Hepatocytes were isolated from knockout liver and cultured overnight prior to transfection. The following day, cells were transiently transfected with the following α_{1b}-AR constructs: pEGFP/human α_{1b}-AR or pmouseα_{1B}-AR/human α_{1b}-AR-GFP. After a further 72 hours in culture to allow for expression of the plasmids, GFP-associated fluoresence was compared to that of non-transfected control cells. Unfortunately expression of the GFP-tagged constructs could not be distinguished from autofluorescence created by extensive culturing of the hepatocytes (figure 2.15).

Discussion

The aim of the work in this chapter was to identify, localise and characterise α_1-ARs expressed in hepatocytes isolated from wildtype and α_{1B}-knockout liver. Several technical limitations arose however, as discussed in the 'introduction to results' section, although a general picture was eventually established, complementing the radioligand binding studies done in the intact liver. In addition, some interesting observations were made, regarding the plasticity of α_1-ARs in cultured hepatocytes.

The ability to draw general conclusions about the nature of QAPB binding to hepatocytes was problematic from confocal analysis alone. A more substantial study was carried out using the ArrayScan™ system, from which an overall picture was formed. Due to low α_1-AR density post-isolation, it was necessary to culture cells for 24hours to allow for inhibition of QAPB-associated fluorescence studies to be carried out. Since the aim of this study was to understand the natural disposition of α_1-ARs in hepatocytes, this prolonged culture period was not ideal, but nonetheless unavoidable.

Comparison of the radioligand binding versus ArrayScan data indicated that prolonged culture had altered the expression of the α_1-AR subtypes. After 24hours in culture, inhibition studies revealed that both wildtype and knockout cells, at 3 and 4months, expressed α_{1A}- and α_{1D}-ARs (figures 2.14b and 2.14c). This was in sharp contrast to studies done on intact liver where a pure population of α_{1B}- and α_{1A}-ARs were found in 4month-old wildtype and knockout tissue, respectively. These observations suggested that although in the intact liver, only one subtype of α_1-AR appeared to be present and therefore presumably required for function, culturing demonstrated the ability of hepatocytes to potentially express all three. Unfortunately, subtyping immediately post-isolation was not feasible.

Another interesting observation was that the up-regulated subtypes, post-isolation, appeared to take over from the α_{1B}-AR population in wildtype cells. The residual fluorescence seen after equilibration of 5nM QAPB ± BMY-7378 or RS100329 (1nM) was specific, as indicated by prazosin-sensitivity (figure 2.9a). The identity of this residual fluorescence was unknown. In the knockout, one would imagine that it represented either α_{1A}- or α_{1D}-ARs, depending on the subtype-selective antagonist used. However, in the wildtype, as to whether a mixture of all three subtypes exists remains to be answered.

Future experiments using a combination of both BMY-7378 and RS100329 will be informative.

α_1-AR subtype transition

Alterations in hepatocyte α_1-AR subtype expression, post-isolation have previously been reported at the mRNA level in guinea pig hepatocytes (González -Espinosa et al., 1999). This study showed that the subtype expressed in the liver was α_{1A}-AR, but when the liver cells were isolated, the mRNA coding for this receptor was degraded and the mRNA for a different subtype (α_{1D}-AR) was markedly induced by the cell isolation procedure (González -Espinosa et al., 1999). Such data indicate that cell isolation can modulate very rapidly, the expression and degradation of α_1-AR subtype mRNAs in hepatocytes. However, in that study, changes in α_1-AR subtype expression were not assessed at the level of receptor protein, and it is well known that mRNA levels do not necessarily correlate with protein expression.

My work is thus the first report to date of changes in the expression of α_1-AR subtype receptor protein upon isolation of hepatocytes. However, it is well documented that post-isolation of rat hepatocytes (best model studied), the α_{1B}-AR

response decreases, and at the same time, a strong β_2-AR response emerges. These responses are accompanied by corresponding changes in receptor density (Kajiyama and Ui, 1994).

It is interesting to note that changes in the expression of α_{1B}- and β_2-ARs during culture are modulated by cell density and that addition of cell membranes to the culture may also modulate such effects (Kajiyama and Ui, 1994). Such data suggests that cell-cell interactions are important in determining the type of AR gene expressed, as shown for other genes (Rana et al., 1994). It could therefore be possible that the changes in expression of α_1-AR subtypes during isolation could be influenced by disruption of cell-cell interactions. This will be discussed in more detail later.

Decline in α_{1B}-ARs in regenerating liver

It is well documented that isolated hepatocytes display a similar response pattern as regenerating liver, in that control of various hepatic functions in the normal, adult male rat are mediated exclusively by the α_{1B}-AR. After partial hepatectomy (Aggerbeck, 1983), the same response is rapidly converted to a predominantly β_2-AR mediated event. A similar conversion from α_1- to β_2-type response occurs in isolation of hepatocytes (Itoh et al., 1984; Kunos et al., 1984; Nakamura et al., 1984; Schwartz et al., 1985; Sandes et al., 1986). Therefore, the down-regulation of the α_{1B}-AR gene expression seen in regenerating liver (Gao et al., 1996) is also likely to occur in isolated hepatocytes. Activation of the α_{1B}-AR is one of the first events that initiate regenerative DNA synthesis after partial hepatectomy (Cruise et al., 1985, 1987; Michalopoulos, 1990). However, the number of α_{1B}-ARs is thereafter down-regulated when hepatocytes de-differentiate.

It has been shown previously that 3 promoters, which generate three mRNA transcripts, control the transcription of the hepatic α_{1B}-AR (Gao et al., 1994). Gao et al., (1996) have shown that partial hepatectomy results in a rapid and marked reduction in all 3 mRNA species, which can be attributed to a corresponding decrease in the rate of transcription of the α_{1B}-AR gene. They also demonstrated for the first time that the tissue level of NF1 (positive activator of α_{1B}-AR in liver), declined, and its binding to the P2 promoter was attenuated in the remnant liver. An analogous decrease of NF1 binding to the P2 promoter was also observed in primary hepatocytes after several hours in culture (Gao, unpublished observations by this group). Since NF1 enhances P2 promoter activity in Hep3B cells and primary hepatocytes, the decline in the tissue level of the NF1 protein is likely to be one of the factors responsible for the decreased expression of the α_{1B}-AR gene during culturing of hepatocytes. Multiple factors, including cell-cell contact (Hartig et al., 1993) are known to regulate the activity of NF1. Interestingly, Kajiyama and Ui. (1994) demonstrated that the transition from an α_1- to β-AR mediated response in cultured hepatocytes did not occur unless the cells were cultured under conditions favourable for cell growth i.e., at low cell density. The switch was prevented progressively as the cell culture density was increased up to 20-fold or a high-density culture was achieved by addition of increasing amounts of liver plasma membranes. Increases in cell-cell interactions were reported to be an important factor in inhibition of cell growth, in favour of cell differentiation, or expression of liver-specific characteristics (Nakamura et al., 1983, 1984). In my own experience, hepatocytes were plated at a maximum density so that a suitable density remained after the initial attachment period, washes, and replacement with culture medium.

So what is the biological significance of this attenuation of α_{1B}-AR transcription?

It has been shown in rat liver that isolation of hepatocytes under conventional conditions results in activation of the immediate-early growth program and an extensive down-regulation of most hepatic functions (Rana et al., 1994).

NA acting through hepatic α_{1B}-ARs is a strong comitogen (Cruise et al., 1985). The decline in α_{1B}-AR expression in the early stages of regenerating liver's response may therefore serve to turn off a mitogenic signal and limit the extent of hepatic proliferation (Gao et al., 1996). A failure to suppress the expression of hepatic α_{1B}-ARs after partial hepatectomy could promote abnormal growth and differentiation. Indeed, in an experimental model, the over-expression of α_{1B}-AR, induced agonist-dependent focus formation and disordered growth (Allen et al., 1991). Thus the decline in the concentration of NF1 may play a part in a tightly regulated cell program aimed to ensure a brief burst of proliferative activity immediately followed by differentiation (Gao et al., 1996). A similar situation seems likely in cultured hepatocyes.

An important consideration for future work would be: Based on the literatue, it appears that the α_{1B}-AR is down-regulated when hepatocytes are put into culture, and a diminished Ca^{++} response is likely. So would that same change happen in the knockout, given that the α_{1B}-AR is replaced by α_{1A}-AR?

In vitro incubation (4hours) of hepatocytes acutely isolated from adult male rats can lead to a 40% decrease in α_{1B}-AR density, and a 70% decrease in α_{1B}-AR mRNA levels (Ishac et al., 1992). Work in this chapter suggests that hepatocytes are capable of increasing their α_1-AR density (α_{1A}- and α_{1D}-AR subtypes) during prolonged culture. Basing our findings on the literature, it appears that activation of the immediate-early growth program, and down-regulation of α_{1B}-AR is likely to be intrinsic to the cell whereas increased expression of α_{1A}- and α_{1D}-ARs was probably

influenced by mitogenic factors in the culture media. For these reasons, information regarding the natural disposition of α_1-ARs at a single cell level could only be hypothesised.

Functional heterogeneity of hepatocytes

In an attempt to understand the reasons for heterogeneous expression of α_1-ARs in isolated hepatocytes, it was necessary to consider the possible existence of functional heterogeneity between individual cells within the liver. An important factor that was obvious from reading all the existing literature was that the phenotype of adult hepatocytes exhibit great plasticity.

Extracellular signals are capable of generating responses that titrate existing gene expression to meet new environmental demands (Higgins and Anderson, 1931; Fausto, 1990; Xanthopoulos and Mirkovitch, 1993). In addition, the structure of the liver lobule ensures that hepatocytes in different parts of the hepatic acinus receive different extracellular signals. This permits heterogeneous local responses to any given systemic signal (Gebhart, 1992). Functional heterogeneity among hepatocytes is instructed by zonal differences in the cellular microenvironment. Factors carried by portal venous and hepatic arterial blood, in turn, regulate many aspects of the microenvironment, as hepatic function is generally varied along the portal (zone 1) to central (3) gradient of afferent blood flow (Coleman et al., 1993). In addition to microenvironmental differences in the 'maturity' of individual hepatocytes, different parts of the liver may also contribute to normal functional heterogeneity within the liver (Sigal et al., 1992). Even though the proliferative activity of adult hepatocytes is low, some hepatocyte replication does occur, which is both time and 'position'

dependent. Proliferation tends to be greater in zone 1 than in zone 3 (Post and Hoffman, 1964). As the α_{1B}-AR has been reported to be involved in proliferation of hepatocytes, are hepatocytes from this area within the liver more densely populated with α_{1B}-ARs? Are α_{1A}-ARs able to perform similar proliferative roles in the knockout?

Mitogenic factors in the culture media

Hepatocytes do not enter into DNA synthesis when kept in chemically defined media or in media supplemented with foetal bovine serum (Michalopoulos, 1990).

Earlier studies have demonstrated that primary culturing of rat hepatocytes plated in serum-containing media leads to a several-fold increase in β_2-AR density (Nakumura et al., 1984; Schwartz et al., 1985; Refsnes et al., 1987) and a parallel marked decrease in α_1-AR density (Nakamura et al., 1984; Schwartz et al., 1985) within 8-48hours. Ishac et al. (1992) found similar but smaller changes detected in hepatocytes suspended in a serum-free buffer after 4hours.

It has recently been reported that epidermal growth factor (EGF), insulin, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), insulin-like growth factor I, and II on their own can rapidly stimulate hepatocyte DNA synthesis and proliferation during short-term culture (4hours) in defined media (Kimura and Ogihara, 1997a-c; Kimura and Ogihara, 1998a,b). The rapid proliferative responses of hepatocytes to these growth factors were found to be dependent on such culture conditions as hormones in the media and initial plating density. The presence of the amino acid proline in the medium (Houck and Michalopoulos, 1985) is also required for DNA synthesis in hepatocytes.

Since there was an increase in α_1-AR density during prolonged culture, it was necessary to assess the possible contribution of the contents of the media used to perfuse, attach and culture the hepatocytes in this study. Unfortunately, the three media used, from the 'Hepatocyte product line' from Life technologies, Inc. were patented and only limited information was available.

Nicotinamide was present in the culture media, and since it is converted to NAD/NADP, endogenous fluorophores, this was likely to be responsible for high autofluorescence observed during extensive incubation periods as seen during transfection. Nicotinamide is also known to enhance cyclic replication of DNA.

Growth hormone was also contained in all three media used and is a known stimulant of protein synthesis.

Similarly, gluococorticoid was present in all three media. Glucocorticoids have been reported to induce the transcription and expression of both the α_{1B}- (Sakaue and Hoffman, 1991) and β_2-AR (Collins et al., 1988; Hadcock and Malbon, 1988; Jazayeri and Meyer, 1988) genes in smooth muscle cells. However, a report by Huff et al. (1991), indicated that glucocorticoid administration *in vivo* was able to program the ontogenic disappearance of hepatic β-ARs, which reaches a peak of sensitivity just before the period in which natural adrenocortical function matures (Miyabo et al., 1980; Lamers and Mooren, 1981). The hypothesis that the development of adrenal steroid secretion provides a normal maturational cue for hepatic AR expression has been supported by preliminary evidence that neonatal adrenalectomy delays the switch from β to α_1-ARs (McMillan, 1983). The failure of glucocorticoids to evoke a similar irreversible shift in the programming of α_1-AR ontogeny suggests that other factors, possibly the development of sympathetic innervation (Slotkin et al., 1986), provides the major input for that subtype.

From these reports, it would seem that the effects of glucocorticoids are cell-type specific and its effects on hepatocytes are non-comparable with smooth muscle cells. They are likely, however, to increase the expression of α_1-ARs, although no direct mitogenic responses have thus far been reported.

The media used to perfuse the liver was a balanced salt solution that initiated the loosening of cell-cell contact. The media used to attach cells to glass cover slips was an enriched Williams E media, supplemented with Foetal Bovine Serum for the adherence of hepatocytes (0-4hours). Hepatozyme-SFM, a serum-free media was used for short-term (4-48hours) and maintenance of hepatocyte phenotypic expression.

Ontogenic differences in expression of α_1-ARs

The studies done at the single cell level contradicted some, but not all, of the radioligand binding data. Regarding overall changes in α_1-AR density at different ages, both techniques reported a significant increase in α_1-AR density in the knockout between 3 and 4months, the opposite of which was observed in the wildtype. Interestingly, at some point between 3 and 4months, the biological discrepancy observed between QAPB binding to hepatocytes from 3 and 4month-old mice seemed to balance out. At 4months, the difference between average fluorescence intensity values for wildtype and knockout cells was no longer statistically significant.

Although not as obvious after prolonged hepatocyte culture, it was evident from radioligand binding studies that the α_1-AR density at 3months in the knockout was considerably lower than in the wildtype liver. Since α_1-ARs play such a major role in

this organ (see general introduction), what compensatory mechanisms are present before the animal reaches 4months?

The only available information on the developmental changes that occur in the liver are based on the rat. In crude membrane preparations from rat liver at different ages, the α_1-AR density increased upon maturation (from birth until 2months), and decreased slightly till the age of 24-26months (Van Ermen and Fraeyman, 1992).

It cannot be assumed that the same ontogenic pattern of α_1-AR expression exists in mice, especially when the 'normal' subtype expressed has been removed from the equation. There may be fundamental differences between wildtype and knockout animals since the α_{1B}-AR gene has been absent since conception onwards. The bulk of evidence from other AR knockout models has revealed that basal physiological functions are not significantly perturbed, and that alternative control points may exist for critical physiological functions such as cardiac rate and contractility, vascular tone, and metabolic state, which can be altered to compensate for the knocked out receptor (Roher and Kobilka, 1998). An example of this was observed in a β_1/β_2-AR double knockout where cardiac muscarinic receptor density was reduced, a counterbalancing reduction in a receptor that is known to functionally antagonise stimulatory β-ARs (Roher et al., 1999). Such knockout models are not likely to reveal the 'true' roles of the subtype in question, as compensatory mechanisms are likely to take effect at specific time points in the development of the animal.

Species heterogeneity of α_1-AR subtype expression

According to the literature, as mentioned in the general introduction, α_1-AR subtype heterogeneity exists between species. These differences may be age-related. With the exception of the guinea pig, animals that are studied early in their life span

(<4months) i.e., rats, mice, hamsters, and chickens, all appear to predominantly express the α_{1B}-AR. In contrast, those species that are studied at a later point, ranging from 6-14months (rabbit, cat, and dog) to several years (monkeys and humans), express the α_{1A}-AR.

Since the α_{1B}-AR has been implicated in growth, it seems logical that this subtype would be present at an early age, with its importance, and hence receptor protein, declining over time. The emergence of the α_{1A}-AR subtype may occur at a specified time point, for example, between 3 and 4months in the mouse.

If the rat liver is capable of altering its α_1- and β-AR populations then it seems plausible that it would also be capable of changing the subtype of α_1-AR expressed *in vivo*. Although this has not yet been reported in any model.

The possibility that the wildtype mouse liver would predominantly express the α_{1A}-AR at a later stage in its life cycle, say after 1 year, would be very interesting. Such age studies should answer these queries regarding the relationship between subtype expression and age of the animal when studied.

Compensatory mechanisms

It appears that α_1-AR subtype redundancy may be involved in the regulation of hepatic functions. The presence of a pure population of α_{1A}-ARs in the knockout liver at 4months would suggest that this subtype is able to compensate for the lack of the α_{1B}-AR present in the wildtype at this age. However, the existence of only a small population of α_1-ARs at 3months in the knockout, compared to the wildtype, suggests that a non-α_1-AR mechanism is compensating for the lack of the α_{1B}-AR subtype, at that time. At a specific time point between 3 and 4months, a replacement

population of α_{1A}-ARs becomes established. In the knockout at 3months, few high affinity α_1-ARs are present (8fmol/mg), at this time, which in the rat, represents the start of post-maturational decline. If the α_{1B}-AR plays such an important role in the growth and development of the rat liver from 3/4 weeks (post-switch from β to α) to 2-3months (mature), then what compensates for the lack of high affinity α_1-ARs in the knockout mouse at 3months? Are events mediated through β-ARs until α_{1A}-ARs are up-regulated? This seems plausible since hepatocytes isolated from adult female rats are responsive to both α_1- and β_2-AR stimulation, whereas adult male rat hepatocytes respond only to α_1-AR stimulation (Studer and Borle, 1982). Perhaps changes in hormone levels compensate accordingly and mediate hepatic functions via a different mechanism. An interesting avenue would be to localise β-ARs in isolated hepatocytes using BODIPY®TMR (±) CGP 12177 (Arribas et al., 1997).

Further investigation is required to determine the α_1-AR subtype expressed at 3months in both strains, in the intact liver, and to establish whether a population of α_{1A}-ARs similar to that in the α_{1B}-knockout is present in the wildtype mouse at 3months. Such future work should shed light on the ontogenic pattern of α_1-AR subtype expression.

'High' and 'low' affinity binding of QAPB to α_1-ARs in hepatocytes

In wildtype and knockout hepatocytes, QAPB bound to high (typical α_1-AR pharmacology) and low affinity populations. The estimated K_D values for these low affinity sites however, did fluctuate. At 3months, they were non-saturable within the concentration range, yet at 4months, they did saturate (<20nM QAPB), and were easily quantified.

The binding of QAPB to α_1-ARs at >5nM, in recombinant cell lines (chapter 1), is normally considered relatively 'non-specific', with specific sites saturating at low concentrations of QAPB (<2nM). It has become obvious, as mentioned in chapter 1, that QAPB will bind 'non-specifically' in cells densely populated with α_1-ARs, as indicated by the similar pattern of binding to prazosin-insensitive sites.

In hepatocytes, a different pattern of QAPB binding was observed compared to recombinant α_1-ARs. QAPB bound to low density, high affinity receptive sites, saturating by 1nM QAPB. A further increase in specific binding of QAPB commenced from 5nM upwards. The identity of these sites remains unknown. As these low affinity sites are also present in wildtype hepatocytes, and partially blocked by RS100329, these results indicate that there is a minor population of α_{1A}-ARs in the wildtype liver. After 24hours in culture, the ArrayScan work demonstrated that both strains were capable of expressing α_{1A}- and α_{1D}-ARs. The confocal studies using BMY-7378 indicated that α_{1D}-ARs were present after 4hours in culture. However, this was only evident from one cell, and when average fluorescence intensity values from several cells were compared, BMY-7378 showed minimal effect.

If the α_{1A}-AR is expressed in small amounts in the wildtype, then perhaps this represents the small population of α_1-ARs observed in the knockout at 3months, yet to be characterised by radioligand binding.

Is the α_{1A}-AR perhaps present at all times, in a low affinity state (α_{1L})? Low affinity binding was seen at 3months in both strains. After the mouse had aged by a further month, cells appeared to bind QAPB with higher affinity, thus displaying typical α_{1A}-AR pharmacology. Studies using 1year-old mice will hopefully shed light on

this. It is obvious from the work in this chapter that a more substantial, higher density α_{1A}-AR population appears between 3 and 4months.

Does low affinity binding of QAPB represent α_{1L}-ARs?

Ohmura and Muramatsu, (1995) reported prazosin-sensitive low affinity binding sites in rabbit liver plasma membranes. This group considered these receptors belonged to the α_{1L}-AR subtype. The same study showed the coexistence of 2 distinct α_1-AR subtypes (α_{1A} and α_{1L}) in the rabbit liver and the higher density of α_{1L}-AR compared with the α_{1A}-AR subtype.

Similarly, in the present study, QAPB bound to high density, low affinity and low density, high affinity sites in mouse hepatocytes.

α_1-ARs with low affinity for prazosin (α_{1L}) are evident from pharmacological studies in many tissues (Muramatsu et al., 1995). Therefore it is interesting to compare the characteristics of α_{1L}-ARs between liver and other tissues. Affinity estimates for prazosin are similar in a wide range of tissues, whereas the affinities of WB4101 (pKi 7.57) and 5-MU (6.75) determined in rabbit liver are much lower than those determined in rabbit aorta or human prostate (8.2-8.5) (Oshita et al., 1993; Muramatsu et al., 1994). This may suggest heterogeneity of α_{1L}-ARs.

Supporting the theory that the α_{1L}-AR is in fact a conformational variation of the α_{1A}-AR (Williams et al., 1996; Ford et al., 1997), and that further subdivisions may exist, it would not be surprising that conformational variants of the α_{1L}-AR exist, when you consider the existence of splice variants of the α_{1A}-AR (Chang et al., 1998) which can lead to functional variations.

Perhaps there are sites in the liver that have low affinity for ligands in general?

Effects of temperature on hepatic α_1-ARs

Using liver plasma membrane fractions, Borst and Scarpace, (1990) observed, as did Lynch et al. (1985), a mixed population of high and low affinity α_1-ARs in binding studies conducted at 25°C. Both groups report conflicting data however at 2°C (Lynch) and 4°C (Borst and Scarpace), where they found a homogeneous population of high affinity and mixed population of α_1-ARs, respectively. At 37°C, a homogeneous population of low affinity binding sites was observed (Borst and Scarpace, 1990; Schwartz et al., 1986). However, an artifactual partial reduction in affinity is also seen when tissue is incubated at 37°C in the absence of agonist. Post incubation at 37°C, hepatocyte analysis in this study was at room temperature.

Subcellular distribution of α_1-ARs in hepatocytes

The majority of studies in the literature, regarding α_1-ARs in the liver, are based on radioligand binding using plasma membranes. The general consensus regarding the functional-location of α_{1B}-ARs is that they reside at the plasma membrane. The work detailed in this chapter demonstrates that α_1-ARs are distributed throughout the cytoplasm in hepatocytes, with densely populated areas surrounding nuclei, presumably networks of golgi apparatus (Nagata, 2001). The functional role of α_1-ARs in hepatic metabolism suggests that perhaps their cytoplasmic distribution is relevant to the distribution of glycogen granules, which are similarly distributed diffusely throughout the cytoplasm of the cell (Stryer, 1988). Contained within each granule are the enzymes that catalyse the synthesis and degradation of glycogen, and also those that regulate these processes (Stryer, 1988).

The consequence of such a distribution is that ligands have to gain access to such sites by diffusion through the membrane, an endocytic mechanism or via a

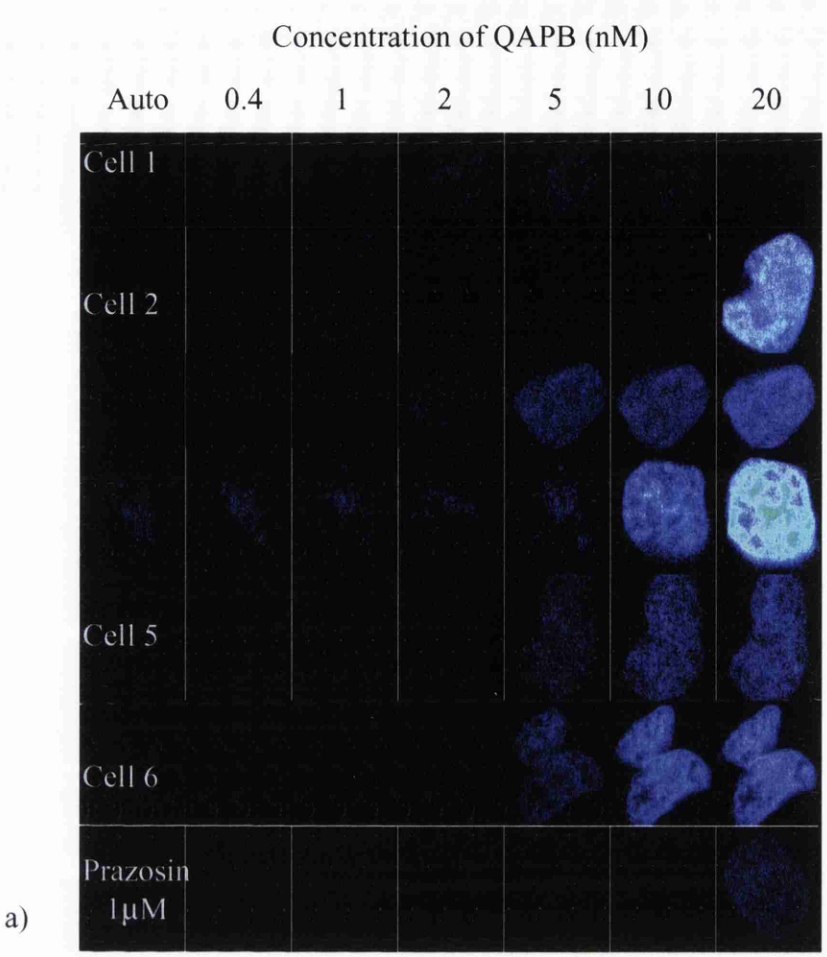
transporter. Interestingly, the distribution of α_1-AR subtypes is the same in wildtype (α_{1B}) and knockout (α_{1A}) hepatocytes, the subtypes indicated from radioligand binding data (4months), suggesting subtype redundancy in this tissue. An important point to note however, is the appearance of both α_{1A} and α_{1D}-ARs in both strains after culturing hepatocytes for 24hours, as evident from the ArrayScan study. Since the QAPB binding sites are mainly α_{1A} and α_{1D}-ARs, could this explain why no differences were observed in the distribution of α_1-ARs between the two strains at the single cell level?

A recent report, regarding the interaction of the α_{1B}-AR with the multifunctional protein, gC1q-R, has created a route for us to locate the α_{1B}-AR in wildtype cells after acute isolation. We hope to use a rabbit polyclonal antibody to gC1-qR (Sigel and Schaerer) and colocalise with QAPB binding, combined with subtype-selective antagonists. gC1-qR, isolated from rat liver, has been reported to retain the α_{1B}-AR inside the cell and mediate the down-regulation of α_{1B}-AR receptor protein (Xu et al., 1999; Hirasawa et al., 2001). In this report, although the K_D value was not significantly altered by co-expression of the α_{1B}-AR with gC1-qR, the B_{max} value of the α_{1B}-AR dramatically decreased. So what is the role for this previously identified complement regulatory molecule in the regulation of the cellular localisation and expression of the α_{1B}-AR?

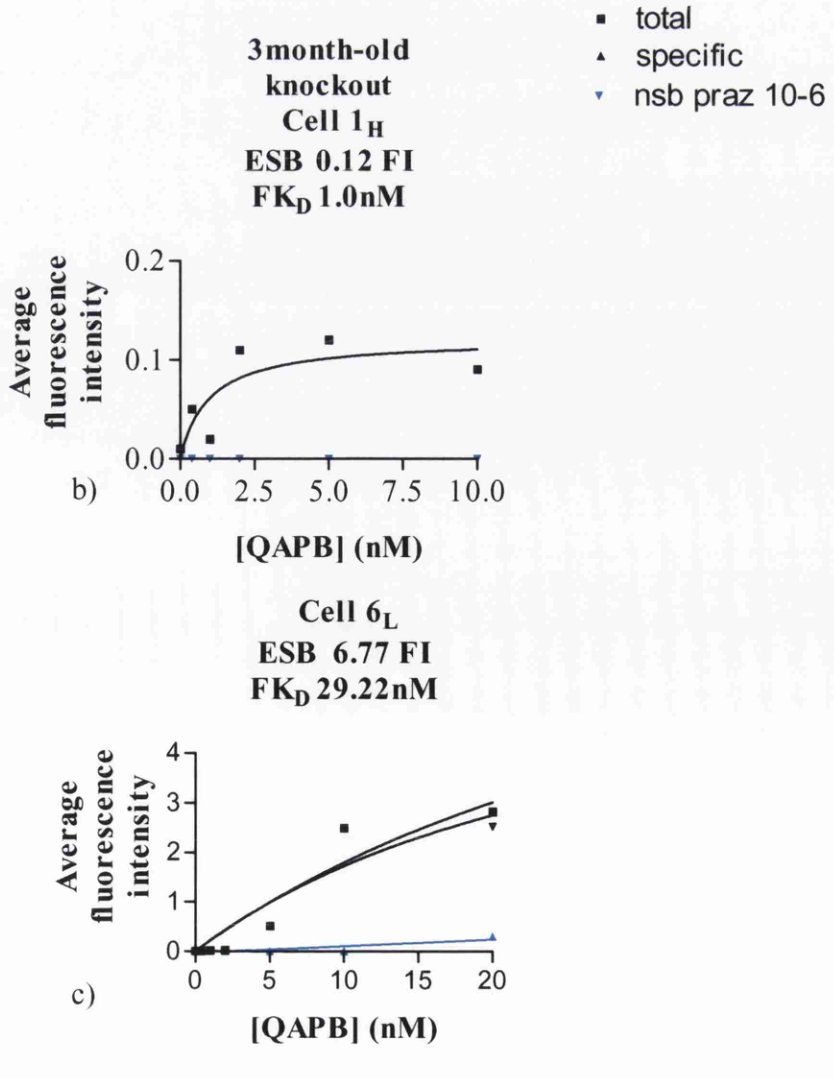
An important consideration is that this protein-α_1-AR complex was isolated from rat liver, which has been reported to express a pure population of α_{1B}-ARs. In view of the α_{1B}-knockout mouse expressing α_{1A}-ARs, how does this compare? Is this protein specific to the α_{1B}-AR, or is it liver-specific, and able to regulate the α_1-AR component, irrespective of subtype?

It is interesting to note that data from radioligand binding studies using liver plasma membranes indicates that α_1-ARs are located at the plasma membrane. The work in this chapter, at the single cell level, suggests otherwise. However, basing our interpretation on the literature regarding hepatic α_1-ARs, it seems plausible that whilst *in vivo*, hepatocytes, up to a certain age, may express α_{1B}-ARs, which may be situated at the plasma membrane.

Figure 2.1 (a) QAPB binding on hepatocytes isolated from livers of 3month-old knockout mice, cultured for ~4hours. Non-specific binding was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b-d) The QAPB-associated fluorescence intensity was calculated by Metamorph software and plotted against increasing concentrations of QAPB to estimate the levels of specific binding of QAPB in sample cells. The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) was measured as fluorescence intensity (FI). Estimates for cells 1,2,5 and 6 were (0.976, 0.121), (0.097, 0.032), (21.80, 1.496) and (29.22, 6.77) respectively. (e) Bar chart representing 20nM QAPB binding to 3month-old knockout hepatocytes cultured for 4hours pre-incubated with 1μM prazosin. Cells were plated on coverslips, incubated for 30minutes with non-fluorescent antagonist, then equilibrated with 20nM QAPB. Inhibition of QAPB binding was measured as average fluorescence intensity of the cell compared to the absence of inhibitor (control).


Concentration of QAPB (nM)
Auto
0.4
1
2
5
10
20
Cell 1
Cell 2
Cell 5
Cell 6
Prazosin 1μM

a)


total
specific
nsb praz 10-6
3month-old knockout
Cell 1H
ESB 0.12 FI
FKD 1.0nM
Average fluorescence intensity
0.2
0.1
0.0
0.0
2.5
5.0
7.5
10.0
[QAPB] (nM)
Cell 6L
ESB 6.77 FI
FKD 29.22nM
Average fluorescence intensity
4
3
2
1
0
0
5
10
15
20
[QAPB] (nM)

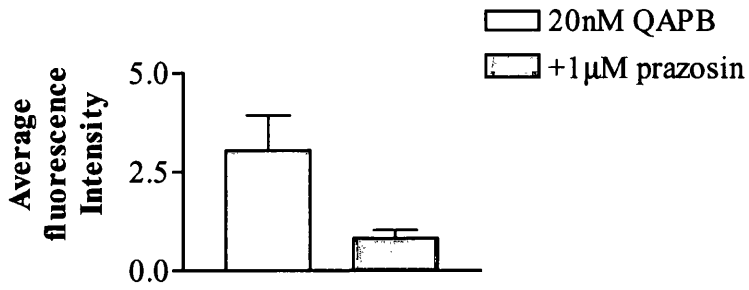
b)

c)

d)

3 month knockout ~4 hours culture	ESB (FI)	FK_D (nM)
cell 2_H	0.03	0.10
cell 5_L	1.50	21.80

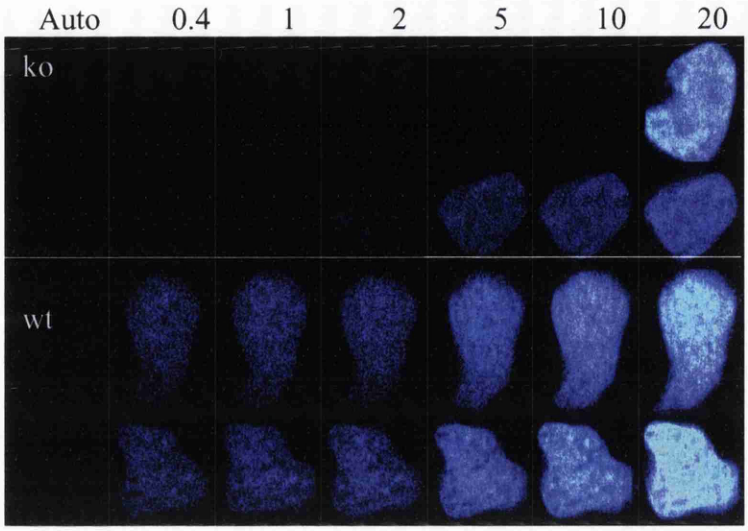
e)



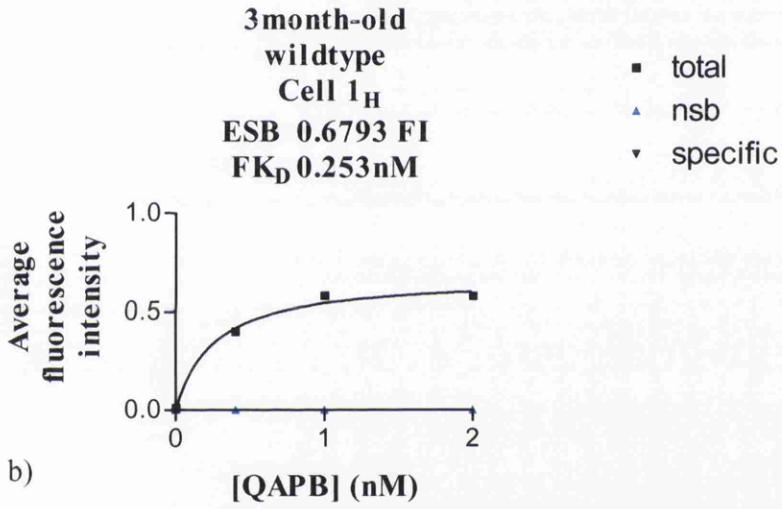
3month-old knockout, 4hours P=0.0157

Figure2.2 (a) Comparison of QAPB binding to 3month-old wildtype and knockout hepatocytes cultured for ~4hours. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b) The QAPB-associated fluorescence intensity was calculated by Metamorph software and plotted against increasing concentrations of QAPB to estimate the levels of specific binding of QAPB in a sample cell. (c) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells are presented in the table.

a)



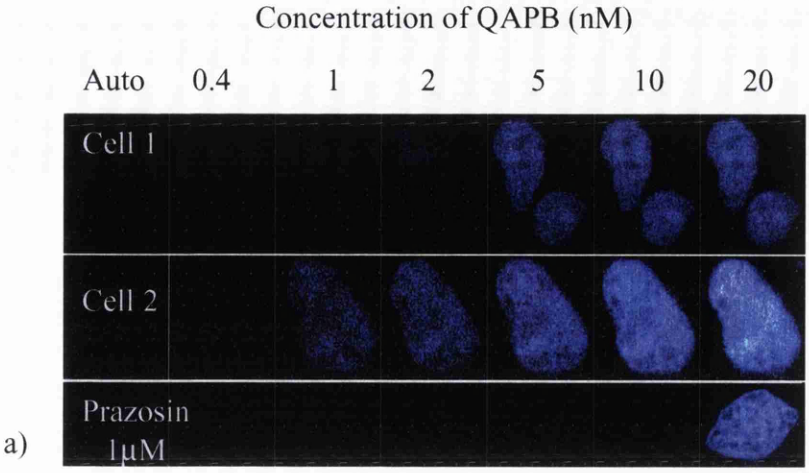
b)



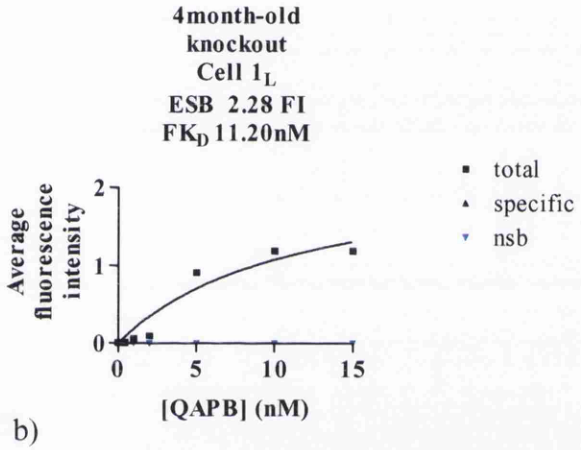
c)

3 month ~4 hours culture	ESB (FI)	FK_D (nM)
knockout Cell 1_H Cell 2_L	0.03 -	0.10 -
wildtype Cell 1_H Cell 2_L	0.68 -	0.25 -

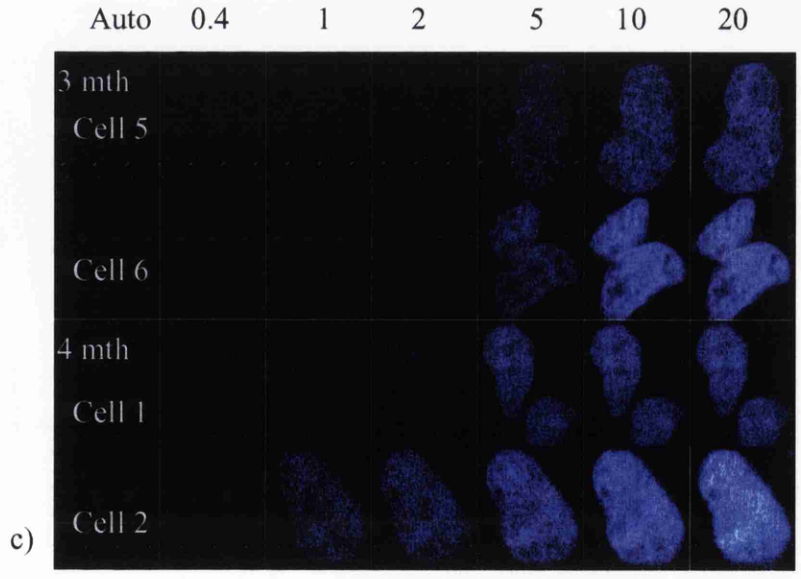
Figure 2.3 (a) QAPB binding on hepatocytes isolated from livers of 4month-old knockout mice, cultured for ~4hours. Non-specific binding was defined by 1μM prazosin. (c) Comparison of QAPB binding on hepatocytes isolated from 3 and 4month-old animals, cultured for ~4hours. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b) The QAPB-associated fluorescence intensity was calculated by Metamorph software and plotted against increasing concentrations of QAPB to estimate the levels of specific binding of QAPB in a sample cell. (d) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells are presented in the table.


Concentration of QAPB (nM)
Auto 0.4 1 2 5 10 20
Cell 1
Cell 2
Prazosin 1μM

a)


4month-old knockout
Cell 1L
ESB 2.28 FI
FKD 11.20nM
Average fluorescence intensity
2
1
0
0
5
10
15
[QAPB] (nM)
total
specific
nsb

b)

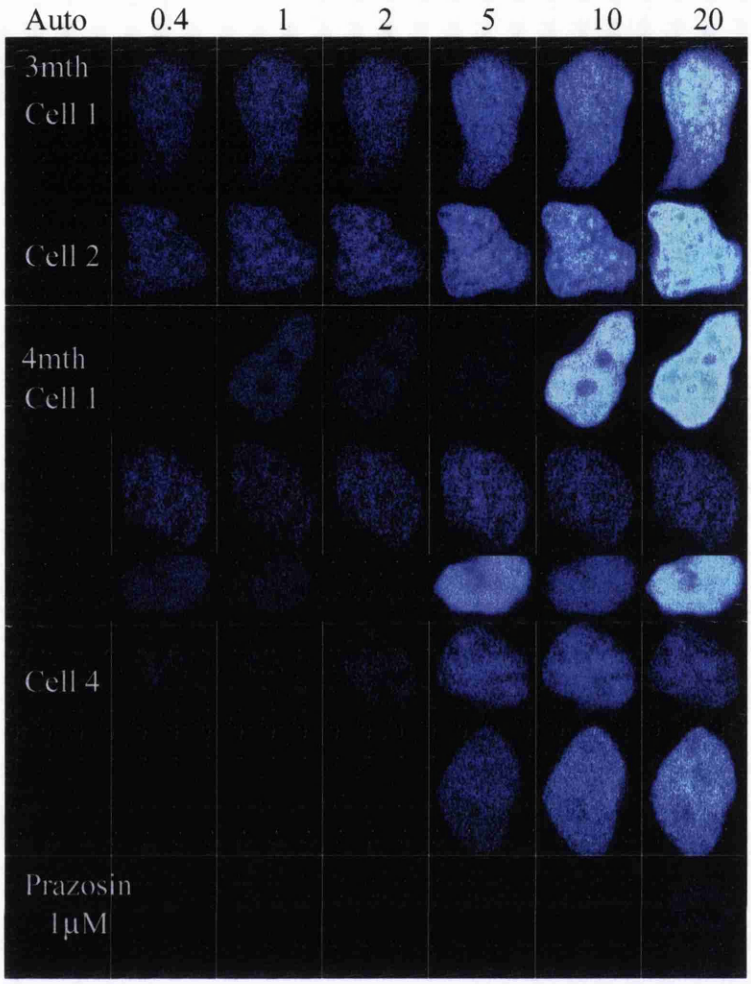

Auto 0.4 1 2 5 10 20
3 mth
Cell 5
Cell 6
4 mth
Cell 1
Cell 2

c)

~4 hours culture	ESB (FI)	**FK_D (nM)**
3 month knockout		
Cell 5_L	1.50	21.80
Cell 6_L	6.77	29.22
4 month knockout		
Cell $1t_L$	2.28	11.18
Cell $1b_L$	1.50	11.55
Cell 2_L	3.94	9.47

d)

a)



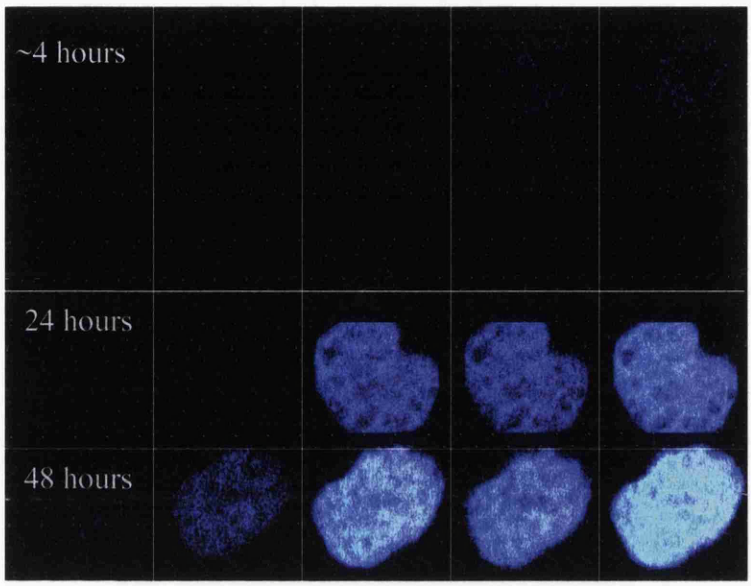
b)

wildtype ~4 hours culture	ESB (FI)	FK_D (nM)
3 months		
Cell 1_H	0.68	0.25
Cell $1/2_L$	-	-
4 months		
Cell 1_H	0.20	0.31
Cell 4_L	1.57	4.30

Figure 2.4 (a) QAPB binding to 3 and 4month-old wildtype hepatocytes cultured for 4 hours. Non-specific binding was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells are presented in the table.

Figure 2.6 (a) Comparison of QAPB binding to 'high' affinity sites on hepatocytes isolated from livers of 3month-old knockout mice, cultured for 4, 24 and 48 hours. (c) Comparison of QAPB binding to 'low' affinity sites on hepatocytes isolated from livers of 3month-old knockout mice, cultured for 4, 24 and 48 hours. (d) Non-specific binding after 48 hours in culture was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells expressing 'high' affinity sites are presented in the table.

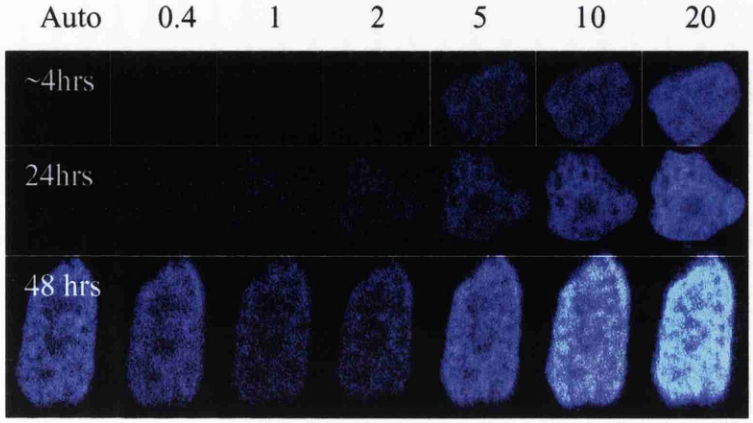
a)



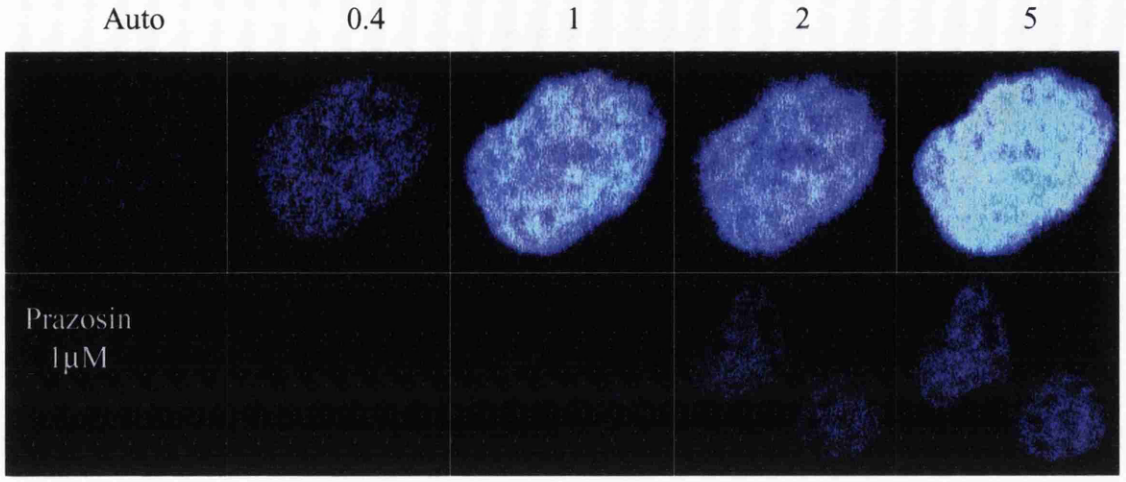
b)

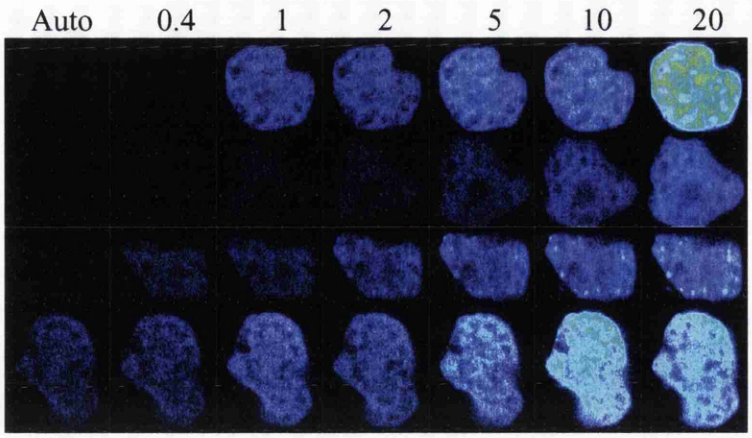
3 month knockout	ESB (FI)	FK_D (nM)
~4 hours	0.12 0.03	1.00 0.10
24 hours	2.79	1.46
48 hours	7.58	0.90

c)



d)





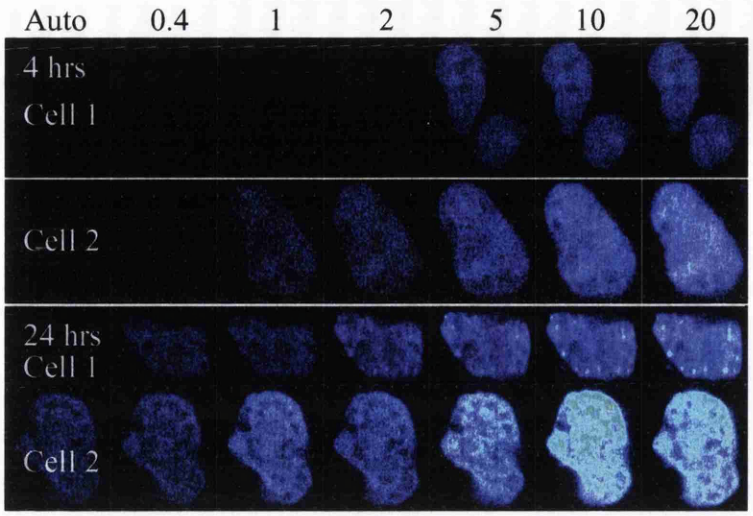
a)

24 hours Culture	ESB (FI)	**FK_D (nM)**
3 month		
cell 1_H	2.79	1.46
cell 1_L	-	-
cell 2_L	-	-
4 month		
cell 1_L	5.81	9.01
cell 2_H	3.10	0.56
cell 2_L	11.57	6.38

b)

Figure 2.7 (a) Comparison of QAPB binding to 3 and 4month-old knockout hepatocytes cultured for 24hours. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells are presented in the table.

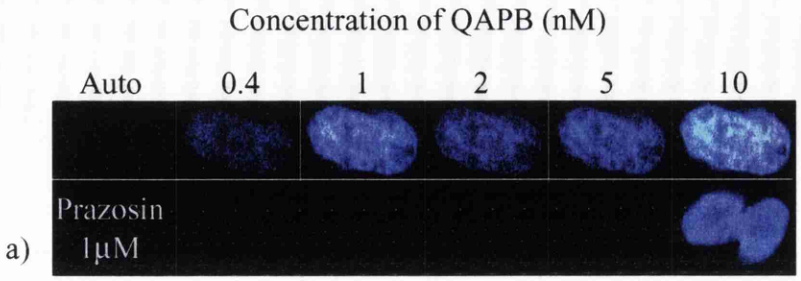
a)

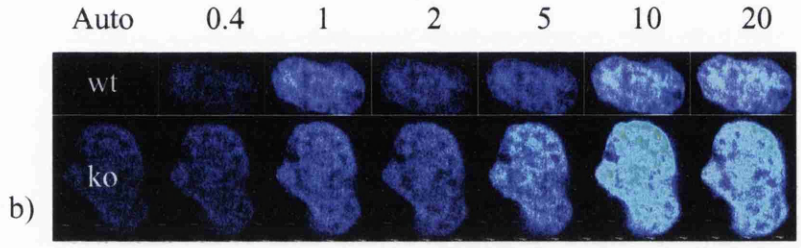


b)

4 month knockout culture	ESB (FI)	FK_D (nM)
~4 hours		
cell $1t_L$	2.28	11.18
cell $1b_L$	1.50	11.55
cell 2_L	3.94	9.47
24 hours		
cell 1_L	5.81	9.01
cell 2_H	3.10	0.56
cell 2_L	11.57	6.38

Figure 2.8 (a) Comparison of QAPB binding to 4-month knockout hepatocytes cultured for 4 and 24hours. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells expressing 'high' and 'low' affinity receptors are presented in the table.



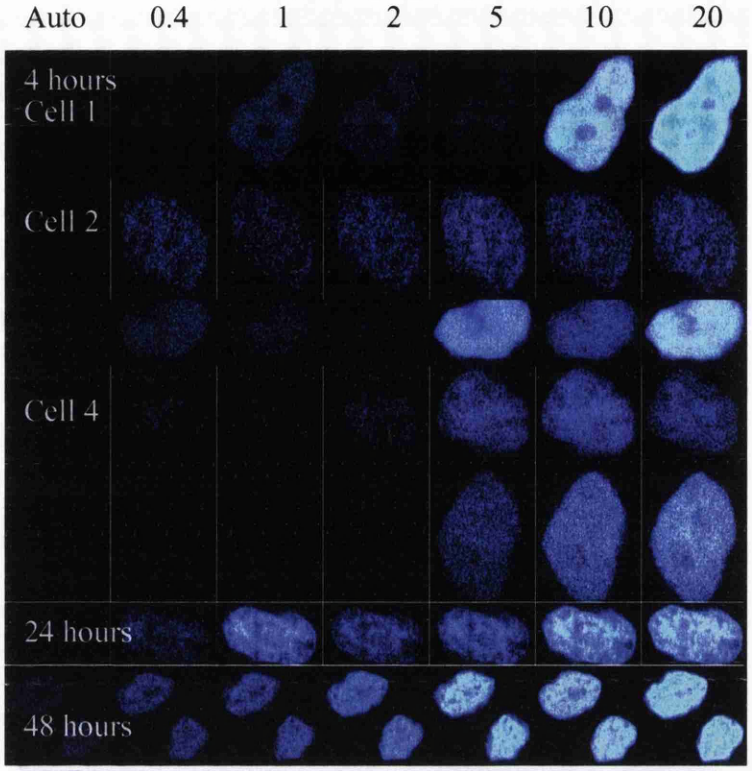


c)

4 months **24 hours**	ESB (FI)	FK_D (nM)
Wildtype		
Cell 1_H	2.20	0.37
Cell 1_L	-	-
Knockout		
Cell 1_H	3.10	0.56
Cell 1_L	10.86	3.92

Figure 2.9 (a) QAPB binding to 4month-old wildtype hepatocytes cultured for 24hours. Non-specific binding was defined by 1μM prazosin. (b) Comparison of QAPB binding to 4month-old wildtype and knockout hepatocytes cultured for 24hours. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (c) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells are presented in the table.

a)



b)

4month wildtype	ESB (FI)	FK_D(nM)
~4hours		
Cell 1_H	0.20	0.31
Cell 2_H	0.67	0.63
Cell 4_L	1.57	4.30
24hours		
Cell 1_H	2.20	0.37
48hours		
Cell 1_L	17.56	6.75

Figure 2.10 (a) Comparison of QAPB binding on 4month-old wildtype hepatocytes cultured for 4, 24 and 48hours. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (b) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells are presented in the table.

Figure 2.11 (a) Inhibition of QAPB binding to native α_1-ARs on 3month-old knockout hepatocytes cultured for 24hours. Figure illustrates inhibition of QAPB-associated fluorescence from specific α_1-AR binding sites using the α_{1A}-AR selective antagonist, RS100329 (1nM). The α_{1D}-AR selective antagonist, BMY-7378 (1nM) was without effect. (b, c) Inhibition of QAPB binding to native α_1-ARs on 4month-old knockout hepatocytes cultured for 4 and 24hours, respectively. Figure illustrates inhibition of QAPB binding to specific α_1-AR binding sites using the α_{1A}-AR selective antagonist, RS100329 (1nM). Cells were plated on coverslips, pre-incubated for 30minutes with antagonist, then examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore.

Concentration of QAPB (nM)

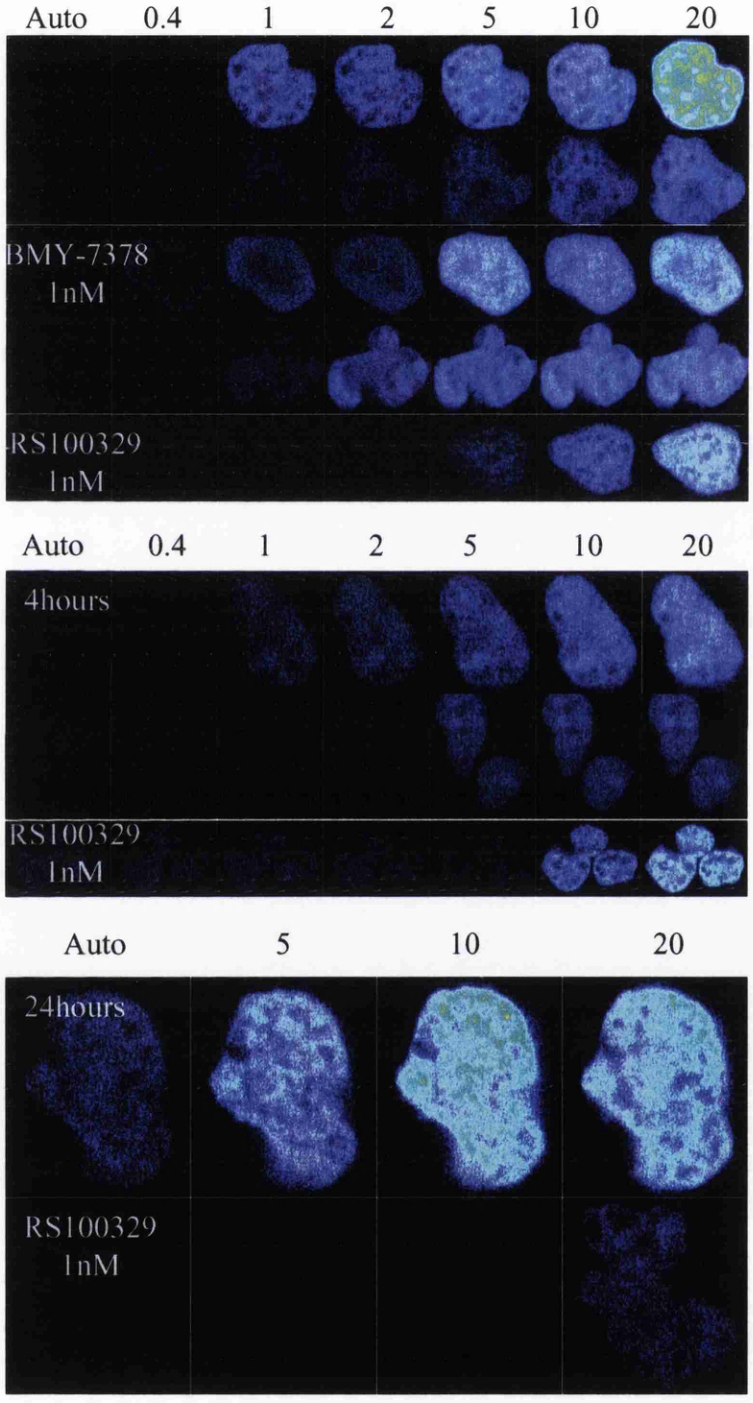
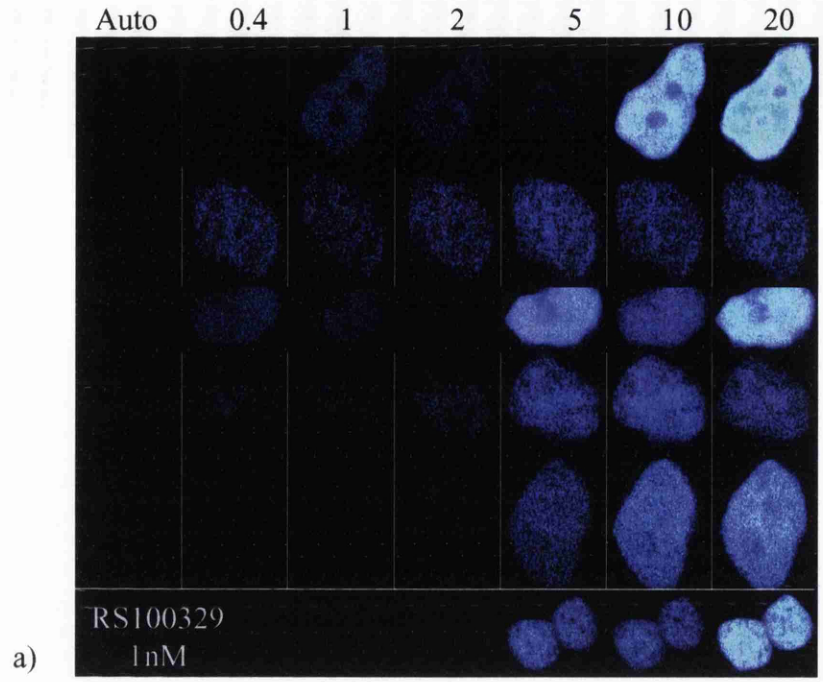
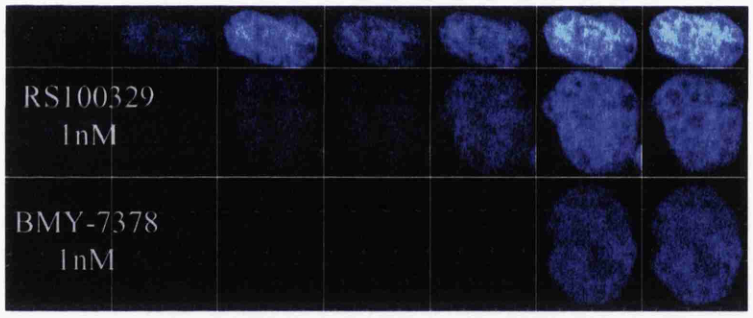


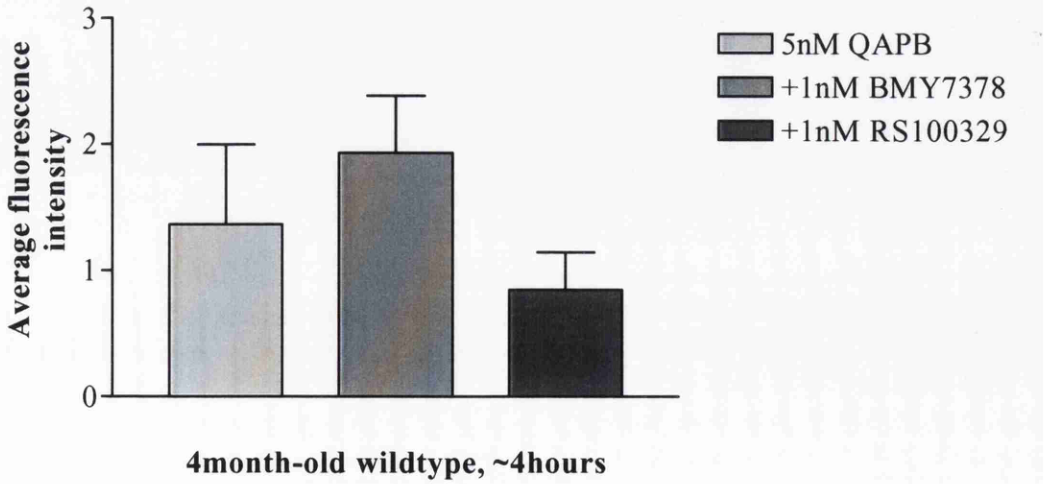
Figure 2.12 (a) Inhibition of QAPB binding to 4month-old wildtype hepatocytes cultured for 4hours by 1nM RS100329. (b) Inhibition of QAPB binding to 4month-old wildtype hepatocytes cultured for 24hours by 1nM RS100329 and BMY-7378. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (c) Bar chart representing 5nM QAPB binding to 4month-old wildtype hepatocytes cultured for 4hours pre-incubated with 1nM BMY7378 or RS100329. Cells were plated on coverslips, incubated for 30minutes with non-fluorescent antagonist, then equilibrated with 5nM QAPB. Inhibition of QAPB binding was measured as Average Fluorescence Intensity of the cell compared to the absence of inhibitor (control).


Concentration of QAPB (nM)
Auto
0.4
1
2
5
10
20
RS100329
1nM

a)


RS100329
1nM
BMY-7378
1nM

b)


Average fluorescence intensity
3
2
1
0
5nM QAPB
+1nM BMY7378
+1nM RS100329
4month-old wildtype, ~4hours

c)

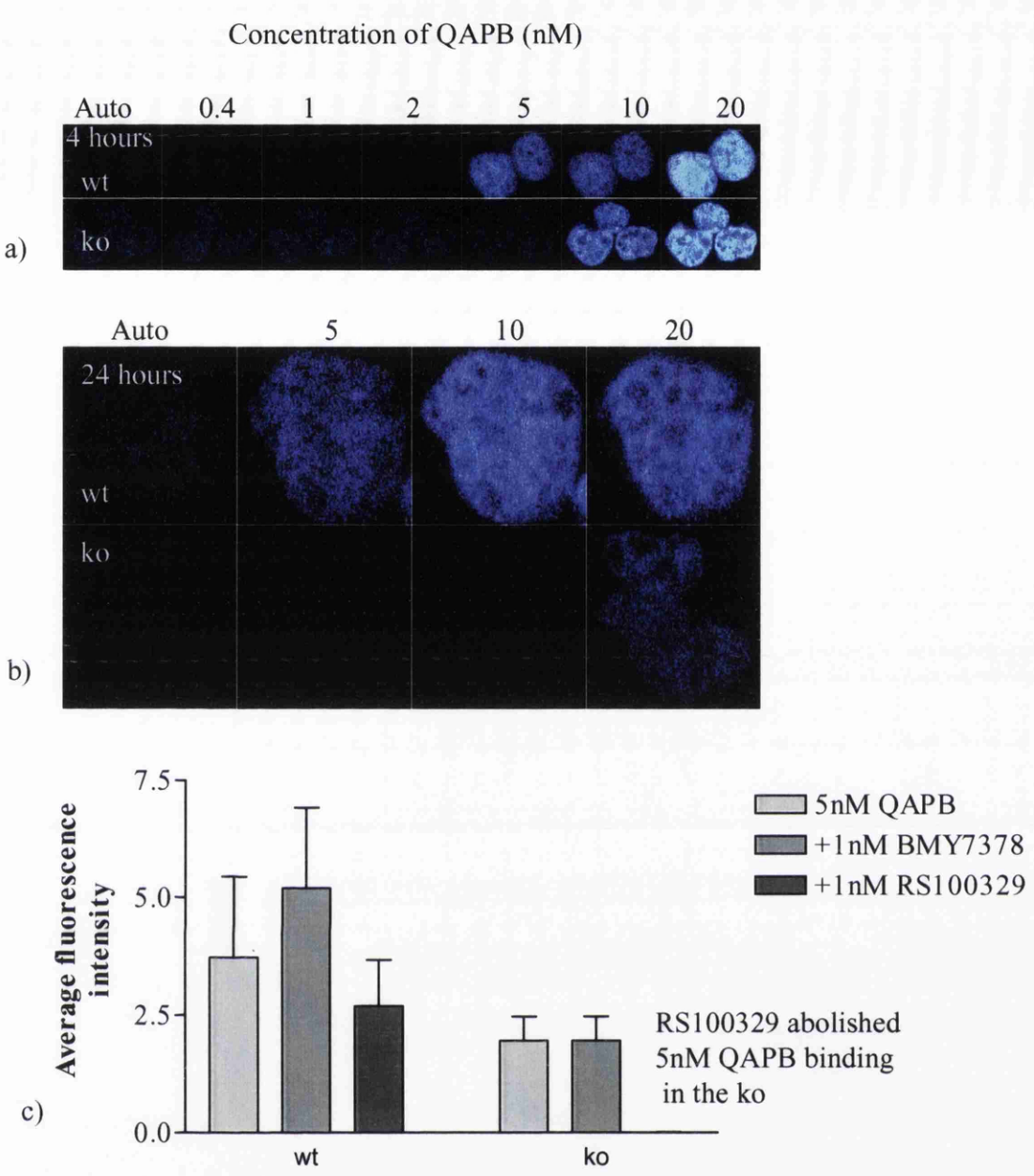
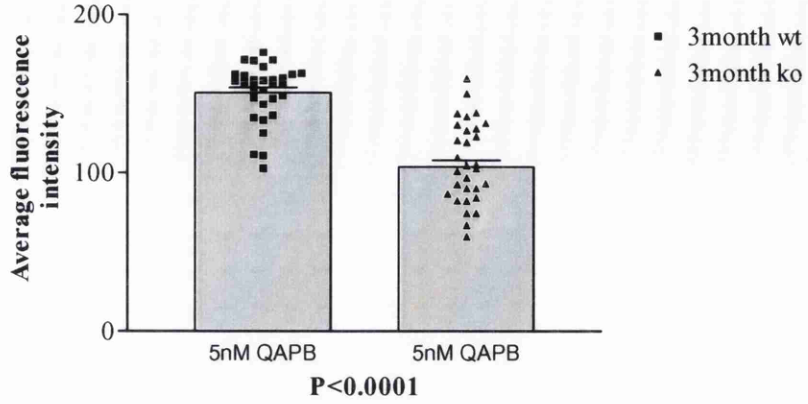
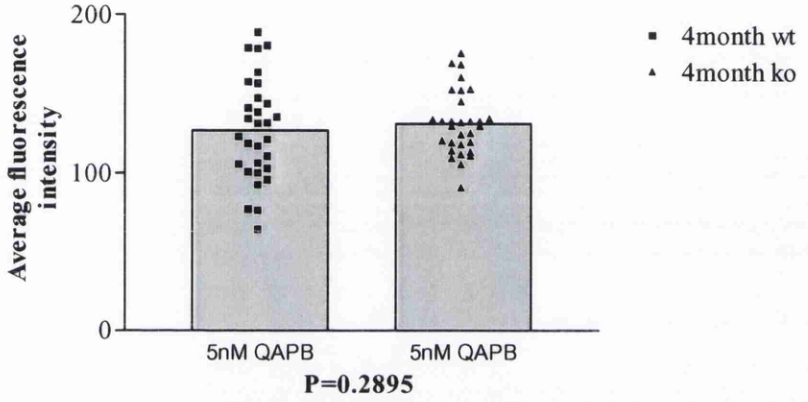
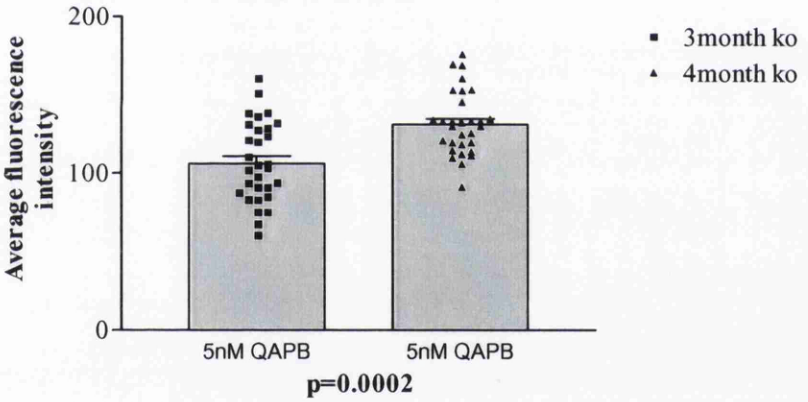


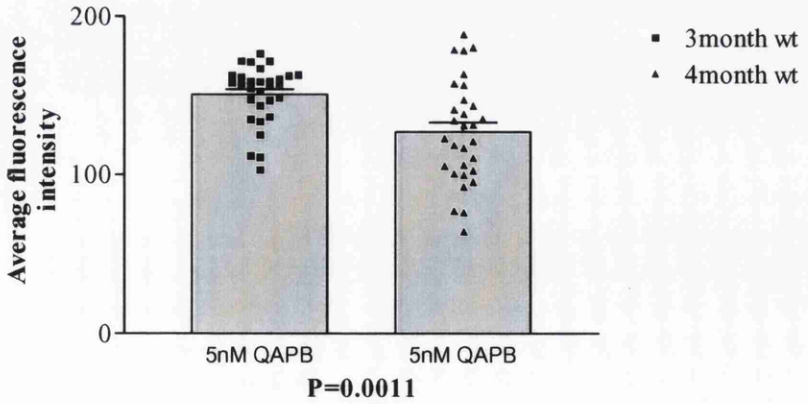
Figure 2.13 (a, b) Inhibition of QAPB binding to 4month-old wildtype and knockout hepatocytes cultured for 4 and 24hours by 1nM RS100329. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations (0.4-20nM) were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (c) Bar chart representing 5nM QAPB binding to 4month-old wildtype and knockout hepatocytes cultured for 24hours pre-incubated with 1nM BMY7378 or RS100329. Cells were plated on coverslips, incubated for 30minutes with non-fluoresent antagonist, then equilibrated with 5nM QAPB. Inhibition of QAPB binding was measured as Average Fluorescence Intensity of the cell compared to the absence of inhibitor (control).

Figure 2.14 (a-c) Hepatocytes were isolated and treated as described in methods. Images from the ArrayScan™ system were transferred to Metamorph software and average fluorescence intensity values for 30 cells (n=30), representing each of the experimental parameters, were recorded and graphed as shown. The distribution of intensity values within each parameter is also shown (•). P values indicating statistical significance are also presented.

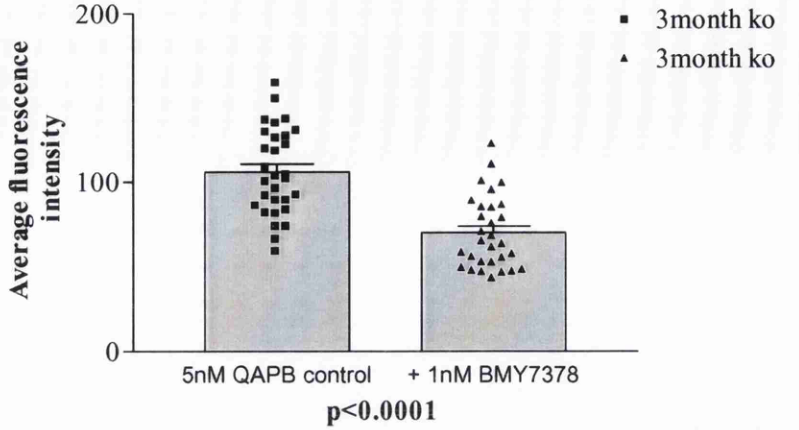

Average fluorescence intensity
200
100
0
5nM QAPB
5nM QAPB
3month wt
3month ko
P<0.0001

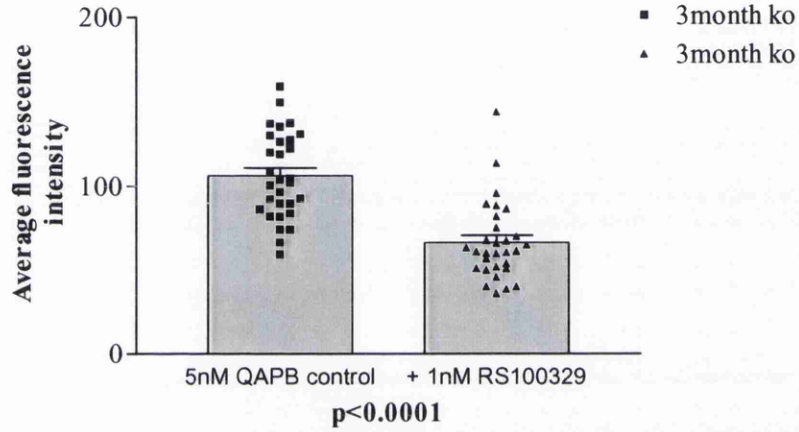

Average fluorescence intensity
200
100
0
5nM QAPB
5nM QAPB
4month wt
4month ko
P=0.2895

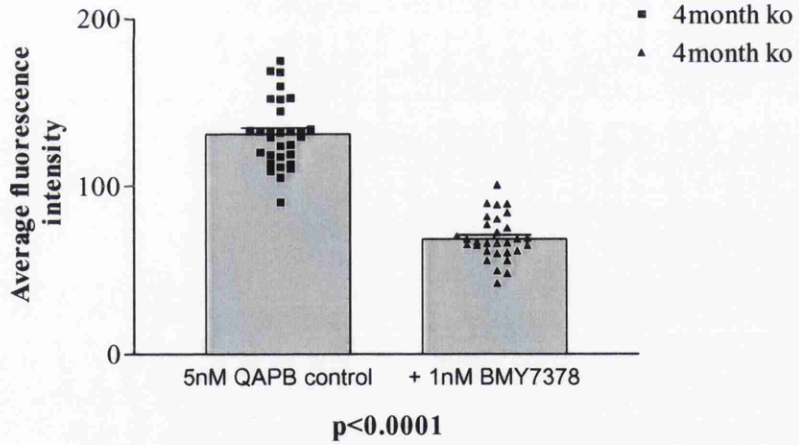

Average fluorescence intensity
200
100
0
5nM QAPB
5nM QAPB
3month ko
4month ko
p=0.0002

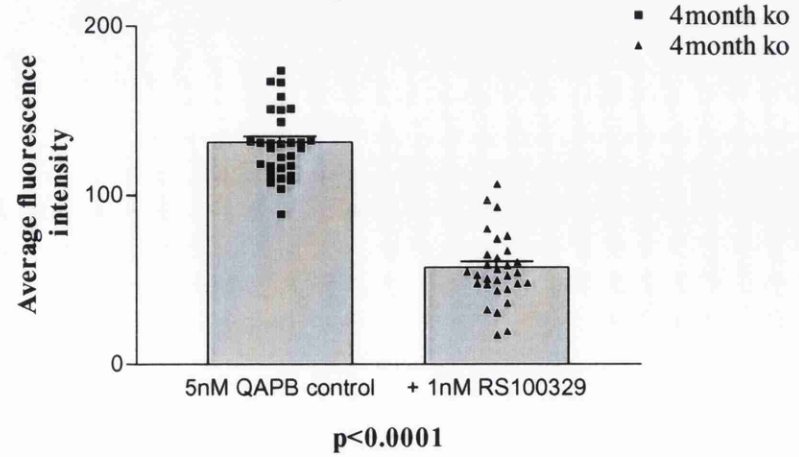

Average fluorescence intensity
200
100
0
5nM QAPB
5nM QAPB
3month wt
4month wt
P=0.0011

a)

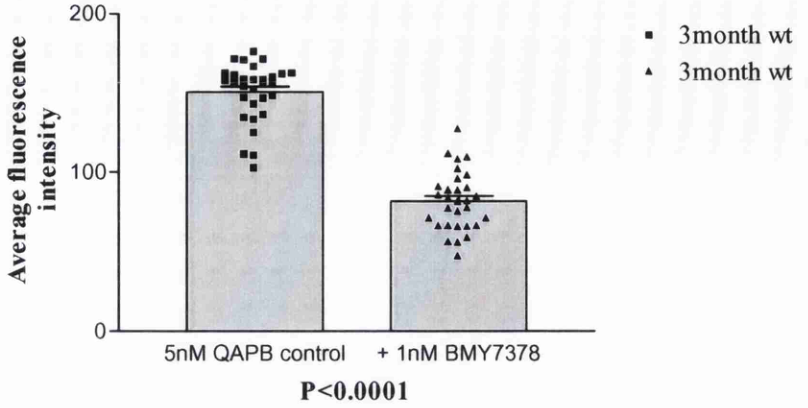

200
100
0
Average fluorescence intensity
3month ko
3month ko
5nM QAPB control
+ 1nM BMY7378
p<0.0001


200
100
0
Average fluorescence intensity
3month ko
3month ko
5nM QAPB control
+ 1nM RS100329
p<0.0001


200
100
0
Average fluorescence intensity
4month ko
4month ko
5nM QAPB control
+ 1nM BMY7378
p<0.0001


200
100
0
Average fluorescence intensity
4month ko
4month ko
5nM QAPB control
+ 1nM RS100329
p<0.0001

b)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100
0
Average fluorescence intensity
3month wt
3month wt
5nM QAPB control
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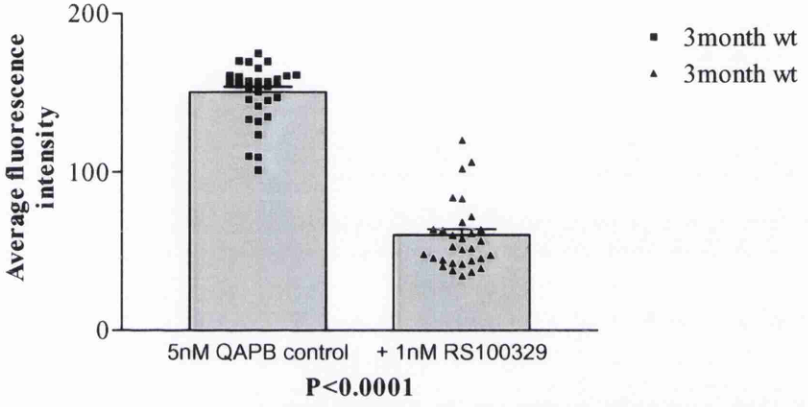
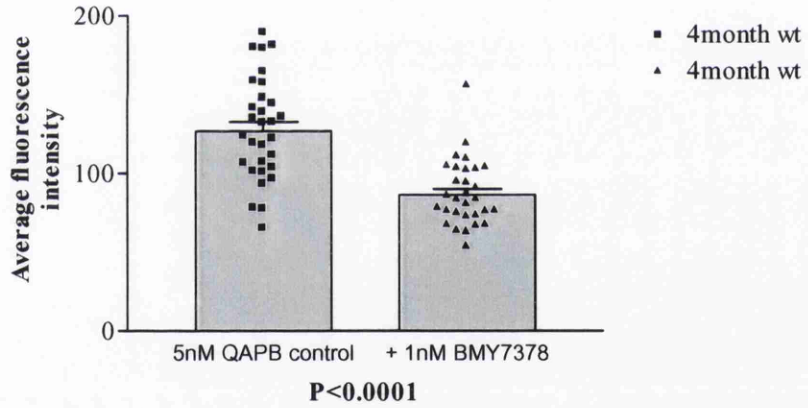
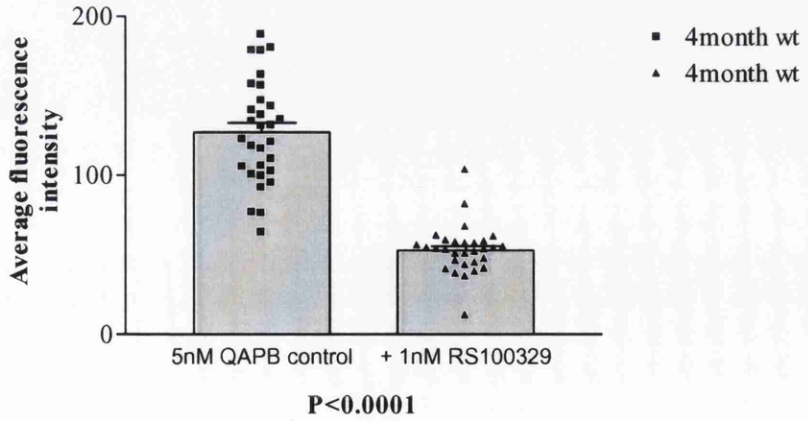
P<0.0001


200
100
0
Average fluorescence intensity
3month wt
3month wt
5nM QAPB control
+ 1nM RS100329
P<0.0001


200
100
0
Average fluorescence intensity
4month wt
4month wt
5nM QAPB control
+ 1nM BMY7378
P<0.0001


200
100
0
Average fluorescence intensity
4month wt
4month wt
5nM QAPB control
+ 1nM RS100329
P<0.0001

c)

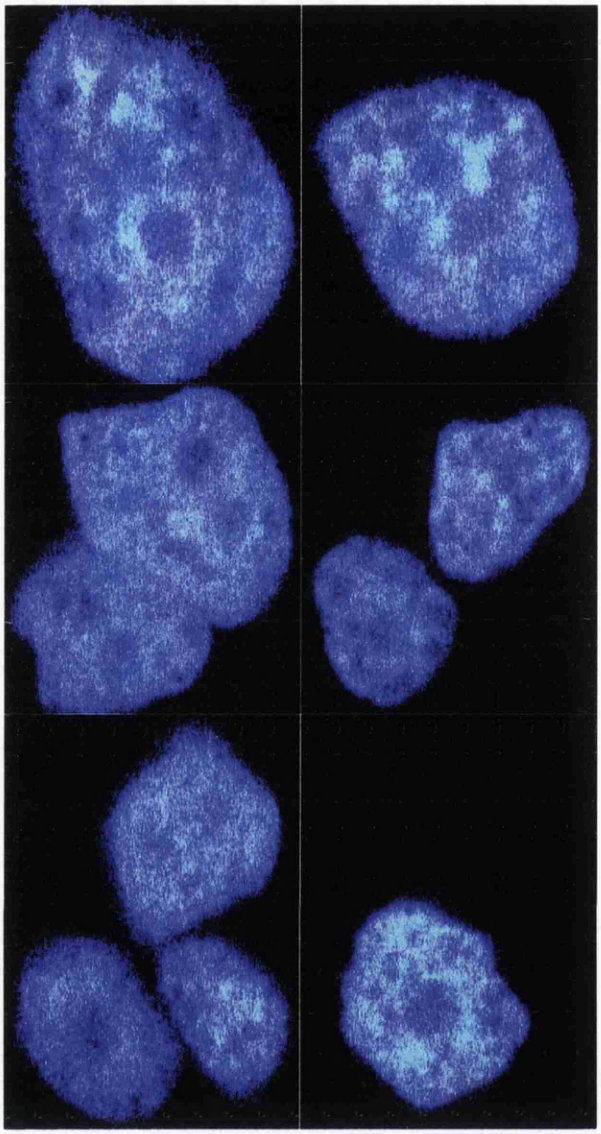


Figure 2.15 Comparison of GFP-associated fluorescence and autofluorescence of knockout hepatocytes transiently transfected with pEGFP/humanα_{1b}AR (top) and pmouseα_{1B}promoter/humanα_{1b}-AR-GFP (middle). Cells were plated on coverslips, transiently transfected, then left for a further 3 days in culture to allow for expression of the plasmid. Control experiment is represented by cells cultured alongside under the same conditions (bottom).

Chapter 3.

Subcellular distribution and characterisation of α_1-ARs in vascular smooth muscle cells.

Chapter 3

Introduction

The work in the previous chapter dealt with the characterisation of α_1-AR subtypes expressed in isolated hepatocytes. Subcellular distribution, age-related differences, and culture-induced changes in expression levels were also assessed. The issues that arose regarding α_1-AR subtype distribution in hepatocytes (see chapter 2 discussion), relates to the work in this chapter, which focuses on the subcellular distribution of α_1-ARs in vascular smooth muscle cells (VSMCs); freshly dissociated, in culture and *in situ*, and addresses the issue of functional location of α_1-AR subtypes. Does each subtype have a common distribution in different cell types, or is the distribution cell specific?

The study of the subcellular distribution of α_1-ARs within smooth muscle cells, has until recently, been restricted, due to technical limitations. Previous work in our laboratory has shown the localisation of α_1-ARs on VSMC, within the media of a rat mesenteric resistance artery, using QAPB (McGrath and Daly, 1995).

Although QAPB is non-selective, the study of α_1-AR subtype distribution in SMCs has since been extended to the subcellular level, and in conjunction with non-fluorescent competitive inhibitors, the characterisation (α_{1A}-AR) and subcellular distribution (40% intracellular) of α_1-ARs, expressed in SMCs dissociated from human prostate tissue (Mackenzie et al., 2000), have been quantified. It is well documented that the α_1-AR subtype responsible for contraction of human prostate is α_{1A}-AR (Marshall et al., 1995; Ford et al., 1996a; Takeda et al., 1997).

In contrast, studies in VSMCs cultured from femoral and renal arteries have demonstrated the expression of all three α_1-AR subtypes using subtype-selective

antibodies (Hrometz et al., 1999; McCune et al., 2000). This group have also shown that despite this, only a single subtype mediates the contractile response in the femoral (α_{1D}-AR) and renal (α_{1A}-AR) artery (Hrometz et al., 1999). So what roles do the other two subtypes play in the function of these blood vessels?

The functional response in the carotid artery and thoracic aorta of the mouse is mediated predominantly via α_{1D}-ARs (Daly et al, in press). Therefore, one would anticipate expression of this subtype at the single cell level.

Such work, and that of others (see general introduction), has indicated a defined subcellular distribution for each of the three subtypes of α_1-AR in SMCs. Interestingly, studies in recombinant cell lines have been shown to represent a similar distribution pattern to that seen in native VSMCs (McCune et al., 2000). This group have shown that α_{1B}- and α_{1D}-ARs are predominantly associated with the cellular periphery and intracellularly, respectively, in both recombinant cells and native VSMCs. Comparisons between SMCs from vascular/non-vascular tissues also show a similar distribution pattern, e.g., α_{1A}-ARs expressed in VSMCs/prostate SMCs are found predominantly intracellularly.

In smooth muscle, α_1-ARs were previously thought to reside at the plasma membrane, where they mediate contraction via activation through extracellular stimuli. The α_{1B}-AR is the only subtype shown to reside predominantly at this location, yet it plays a limited role in mediating contraction.

The overall objective of this chapter was to demonstrate receptor distribution within the walls of small resistance arteries, to extend this to the subcellular level and to determine whether receptor internalisation could be demonstrated in the intact vessel in response to agonist. Using methods developed initially in a recombinant cell line (chapter 1) and primary hepatocytes (chapter 2), the work in this chapter takes on the

study of the subcellular distribution and characterisation of native α_1-ARs in freshly dissociated and cultured VSMCs. Initially, the distribution and binding properties of the α_1-ARs expressed in smooth muscle cells were established. Cells were grown from an explant of thoracic aorta from the α_{1B}-AR knockout mouse, or dissociated from the carotid artery of either wildtype or knockout mice. As already demonstrated in the previous chapter, QAPB binding in the presence of non-fluorescent competitive antagonists allows the identification and visualisation of α_1-AR subtypes within native cells. Does the α_1-AR subtype(s) expressed at the single cell level correlate with the subtype-mediated (Daly et al, in press) functional response in these blood vessels?

Further to this, employing the α_{1B}-AR knockout mouse and *in vitro* transfection of aortic segments, we were able to manipulate the α_{1b}-AR population enabling expression *in situ*. Using GFP-tagged constructs and CLSM, direct visualisation, at a subcellular level was permitted.

The GFP-tagged constructs used were pEGFP/humanα_{1b}-AR (Tsujimoto) and pmouseα_{1B}-AR/human α_{1b}-AR- GFP (Perez). The latter construct is regulated by the mouse α_{1B}-AR promoter, which was cloned recently by Zuscik et al. (1999). As mentioned earlier, this group have confirmed that this mouse promoter fragment is able to regulate reporter gene expression in response to the competency of a given cell to express α_{1B}-ARs, thus able to mimic the distribution of their natural environment.

Chapter 3

Methods

Primary Smooth muscle cell dissociation

Cells were dissociated by a method adapted from Kamishima et al., 1997. Briefly, the arteries (carotid, thoracic aorta) were removed from the animal, dissected free from fat and connective tissue, weighed, briefly doused in 70% ethanol then placed in buffer 1 (NaCl 147mM, KCl 5mM, $MgCl_2$ 1mM, $CaCl_2$ 1.8mM, HEPES 10mM, BSA 0.1%, pH 7.4). Arteries were washed once in buffer 1, resuspended in pre-heated (35°C) buffer 2 (sodium glutamate 80mM, NaCl 54mm, KCl 5mM, $MgCl_2$ 1mM, $CaCl_2$ 0.1mM, HEPES 10mM, glucose 10mM, EDTA 0.2mM, BSA 0.1%, pH 7.3) with papain (28.9units/mg tissue, Sigma) and dithioerythritol (1mg/mg tissue, Sigma) and incubated at 35°C for 30 minutes. Arteries were then centrifuged at 1200g for 2 mins at room temperature and the supernatant discarded. Arteries were then resuspended in buffer 2 with collagenase II (425 units/mg tissue, Sigma) and hyaluronidase (330 units/mg tissue, Sigma) and incubated at 35°C for a maximum of 2-3 minutes, then the SMCs were dispersed immediately with a fire polished Pasteur pipette. Cells were washed in buffer 2 containing 40% foetal bovine serum (Life technologies, Inc), then plated onto cover slips, stored at 4°C and analysed within 24-48hours.

Cultured Vascular Smooth Muscle Cells

Dissociated

Arteries were treated in a similar manner as above, except that the dispersed cells were washed and thereafter cultured in DMEM (Life technologies, Inc)

supplemented with 20% (v/v) foetal calf serum (Life technologies, Inc), penicillin (100 units/ml) / streptomycin (100 μg/ml) (Life technologies, Inc), bovine insulin (10 μg/ml, Sigma), L-glutamine (1mM, Life technologies, Inc), sodium pyruvate (1mM, Life technologies, Inc), Fungizone (2.5μg/ml, Life technologies, Inc), geneticin (knockout cells only) (400μg/ml, Life technologies, Inc) in a 95% air, 5% CO_2 atmosphere at 37°C. Cells were fed every 2-3 days and split at confluency. Smooth muscle cells were immunohistochemically stained with a cy3 conjugated α-actin monoclonal antibody (Sigma). The stained specimens were then examined with a Zeiss Axiphot microscope.

Explant

Briefly, arteries were removed from the animal, aseptically dissected free from fat and connective tissue, cut longitudinally, and spread out flat on a sterile culture dish. After 5 days in culture, SMCs that had grown from the vessel were trypsinised, replated and maintained as above.

Measurement of $[Ca^{++}]_i$

Calcium imaging

Vascular smooth muscle cells were removed from culture flasks using trypsin/EDTA (Sigma), washed by centrifugation/resuspension in fresh DMEM and aliquots of this suspension were plated onto glass cover slips. Experiments were performed on cells that had been passaged a minimum of one and a maximum of ten times. Cells were grown overnight and then loaded (15 min at 37°C), with Fura-2 AM (1.5 μM). Fura-2 loaded cells were then mounted in a chamber attached to the stage of a Nikon Diaphot inverted microscope where the

cells were superfused with physiological saline solution (NaCl, 130mM; KCl, 5mM; $CaCl_2$, 1mM; $MgCl_2$, 1mM; 4-(2-hydroxyethyl)-1-piperazine sulphonic acid (HEPES), 20mM; D-glucose, 10mM; pH adjusted to 7.4 using NaOH). An Optoscan monochromator (Cairn Research, Faversham, Kent U.K.), positioned between a 75W Xenon lamp and the epifluorescence port of the microscope, was used to alternate the excitation wavelength between 340, 380 and 488 nm (band pass 10 nm) and to control the excitation frequency. Excitation light was reflected from a custom designed dichroic mirror through a Nikon 40x oil immersion FLUOR objective (NA=1.3). Fura-2 emitted fluorescence light at 515 nm was monitored either by a low noise COHU CCD camera or a photomultiplier tube with a bialkali photocathode. Images acquired with the CCD camera were stored and analysed digitally under the control of Meta Fluor imaging software (Universal Imaging Corp., West Chester, PA, USA). Images acquired for each excitation wavelength were collected every 1 sec; exposure to excitation light was always 80ms/image and the time interval between the acquisition of each image was ~2 msec. Cells were delimited by producing a mask that contained pixel values above a threshold applied to the 380 nm image and time-dependent changes in $[Ca^{2+}]_i$ was calculated from the ratio of two background subtracted images.

Confocal Microscopy

Identical parameters (brightness, contrast, laser intensity, excitation and emission wavelengths, slit size and frame averaging) were set in every experiment, unless stated otherwise. The objectives used throughout this chapter were Nikon x40, oil

and Zeiss x40, water, in conjunction with the inverted and upright systems, respectively.

Whole cell image analysis

Images were collected and analysed using Universal Imaging's 'Metamorph' software. Cells were grown on cover slips for 24 hours prior to use, unless otherwise stated. Cover slips were mounted in a flow chamber (WPI) and placed on the stage of an invert (Nikon Diaphot) microscope fitted with a Noran Odyssey Laser Scanning Confocal Module. Fluorophores were excited using a 488 nm argon laser and detected with a 515 nm band pass filter. In all experiments, a 15μm slit was used and all other parameters were kept constant. All cells were checked for viability using Propidium Iodide staining prior to every experiment.

QAPB

Using cell autofluorescence, a suitable group of cells was selected and the focal plane fixed by locking the focus motor. The system was then set to acquire images (64 frame averages, unless stated otherwise) at 1minute intervals. After a baseline was established, typically 5-6 minutes, the first concentration of fluorescent ligand was added and allowed to equilibrate (i.e., until no further increase in fluorescence) for at least 5minutes. After equilibration the next concentration of fluorescent ligand was added, without washing, and given time to equilibrate as before. Once saturation had been reached the individual cells were outlined using Metaporph's define-region tool and the fluorescence intensity values representative of each concentration at equilibrium were recorded. Non-specific binding was defined as fluorescence binding in the presence of 10μM prazosin. The composition of the HEPES buffer used in all single cell work was as follows:

NaCl 130mM, KCl 5mM, HEPES 20mM, Glucose 10mM, MgCl 1mM, CaCl 1mM.

Inhibition of QAPB-associated fluorescence binding

Cells were mounted in a flow chamber bath and experiments carried out in a similar manner to that described above. VSM cells were pre-incubated for 30 minutes with inhibitors, BMY-7378 or RS100329 prior to addition of QAPB. Characterisation of α_1-AR subtypes expressed was estimated in terms of the ability of these drugs to inhibit the development of QAPB-associated fluorescence.

Since it was evident from preliminary experiments that BMY-7378 was unable to access intracellular sites in this cell type, Streptolysin O (SLO, Sigma) was employed to permeabilise the plasma membrane permitting cellular uptake of large or charged molecules. Cells plated on cover slips, were covered, drop wise, with SLO (1 unit/cover slip) and BMY-7378 (1nM) in PBS (pH 7.4) and incubated for 30minutes at 37°C. After this incubation period, cell membranes were allowed to reseal in the presence of their culture medium, the active ingredient being; foetal bovine serum, for 5minutes. Following a brief wash with HEPES, cover slips were mounted in the flow chamber as before and allowed a further 15minute incubation with BMY-7378 alone, to allow for any binding on the plasma membrane.

Experiments were performed on cells that had been passaged a minimum of 3/4 and a maximum of 10 times.

Transient transfection

Carotid Artery cultured smooth muscle cells

Smooth muscle cells were grown routinely and plated on cover slips 24 hours prior to transfection. Transient transfections were carried out via a lipofection method with a variety of commercially available reagents. DOTAP (Boehringer Mannheim), Transfectam®, Transfast™ and Tfx™-50 (Promega) were all used according to the manufacturers instructions. To optimise the transfection conditions, various alterations to the protocol were carried out. Promega's 'transfection assistant' database (www.promega.com) was used to try to determine optimal transfection conditions for this cell type. Specific factors determine the efficiency of transfection of smooth muscle cells, including the reagent used, charge ratio (Reagent:DNA), amount of DNA used, ± serum, time of transfection, confluency of cells, medium exchange etc. In all cases, prior to transfection, the medium was aspirated and the cells were washed twice in serum-free DMEM. Post-transfection, cells were overlayed with complete medium and assayed after a further 60 hours in culture.

Thoracic aorta

Briefly, the arteries were removed from the animal, aseptically dissected free from fat and connective tissue, cut longitudinally, and spread out flat on a sterile culture dish. The vessel segment was flushed with serum-free DMEM, then the transfectant mix (2.5μg/ml vector, Tfx™-50 ratio 4:1, 6:1) was added, sufficient to cover the segment for 2 hours and finally overlaid with complete DMEM. After the appropriate incubation period, 5 days, with medium changes every day, the segments were removed from the culture dish, mounted on a microscope slide in fluorescent mounting medium (DAKO) ready for analysis.

Vascular tissue analysis

GFP detection

Arteries (thoracic aorta) were removed from culture dishes and visualised mounted on microscope slides in fluorescent mounting medium (DAKO). The majority of the vessel work was performed on an upright (Nikon Optiphot) microscope. As mentioned previously, all parameters were kept constant to ensure that comparisons could be made between different specimens.

Using GFP-associated fluorescence, a suitable region was chosen, and a stack of images collected by selecting a start plane within the specimen. Setting the stepper motor interval (z) to 0.5μm, serial confocal z-sections of GFP/α_{1b}-AR distribution within SMCs/adventitial cells *in situ* were acquired. Movies and 3D-reconstruction of some transfected cells were made and can be visualised on the CD enclosed.

Results

Fluorescent ligand saturation binding on cultured vascular smooth muscle cells.

Smooth muscle cells grown from an explant of thoracic aorta from the α_{1B}-AR knockout mouse, or dissociated from the carotid artery of either wildtype or knockout mice, were cultured for indicated passages, and the specific binding of QAPB to α_1-ARs was assessed. Cells from wildtype aorta were not studied due to time constraints. Bright field images of the cells were used to fix the focus to allow image capture at one focal point as autofluorescence was negligible. QAPB-associated fluorescence binding was concentration-dependent in smooth muscle cells from carotid artery and aorta (figures 3.1, 3.5 and 3.10). Fluorescence intensity in most cells reached saturation within the concentration range, although some cells displayed little or no QAPB staining indicating heterogeneity in expression levels between individual cells. In the presence of 1μM prazosin, fluorescence due to increasing concentrations of QAPB was significantly lower (figure 3.5a) and this remaining binding was used to estimate non-specific binding for all cultured cells. Specific binding curves were constructed: examples are shown in figures 3.2a, 3.5b, and 3.10b. The estimated specific binding and affinity (FK_D) of QAPB (nM) was estimated where applicable. Heterogeneity in the morphology of individual cells prevented comparisons of FK_D values to be made in this cell type. The calculated FK_D values were consistent with the affinity of QAPB at α_1-ARs in other native cells.

The image of concentration-dependent binding of QAPB detected in cultured smooth muscle cells was dependent on the morphology of individual cells. In

spindle-like cells, binding was composed of diffuse, intense staining throughout the entire cell, excluding the nucleus. In 'fried egg' shaped cells, clusters of QAPB binding were focused around the nuclear membrane. Some cells showed binding predominantly at the plasma membrane only (figure 3.9). No obvious differences between wildtype and knockout cells were observed. Phenotypic heterogeneity did exist within each cell line including α_1-AR expression levels, cell morphology and growth rates, hindering simplistic conclusions.

Cells were stained with a cy3 conjugated α-actin monoclonal antibody to verify SMC origin (figure 3.11).

Fluorescent ligand saturation binding on freshly dissociated vascular smooth muscle cells from mouse carotid artery.

Smooth muscle cells were dissociated from both wildtype and knockout carotid artery. Due to greater availability of these mice, most work was done on wildtype cells. Post dissociation, cells were plated on cover slips and incubated in buffer containing 40% FBS overnight or for 48hours at 4°C. The specific binding of QAPB to native α_1-ARs was assessed using a nanomolar concentration range as indicated. Bright field images of cells that were sufficiently stuck to the cover slip were used to fix the focus to allow image capture at one focal point. QAPB-associated fluorescence binding was concentration-dependent in cells from both wildtype and knockout carotid artery, although heterogeneity did exist between individual cells in their expression levels and in the concentration at which these sites saturated (figures 3.3 and 3.4c). Another interesting observation was that the initial concentration at which QAPB bound to these cells varied quite considerably. If QAPB enters cells via an endocytic mechanism, then it seems

plausible that this may be due to a lag period while cells adjust to the temperature change from 4°C (overnight) to room temperature (HEPES). In the presence of 1μM prazosin, fluorescence due to QAPB was significantly reduced (figure 3.4c) and the remaining binding was used to estimate non-specific binding for all freshly dissociated cells. Specific binding curves were constructed: an example of which is shown in figure 3.4b. The specific affinity (FK_D) of QAPB (nM) and average fluorescence intensity for cells was estimated where applicable (figure 3.4a). The calculated FK_D values for most of the fresh cells were consistent with the affinity of QAPB at α_1-ARs in other native cells (Mackenzie et al., 2000), although there was variation between cells.

Two patterns of QAPB-associated fluorescence were observed over a cumulatively increasing concentration range. At the lowest concentration at which QAPB bound to specific sites, binding appeared to be diffuse throughout the cell. At higher concentrations, diffuse staining became stronger in an intracellular location (figure 3.3 and 3.4c). In a patch of wildtype cells, equilibrated with 10nM QAPB, this intracellular binding was clearly distributed in a perinuclear orientation (figure 3.9c). In a knockout cell (figure 3.4c), a similar specific binding pattern was observed.

Identification of native α_1-AR subtypes on cultured smooth muscle cells by inhibition of QAPB-associated fluorescence.

Cultured aortic and carotid artery smooth muscle cells were passaged as indicated in the figure legends. Cells were pre-incubated with 1nM RS100329 or BMY-7378. Initial studies using BMY-7378 and knockout carotid artery SMCs indicated that no α_{1D}-ARs were expressed in these cells (figure 3.6b). Since this

did not correlate with the functional response from this vessel (Daly et al, in press), I presumed that BMY-7378 was unable to access intracellular sites. Therefore, streptolysin O (SLO) was employed to form pores within the plasma membrane. Post incubation with antagonist, the membrane was resealed in the presence of foetal bovine serum. QAPB binding was unaffected by the action of SLO alone (figure 3.6a).

On addition of cumulative concentrations of QAPB to knockout cells from both types of vessel, the appearance of fluorescence was significantly inhibited in the presence of BMY-7378 (five of out five tested) (figures 3.6 and 3.7 and 3.10), with the occasional cell (one out of two tested) showing blockade by RS100329 (figure 3.8). Addition of a single concentration of QAPB to patches of knockout carotid artery smooth muscle cells, pre-incubated as above, also illustrated a significant reduction of QAPB-associated fluorescence in the presence of 1nM BMY-7378 yet in this case RS100329 was not effective (figure 3.8).

In knockout aortic cells, the overall fluorescence intensity was lower than in identically treated cells from the carotid artery, hindering interpretation of antagonist experiments, although BMY-7378 appeared to inhibit to a greater extent than RS100329. Collectively, these data suggest that in knockout smooth muscle cells cultured from both the thoracic aorta and carotid artery, the α_1-AR subtype expressed is predominantly α_{1D}-AR, although the presence of the α_{1A}-AR cannot be completely ruled out.

Cultured wildtype carotid artery smooth muscle cells were pre-incubated for 30minutes with 1nM RS100329 or BMY-7378. On addition of cumulative concentrations of QAPB, the appearance of fluorescence was reduced, but not completely blocked, in all cells in the presence of BMY-7378 (figure 3.1).

RS100329 significantly reduced the binding of QAPB in the chosen experimental cell in figure 3.1. Addition of 30nM QAPB to patches of wildtype cells pre-incubated as above, resulted in a similar reduction of QAPB-associated fluorescence in the presence of either BMY-7378 or RS100329, indicating that at least two, namely α_{1A}-AR and α_{1D}-AR are predominantly expressed in wildtype cells (figure 3.2). The question of whether individual cells express one or the other or perhaps all three subtypes remains unanswered. Such inhibition experiments are open to misinterpretation as differences in expression levels can suggest that the antagonist in question is lowering or blocking QAPB binding, when actually the α_1-AR complement of that cell could be low or negative.

Comparison of bar charts representing QAPB binding to both wildtype and knockout smooth muscle cells in the presence of BMY-7378 (figures 3.8) demonstrated the ability of this antagonist to reduce specific QAPB binding to a greater extent in knockout cells. This suggests that the residual fluorescence seen in wildtype cells is due to binding to α_{1B}-ARs.

Collectively, these results suggest that α_1-AR subtype expressed in knockout cells is predominantly α_{1D}-AR, with the possibility that some cells express the α_{1A}-AR subtype. In the wildtype there is evidence for α_{1A}-AR and α_{1D}-AR plus a residual binding after antagonists greater than in α_{1B}-AR knockout that is likely to be α_{1B}-AR.

This illustrates that, when comparing wildtype and knockout cells, removing one subtype may lead to up-regulation of another subtype. In this case α_{1D}-AR replacing α_{1B}-AR. There was no obvious differences in the distribution of QAPB in either cell type, indicating that α_1-ARs could be localised in a defined region in

cultured vascular smooth muscle cells, irrespective of their subtype, yet influenced by their morphology in culture.

Expression of GFP-tagged α_{1b}-ARs in situ.

Tfx™-50 mediated transfection facilitated the delivery of the GFP-tagged α_{1b}-AR gene into segments of thoracic aorta from the α_{1B}-AR knockout mouse. Analysis of vessel segments, mounted in fluorescent mounting medium, 5days post-transfection showed patches of cells displaying GFP-associated fluorescence (figure 3.12). The GFP-expression in SMCs was distributed throughout the cell, with the non-fluorescent nucleus being clearly defined in most cells. Adventitial cells within the vessel wall also expressed the construct that was controlled by the viral promoter (figure 3.13a, bottom). Dividing cells in the culture media surrounding the transfected vessel also appeared to express the GFP-tagged construct, but to varying degrees (figure 3.13a, top). Movies representing 0.5μm sections through the blood vessel wall encompassing transfected SMCs (figure 3.12), and an adventital cell (figure 3.13b) are presented. 3D reconstructions representative of these cells are also shown (figures 3.12 and 3.13b). Culturing of vessel sections alone increased autofluorescence levels (figure 3.14), although GFP-associated fluorescence was sufficiently more intense to allow transfected cells to be distinguished from non-transfected cells. For this reason the laser power used to detect GFP was much lower, explaining the low levels of autofluorescence apparent in the transfected vessel images.

Chapter 3

Discussion

The work in this chapter took on the study of the subcellular distribution and characterisation of native α_1-ARs in VSMCs; freshly dissociated, in culture, and *in situ*. Initially, the distribution and binding properties of the α_1-ARs in SMCs grown from an explant of thoracic aorta from the knockout mouse, or dissociated from the carotid artery of either wildtype or knockout mice were established.

The difficulty of studying VSMCs *in vitro,* and in interpreting such data, is due to their ability to modulate phenotype rapidly when removed from *in vivo* conditions. The degree of alteration is dependent on the methods used for isolating the cells, length of time in culture, and culture conditions. Bower and Dahm, (1993) have carefully examined these factors in cultured avian amniotic SMCs, and concluded that serum-derived adhesion factors alter SMC morphology, causing cells to take on a flattened phenotype and lose contractile ability. In contrast cells cultured in the absence of serum maintain a spindle-shaped morphology and the ability to contract. Additionally, individual VSMCs from different vessels and organisms may not respond similarly to identical *in vitro* conditions (Owens, 1995).

QAPB-asssociated fluorescence binding demonstrated that specific, high affinity α_1-ARs were present on mouse carotid artery and thoracic aorta VSMCs, both freshly dissociated (carotid artery only) and cultured up to a maximum of ten passages. Cultured cells were used for quantitative, competitive analysis of subtypes since this provided a population of cells that could withstand prolonged protocols giving reliable, high affinity binding.

QAPB binding to freshly dissociated cells was informative in that it showed specific, predominantly intracellular binding. The distribution of α_1-ARs was consistent in all cells that were analysed. Similarly, no differences were observed between wildtype or knockout cells, indicating a similar pattern was likely within the vessel wall, irrespective of subtype. In contrast, the distribution of QAPB binding in the cultured cells differed significantly, and was influenced by the morphology of individual cells. These differences were observed in both wildtype and knockout cells.

Information on native α_1-AR subtype distribution is very limited. A recent study looked at the distribution of the α_{1A}-AR in freshly dissociated human prostatic SMCs (Mackenzie et al., 2000). Quantitative imaging revealed that 40% of QAPB binding sites were intracellular, particularly around the nucleus, with the remainder residing at the plasma membrane. Equilibration of prostatic SMCs with low concentrations of QAPB (<2nM) revealed fluorescence predominantly associated with the cell membrane. At higher concentrations, intracellular binding sites around the nucleus were identified.

These observations were also made in the specific binding of QAPB to freshly dissociated carotid artery SMCs. Two patterns of QAPB-associated fluorescence were observed over a cumulatively increasing concentration range. At the lowest concentration at which QAPB bound to specific sites, binding appeared to be diffuse throughout the cell. At higher concentrations, diffuse staining became stronger in an intracellular location (figure 3.3 and 3.4c). This intracellular binding was clearly distributed in a perinuclear orientation (figure 3.9c).

Due to technical limitations, SMCs had to be cultured in order to establish the subtype(s) of α_1-AR present on these cells.

The compound used to discriminate between the α_{1A}-AR and the other two subtypes was RS100329. The compound was used at 1nM, a concentration 5 times higher than its inhibitory constant versus α_{1A}-ARs but still 10 times lower than versus α_{1B}-ARs or α_{1D}-ARs and thus it should inhibit only α_{1A}-ARs. This concentration of RS100329 reduced the specific binding of QAPB in some cells from both blood vessels, indicating the presence of this subtype in selected cells.

The compound used to discriminate between the α_{1D}-AR and the other two subtypes was BMY-7378. It was used at 1nM, a concentration slightly higher than its inhibitory constant versus α_{1D}-ARs (pKi 8.5-9.5) but still 100 times lower than versus α_{1A}-AR or α_{1B}-ARs and thus it should inhibit only α_{1D}-ARs. This concentration of BMY-7378 greatly reduced QAPB in knockout SMCs from both aorta and carotid artery, but reduced it less in wildtype carotid artery cells.

The α_1-AR subtype identified in prostate SMCs was the α_{1A}-AR (Mackenzie et al., 2000). In carotid artery SMCs, the predominant subtype was α_{1D}-AR in the knockout, and a mix of all three in the wildtype is likely. The current situation in the literature leads us to believe that differences in receptor distribution might allow subtypes to be distinguished. However, in this case, the characterisation of α_1-AR subtypes and analysis of their distribution, suggests that different subtypes adopt a common location in freshly dissociated SMCs.

The pattern of QAPB binding in cultured carotid artery SMCs was dependent on the morphology of the differentiated cells. In spindle-like cells, binding was composed of diffuse, intense staining throughout the entire cell, excluding the nucleus. In 'fried egg' shaped cells, clusters of QAPB binding were focused around the nuclear membrane, with some cells showing binding predominantly at the plasma membrane (figure 3.9).

The only other work that has been done on α_1-AR subtype distribution in cultured VSMCs, used α_1-AR subtype selective antibodies (Hrometz et al., 1999; McCune et al., 2000). VSMCs cultured from renal and femoral artery stained positive with all three antibodies, but to varying degrees. Staining with the α_{1A}-AR and α_{1D}-AR antibodies were predominantly focused around the nuclear membrane. Staining with the α_{1B}-AR antibody was detected around the cell periphery and also in a perinuclear orientation. The morphology of VSMCs in this study was similar to the 'fried egg' appearance seen in some of the cells cultured from the carotid artery/aorta. Similarly, the distribution of QAPB binding to α_1-AR subtypes in cells with such morphology was predominantly associated with the nuclear membrane.

Our objective was to understand the natural disposition of α_1-ARs in VSMCs, but prolonged incubation, and culture, leads to de-differentiation with possible changes in distribution and receptor types. In fact, using the same cells as mentioned previously, Hrometz et al. (1999) observed differences in the immunostaining pattern that were dependent on the receptor, cell type and the time in culture. This indicates that the expression of the α_1-ARs can be affected by culture conditions, and that caution should be taken in using cultured SMCs to delineate α_1-AR function *in vivo*. Strong immunostaining was obtained in frozen sections from intact blood vessels using all three antibodies. In cultured cells, strong immunostaining was observed only when using the α_{1B}-AR antibody. The indication that culturing of SMCs can depress α_1-AR subtype expression is not ideal, since our objective is to understand their natural distribution.

The observed *in vitro* cellular diversity of SMCs in culture is now thought to be attributed to the existence of intrinsic heterogeneity rather than to the process of

phenotypic modulation. The work of several groups (reviewed by Frid et al., 1997), supports the idea that heterogeneity of different SMC subpopulations exists and that these differences are intrinsic to the cell type. Evidence demonstrates that the arterial media in systemic vessels is not composed of a phenotypically homogeneous population of SMCs but rather of heterogeneous subpopulations of cells with unique developmental lineages.

It has been demonstrated that structural and cellular heterogeneity exists between three layers of bovine arterial media (Bochaton-Piallat et al., 1996). Therefore it seems plausible that the difference in distribution of α_1-ARs seen in cells with varying morphologies could be explained by their orgin from within the vessel wall. Perhaps cells belonging to a particular layer have a specific role within the vasculature and therefore the functional distribution of α_1-ARs may be cell-specific. An example of this would be the 'fried egg' shaped cells, described previously as senescent, characterised by large vacuoles and unable to reach confluence (Bochaton-Piallat et al., 1996), the localisation of QAPB binding in such cells is perinuclear, and from traditional models of GPCR signalling, this would suggest that these cells are not primarily involved in contraction. So what purpose do they serve? The spindle-shaped cells, form tightly packed monolayers, divide at a much faster rate than 'fried egg' cells, and have a distribution of α_1-ARs in close proximity to the plasma membrane, suggesting a possible role for these cells in contraction. Another interesting observation in this study was that SMCs cultured from the wildtype carotid artery were almost quiescent, in that after passage 1/2, no further proliferation was obvious to the eye. This was in sharp contrast to knockout SMCs, which grew at an enormous rate. Unfortunately,

the growth rates could not be quantified due to the quiescent nature of the wildtype cells.

A common technique that has been used to assess growth potential of VSMCs has been to isolate VSMCs from different regions along the length of one vessel (e.g. the aorta) and/or to remove cells from one particular vessel type at different developmental time points and culture them separately. Results suggest that cells derived from different regions have distinct growth patterns and different metabolic needs in addition, authors suggest that mitogenic factors regulate the growth of SMCs dependent on the stage in development of the blood vessel (see review Hungerford and Little, 1999).

Studies by Paglin et al. (1987) indicated a heterogeneous distribution of ANG II receptor density in enriched VSMCs from rabbit aorta, and Absher et al. (1989) reported a high degree of heterogeneity in proliferative behaviour among cell lines derived from bovine carotid artery. The present findings with α_1-ARs are consistent with this.

The phenotypic diversity displayed here includes cell size, proliferation rate, and α_1-AR density. In the light of previous work, and the work presented in this chapter, it is possible that the cells described represent the heterogeneity of VSM tissue found *in vivo*. These features may reflect a specialised function related to location in the blood vessel wall. However, because these analyses have been performed *in vitro*, phenotypic modulation of the individual cells remains uncontrolled and it is impossible to draw direct conclusions regarding the parental cells *in situ*.

A possible solution to this problem was to employ the α_{1B}-AR knockout mouse and *in vitro* transfection of intact blood vessel. It was possible to manipulate the

α_{1b}-AR population enabling expression and visualisation of GFP-tagged constructs *in situ*. Using GFP-tagged constructs and CLSM, direct visualisation, at a subcellular level was permitted.

The efficiency of successful gene transfer using cationic liposomes, however, is variable and highly dependent on the cell type. Previous studies have established that the efficacy of liposome-facilitated transfection may vary up to 100-fold, depending on the cell line used (Felgner et al., 1987). Most *in vitro* experience to date has been with continuous/immortal animal cell lines. The results of studies using these types of cells, however, have uncertain implications for the likelihood of success in native cells.

In this study, it has been shown that using the commercially available liposome, Tfx™-50, it was possible to transfer and express GFP-tagged α_{1b}-ARs into VSMCs *in situ*. The DNA concentration, the ratio of liposome to DNA (which affects the net charge of the complexes formed), the transfection time and the effect of serum were critical factors in these transfections. The efficiency of GFP-expression following the transfection of aortic segments is encouraging but raises a number of questions: why do certain cells express high levels of GFP while their neighbours appear untransfected? The majority of transfected cells have morphology typical of the contractile phenotype, being spindle-shaped, corresponding to the hills-and-valleys appearance. It may be possible that the efficiency of transfection is clonal in nature. This will be investigated further. In order for direct comparisons to be made between the expression of both constructs, it would be essential that the transfection protocol is optimal and identical in both cases. Further work is also required to establish specific regions within the vessel wall that express the α_{1B}-AR.

In summary, the objective in this chapter was to understand the natural disposition of α_1-ARs in VSMCs. Initially, the subcellular distribution of native α_1-ARs from freshly dissociated carotid artery SMCs was assessed. Characterisation of native α_1-ARs in VSMCs from carotid artery and thoracic aorta were thereafter enabled post-differentiation, in culture. Further to this, *in vitro* transfection enabled the manipulation of the α_1-AR population in segments of thoracic aorta, allowing visualisation of GFP-tagged α_{1b}-ARs expressed in VSMCs *in situ.* Information from this work, and that of others, showed that the 'natural' disposition of α_1-ARs, irrespective of subtype, is similar in freshly dissociated SMCs from vascular/non-vascular tissue, but phenotypic modulation does alter this distribution. The question that remains unanswered is whether this difference in α_1-AR distribution is solely due to their differentiated state, or whether it is intrinsic to the cell and therefore represents heterogeneity within the vessel wall.

Figure 3.1 QAPB binding on cultured VSMCs dissociated from the carotid artery of wildtype mice. Cells had been passaged a maximum of two times. Non-specific binding was defined by 1μM prazosin. Inhibition of QAPB binding to specific α_1-AR binding sites was assesed by pre-incubation for 30minutes with 1nM of the α_{1A}-AR and α_{1D}-AR selective antagonists RS100329 and BMY-7378, respectively. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations of QAPB, as indicated, were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore.

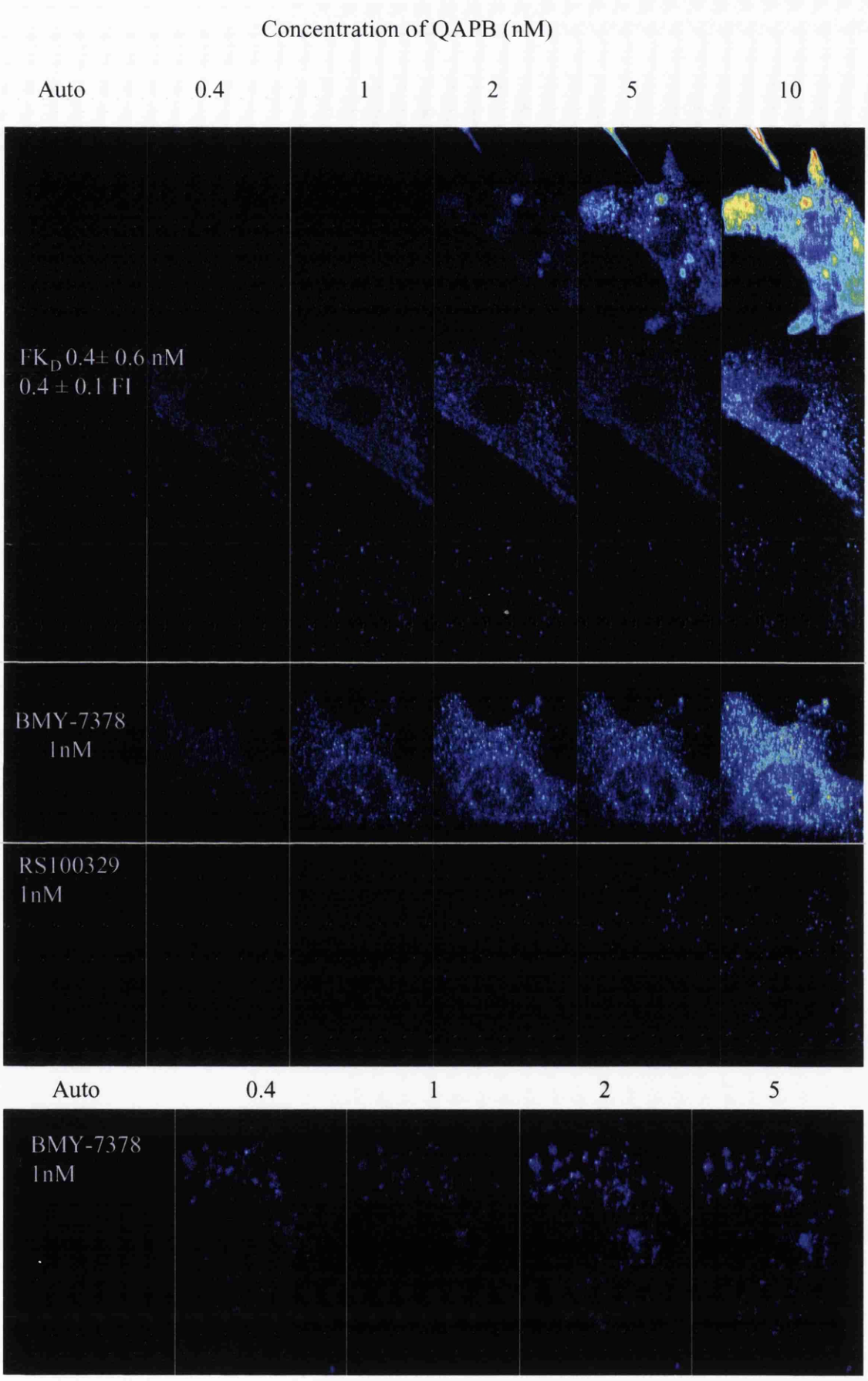
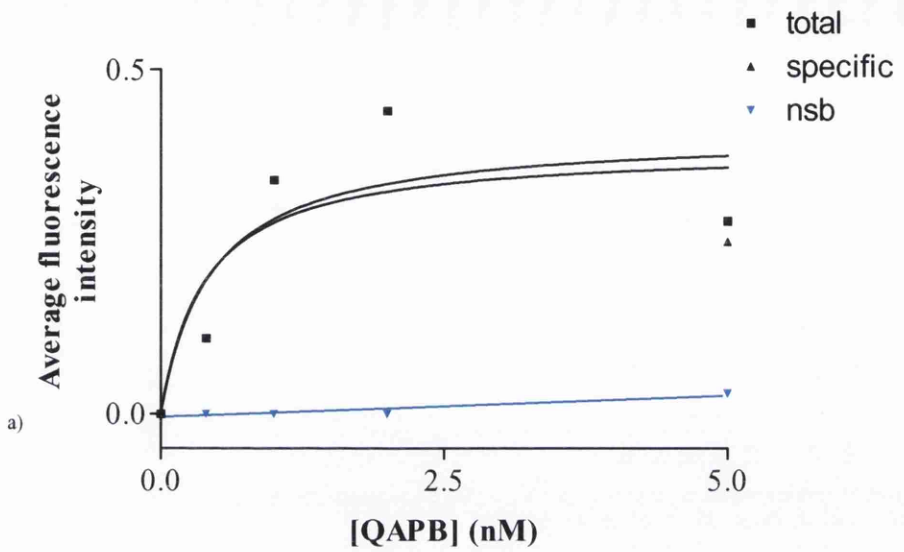
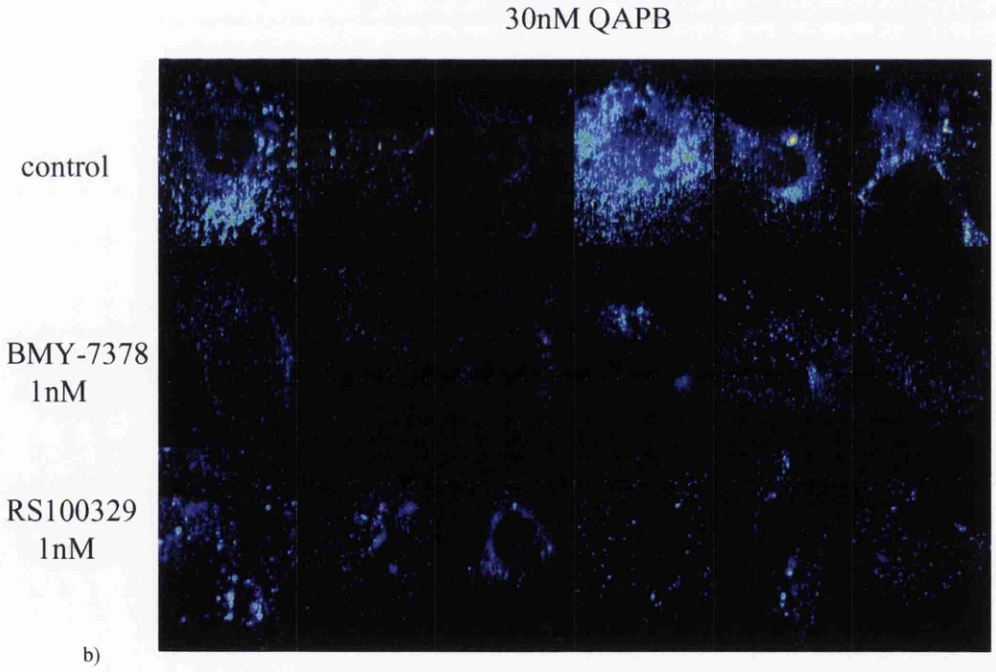

Concentration of QAPB (nM)
Auto
0.4
1
2
5
10
FK$_D$ 0.4± 0.6 nM
0.4 ± 0.1 FI
BMY-7378
1nM
RS100329
1nM
Auto
0.4
1
2
5
BMY-7378
1nM

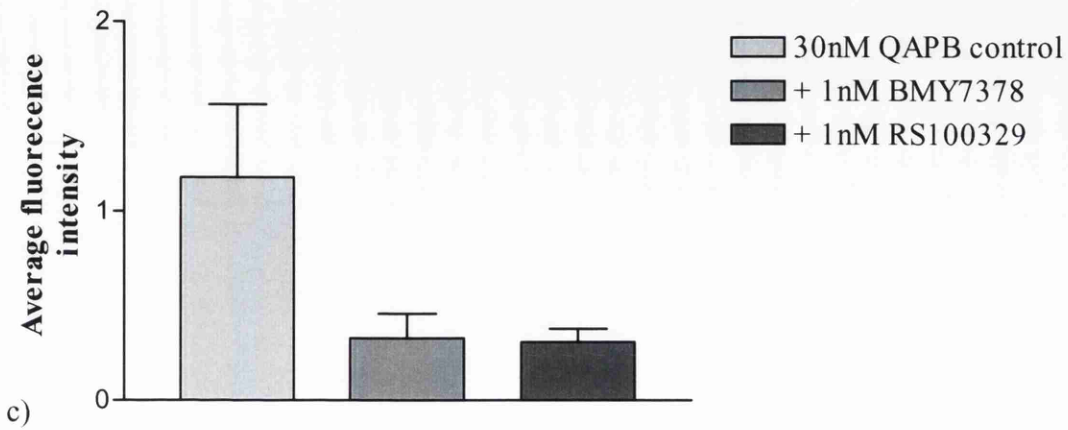
Figure 3.2 (a) The QAPB-associated fluorescence intensity was calculated by Metamorph software and plotted against increasing concentrations of QAPB to demonstrate the levels of specific binding of QAPB in a sample wildtype cell. The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) was measured as fluorescence intensity (FI). (b) Inhibition of QAPB binding on cultured vascular smooth muscle cells dissociated from wildtype carotid artery pre-incubated with 1nM RS100329 and BMY7378. Cells were plated on coverslips and examined by confocal microscopy. Images were captured and image data collected after equilibration of 30nM QAPB. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (c) Bar chart representing 30nM QAPB binding to VSMCs pre-incubated with 1nM BMY7378 or RS100329. Cells were plated on coverslips, incubated for 30minutes with non-fluorescent antagonist, then equilibrated with 30nM QAPB. Inhibition of QAPB binding was measured as average fluorescence intensity of the cell compared to the absence of inhibitor (control).



a)



b)



c)

wildtype CA SMC #2

Figure 3.3 (a) QAPB binding on VSMCs freshly dissociated from the carotid artery of wildtype mice. Post dissociation, cells were incubated in buffer at 4°C overnight (Cells 1-4) and for 48hours (Cells 5-7) prior to experiments. Non-specific binding was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations of QAPB, as indicated, were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore.

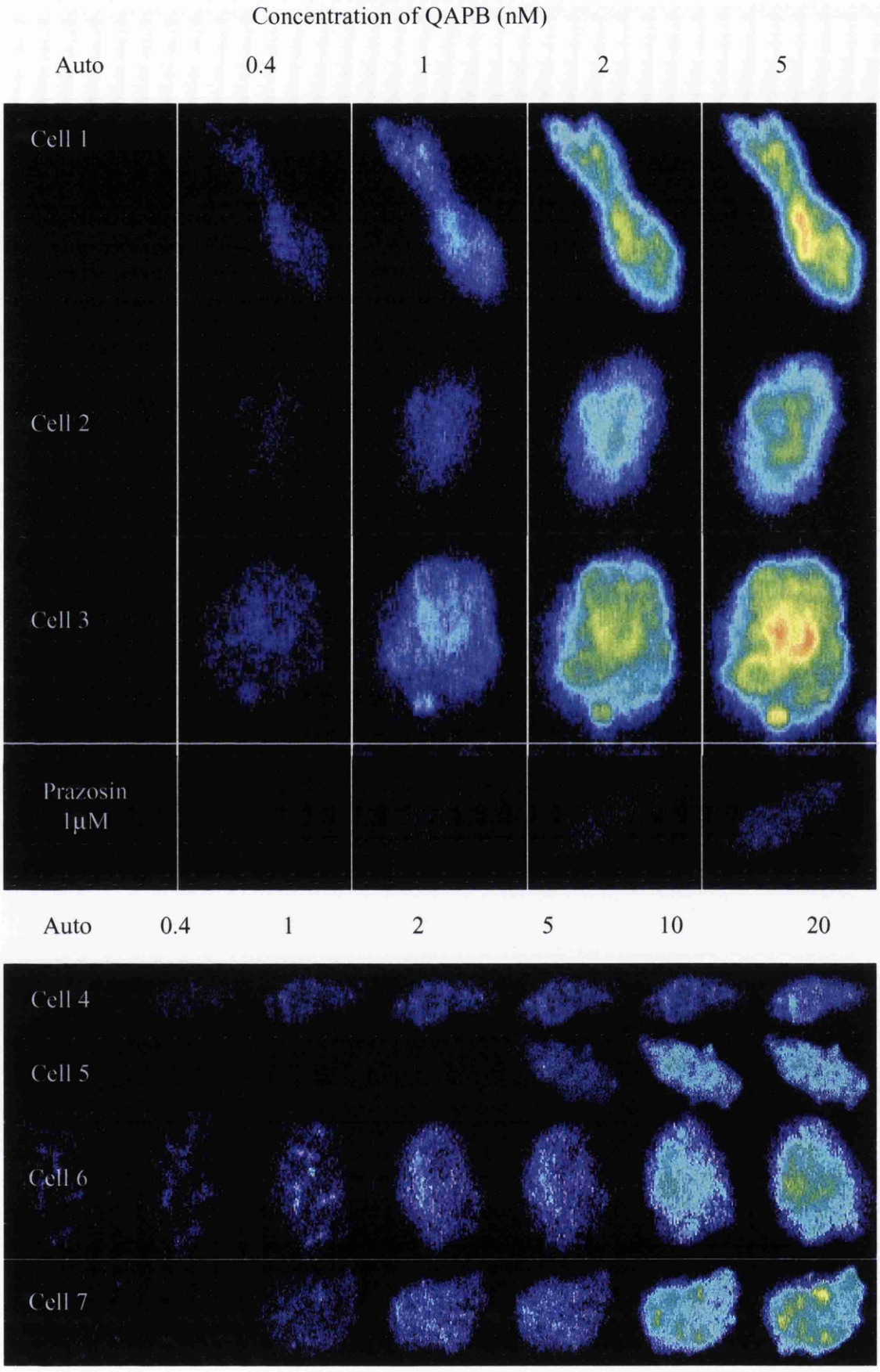
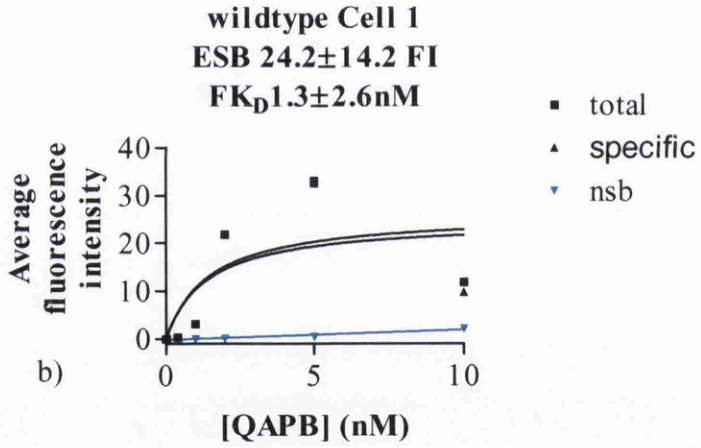

Concentration of QAPB (nM)
Auto
0.4
1
2
5
Cell 1
Cell 2
Cell 3
Prazosin
1μM
Auto
0.4
1
2
5
10
20
Cell 4
Cell 5
Cell 6
Cell 7

Figure 3.4 (a) The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Values for freshly dissociated wildtype carotid artery SMCs were estimated where applicable and presented in the table. (b) The QAPB-associated fluorescence intensity was calculated by Metamorph software and plotted against increasing concentrations of QAPB to demonstrate the levels of specific binding of QAPB in a sample cell. (c) QAPB binding on VSMCs dissociated from the carotid artery of knockout mice. Post dissociation, cells were incubated overnight in buffer at 4°C prior to experiments. Non-specific binding was defined by 1μM prazosin. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations of QAPB, as indicated, were added cumulatively and images were collected at 1-minute intervals. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore.

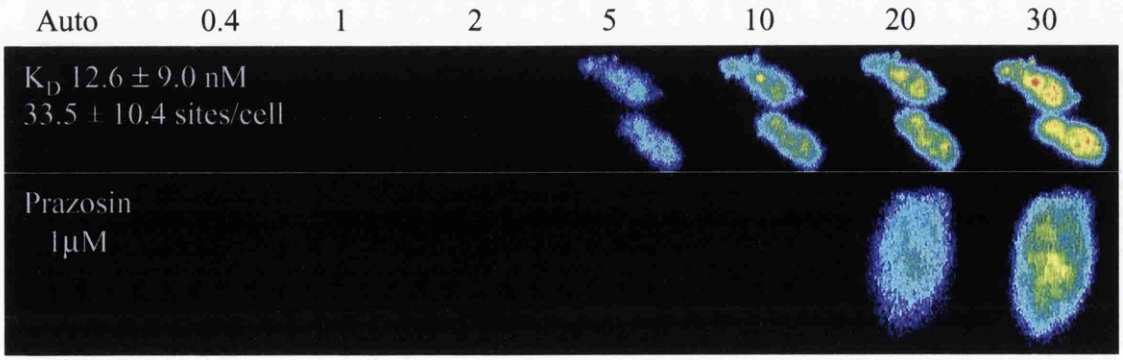
a)

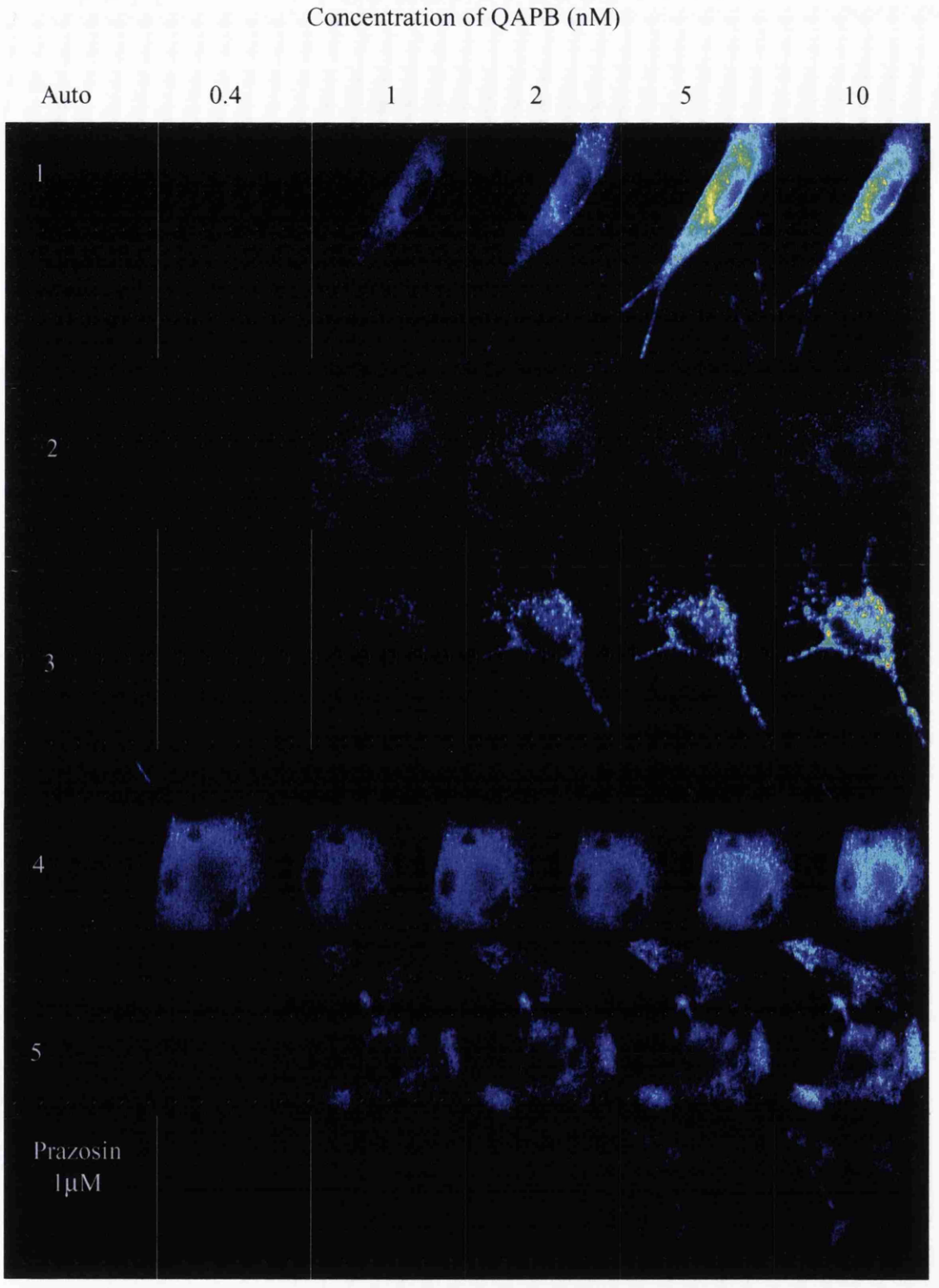
wildtype	FK_D (nM)	ESB (FI)
Cell 1	1.3±2.6	24.2±14.2
Cell 2	1.1±3.1	7.7±6.2
Cell 3	1.6±2.8	35.0 ±19.5
Cell 4	1.8±0.6	2.1±0.2
Cell 5	4.5±14.9	3.2 ±3.8
Cell 6	2.1±2.4	3.5±1.8
Cell 7	1.6±1.7	3.0±1.3

b)

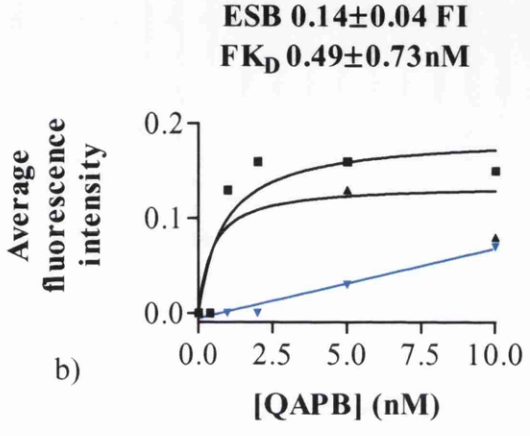


c)





a)



b)

c)

ko	FK$_D$ (nM)	ESB (FI)
Cell 1	5.7±11.5	25.5 ±25.1
Cell 2	0.5 ±0.8	0.1 ±0.04
Cell 4	1.3 ±1.8	2.4 ±1.0

Figure 3.6 (a) QAPB binding to specific α_1-AR binding sites on cultured VSMCs dissociated from the carotid artery of knockout mice was assesed in cells that had been pre-treated with Streptolysin O (SLO). Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations of QAPB (0.4-10nM) were added cumulatively and images were collected at 1-minute intervals. (b, c) Demonstration that BMY7378 was unable to access intracellular binding sites in the absence of streptolysin O. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (d) Transmission images of cultured VSMCs dissociated from the carotid artery of knockout mice.

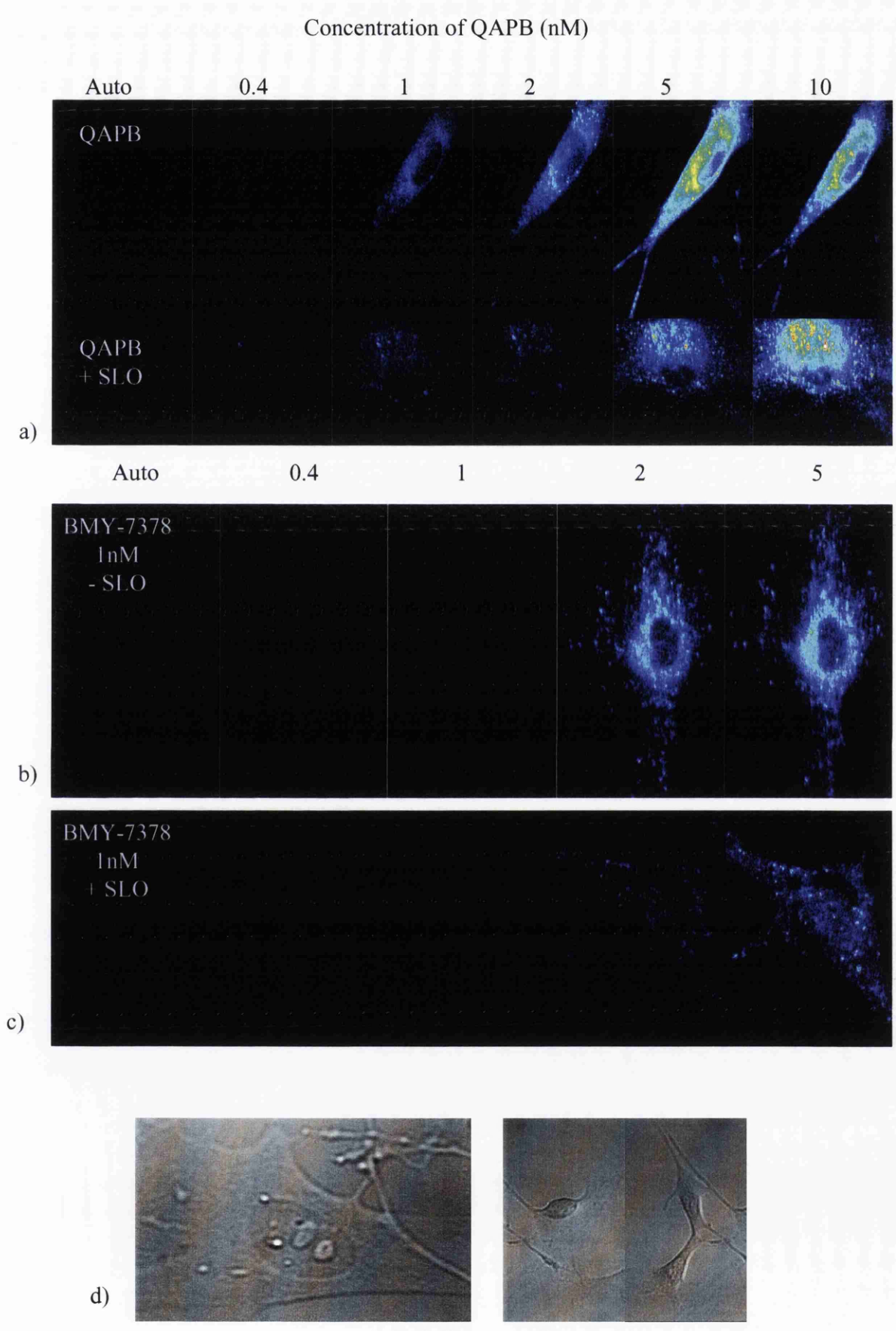

Concentration of QAPB (nM)
Auto
0.4
1
2
5
10
QAPB
QAPB
+ SLO
a)
Auto
0.4
1
2
5
BMY-7378
1nM
- SLO
b)
BMY-7378
1nM
+ SLO
c)
d)

Figure 3.7 (a) Inhibition of QAPB binding to specific α_1-AR binding sites on cultured VSMCs dissociated from the carotid artery of knockout mice was assesed by pre-incubating cells for 30minutes with 1nM of the α_{1D}-AR selective antagonist BMY-7378. Cells that had been passaged a minimum of three and maximum of ten times were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations of QAPB, as indicated, were added cumulatively and images were collected at 1-minute intervals. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore.

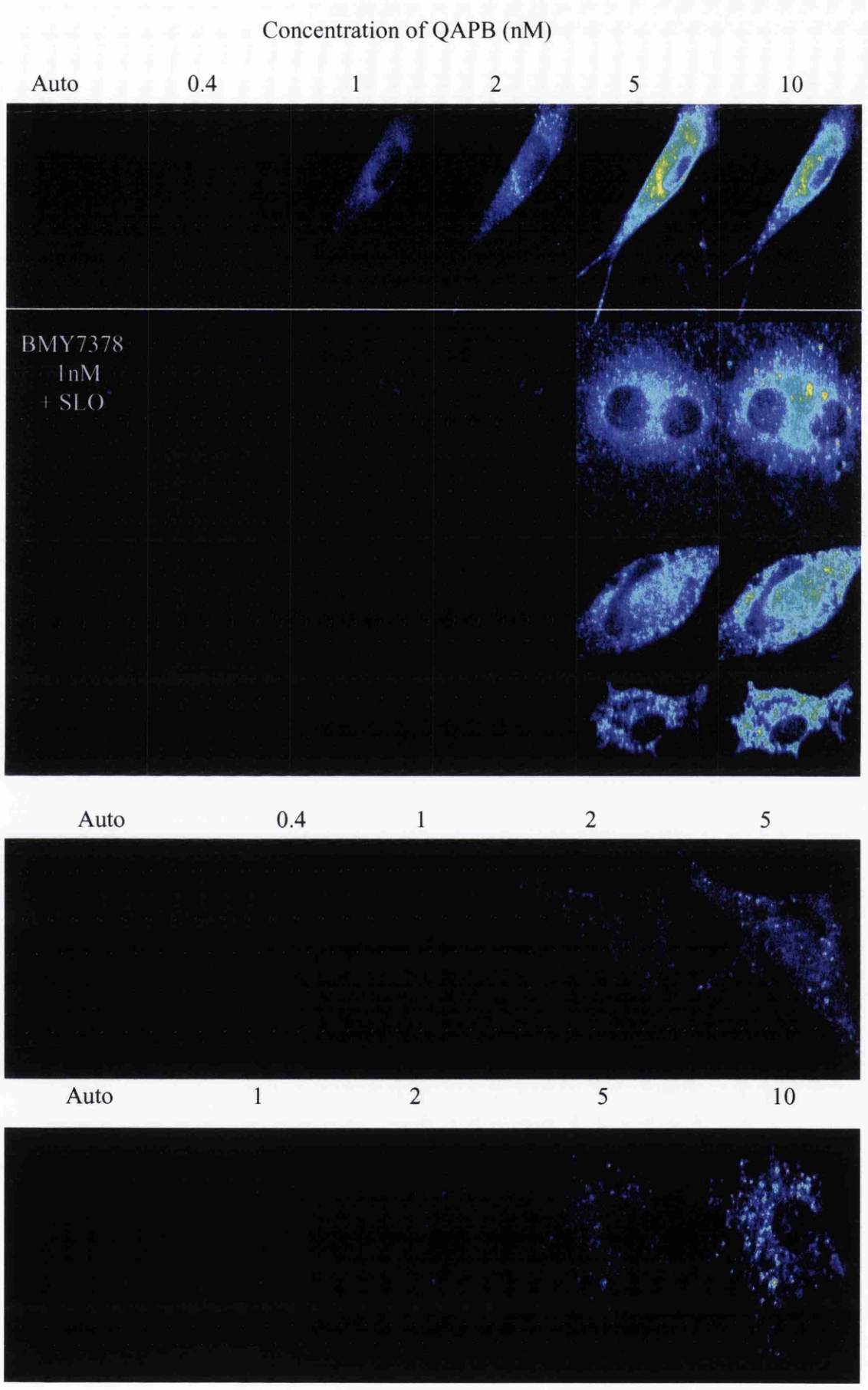

Concentration of QAPB (nM)
Auto
0.4
1
2
5
10
BMY7378
1nM
+ SLO
Auto
0.4
1
2
5
Auto
1
2
5
10

Figure 3.8 (a) Inhibition of QAPB binding to specific α_1-AR binding sites on cultured VSMCs dissociated from the carotid artery of knockout mice was assesed by pre-incubating cells for 30minutes with 1nM of the α_{1A}-AR selective antagonist RS100329. Cells that had been passaged a minimum of three and maximum of ten times were plated on coverslips and examined by confocal microscopy with timelapse photography. (a) Increasing concentrations of QAPB (0.4-10nM) were added cumulatively and images were collected at 1-minute intervals. (b) Images were captured and image data collected after equilibration of 20nM QAPB. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. (c, top) Bar chart representing 30nM QAPB binding to wildtype VSMCs pre-incubated with 1nM BMY7378 or RS100329. (c, middle) Bar chart representing 20nM QAPB binding to knockout VSMCs pre-incubated with 1nM RS100329. (c, bottom) Bar chart representing 30nM QAPB binding to knockout VSMCs pre-incubated with 1nM BMY7378. Cells were plated on coverslips, incubated for 30minutes with non-fluorescent antagonist, then equilibrated with the indicated concentration of QAPB. Inhibition of QAPB binding was measured as average fluorescence intensity of the cell compared to the absence of inhibitor (control).

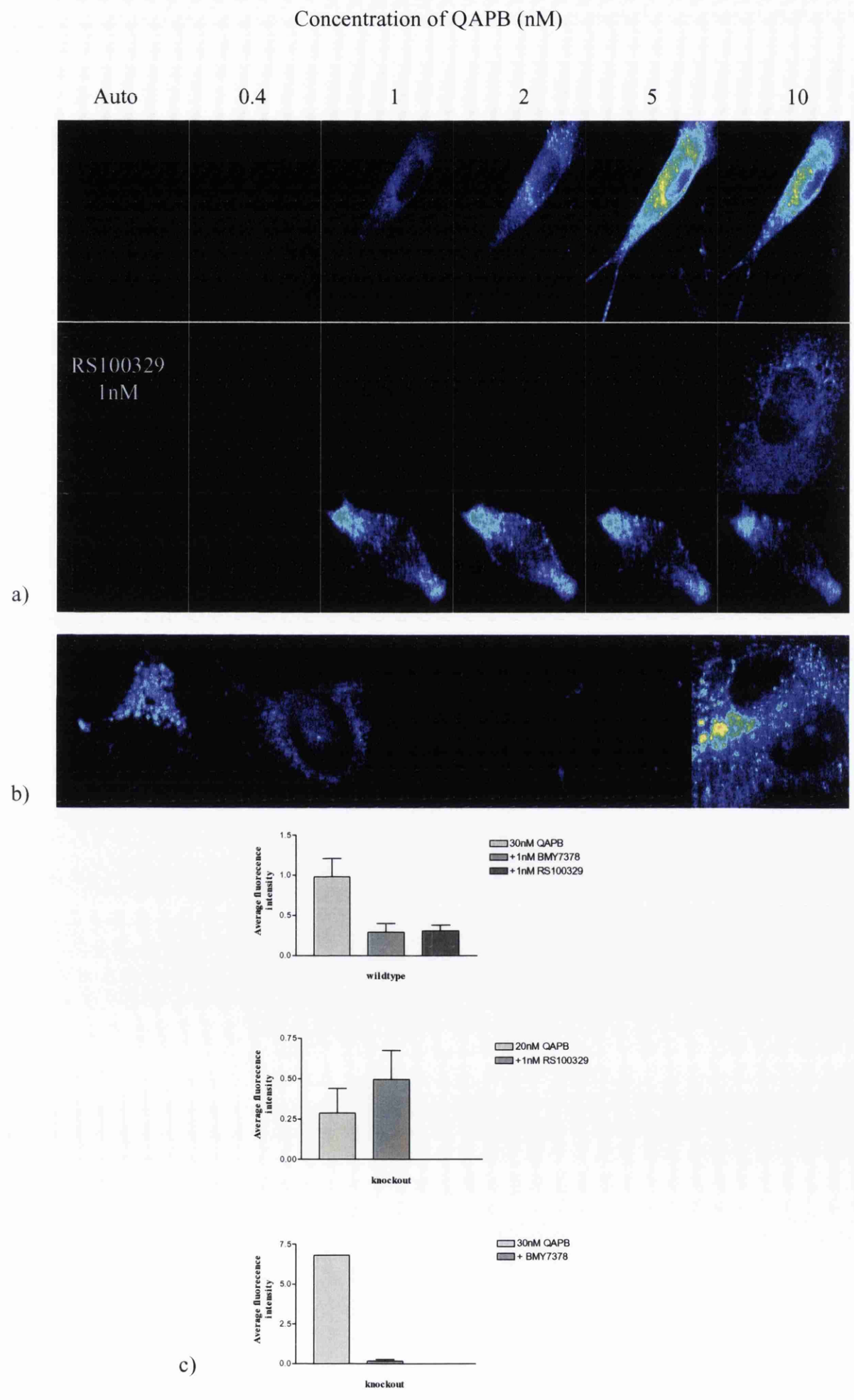
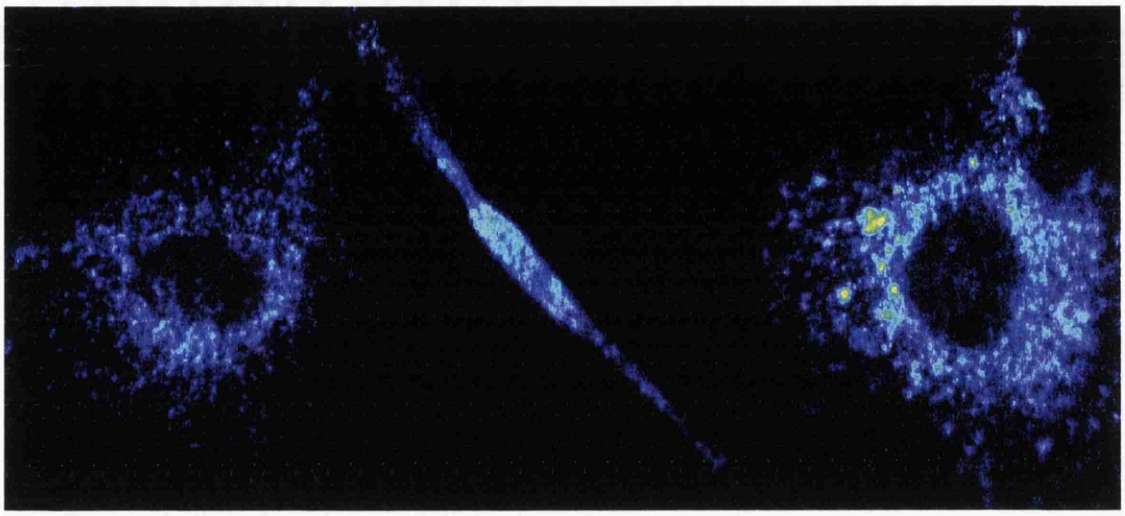
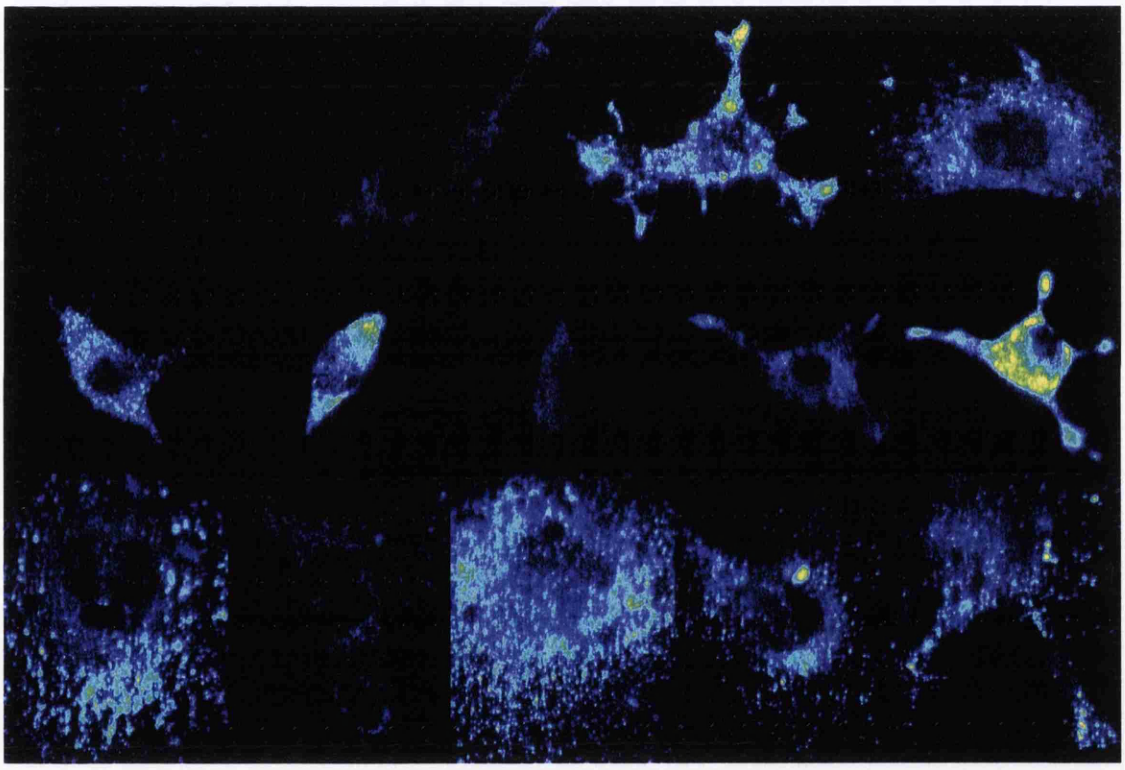

Concentration of QAPB (nM)
Auto
0.4
1
2
5
10
RS100329
1nM
a)
b)
Average fluorecence intensity
30nM QAPB
+1nM BMY7378
+1nM RS100329
wildtype
20nM QAPB
+1nM RS100329
knockout
30nM QAPB
+ BMY7378
knockout
c)

Figure 3.9 (a, d) 10nM QAPB binding on cultured VSMCs dissociated from the carotid artery of knockout mice. Cells had been passaged four times. (b, top and middle) 10 and 20nM, QAPB binding respectively on cultured vascular smooth muscle cells dissociated from the carotid artery of knockout mice. Cells had been passaged ten times. (b, bottom) 30nM QAPB binding on cultured VSMCs dissociated from the carotid artery of wildtype mice. Cells had been passaged a maximum of two times. (c) QAPB binding on VSMCs freshly dissociated from the carotid artery of wildtype mice. Post dissociation, cells were incubated in buffer at 4°C overnight prior to experiments. (e) 20nM QAPB binding on cultured VSMCs dissociated from the thoracic aorta of knockout mice. Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore.

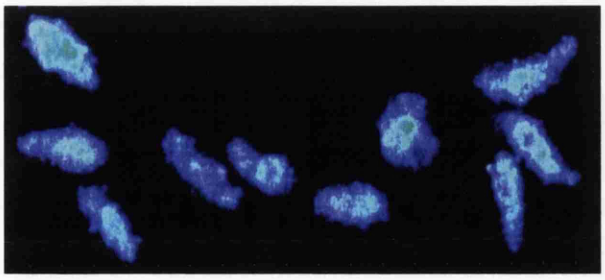
a)



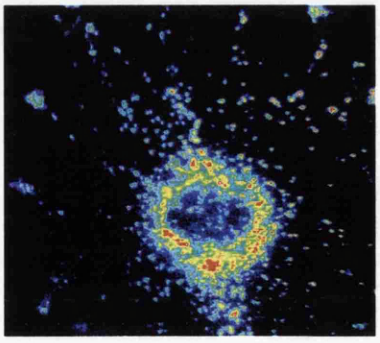
b)



c)



d)



e)

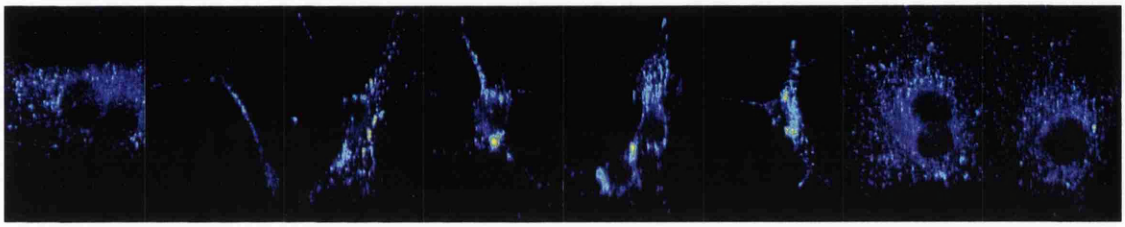
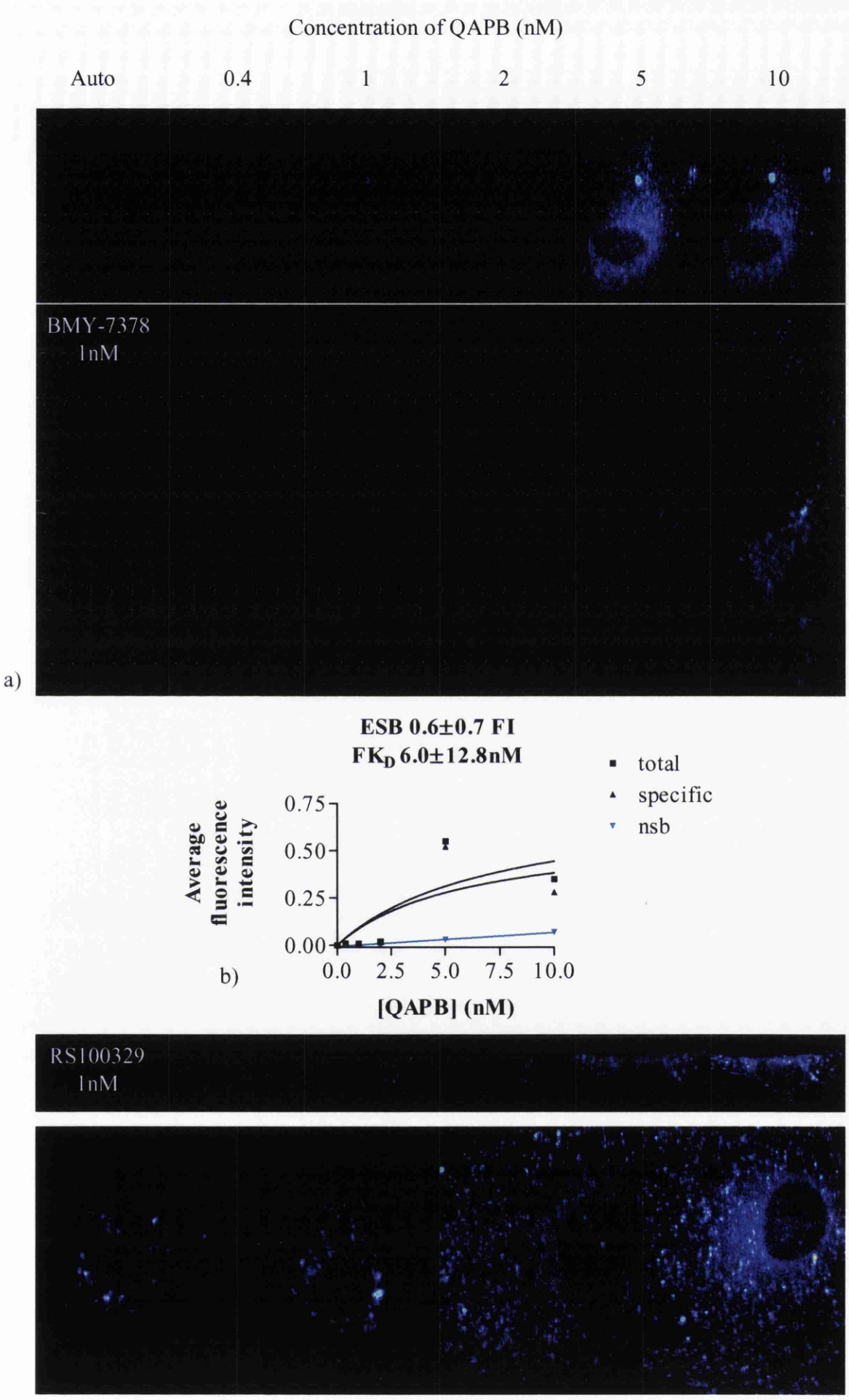
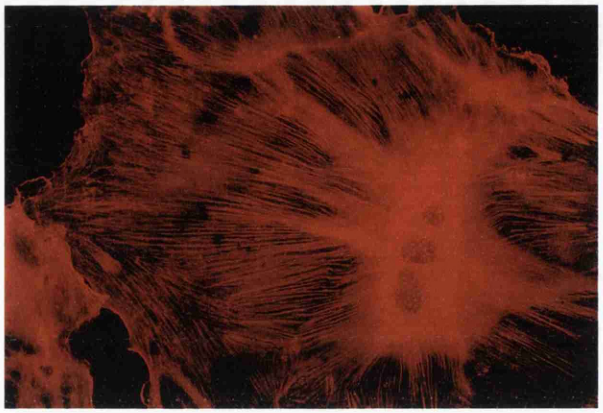
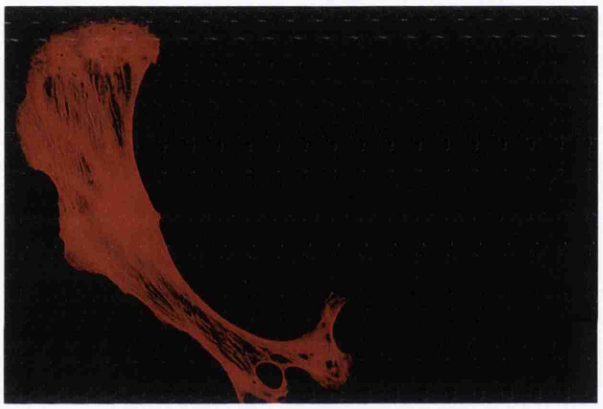


Figure 3.10 QAPB binding on cultured VSMCs dissociated from the thoracic aorta of knockout mice. Cells had been passaged a maximum of four times. Inhibition of QAPB binding to specific α_1-AR binding sites was assesed by pre-incubation for 30minutes with 1nM of the α_{1A}-AR and α_{1d}DAR selective antagonists RS100329 and BMY-7378, respectively. Cells were plated on coverslips and examined by confocal microscopy with timelapse photography. Increasing concentrations of QAPB, as indicated, were added cumulatively and images were collected at 1-minute intervals. Image data was collected at equilibrium time points for each concentration of QAPB (added at 5minute intervals). Images are shown in pseudocolour, where black indicates no staining and blue, green, yellow and red indicate increasing levels of saturation of the fluorophore. The QAPB-associated fluorescence intensity was calculated by Metamorph software and plotted against increasing concentrations of QAPB to demonstrate the levels of specific binding of QAPB in sample cells. The estimated specific binding affinity (FK_D) of QAPB was measured as nanomolar (nM) and the estimate of specific binding (ESB) measured as fluorescence intensity (FI). Estimates made for cells are presented in the table.


Concentration of QAPB (nM)
Auto
0.4
1
2
5
10
BMY-7378
1nM
a)
ESB 0.6±0.7 FI
FK$_D$ 6.0±12.8nM
total
specific
nsb
Average fluorescence intensity
0.75
0.50
0.25
0.00
0.0
2.5
5.0
7.5
10.0
[QAPB] (nM)
b)
RS100329
1nM
c)



Wildtype
Carotid artery



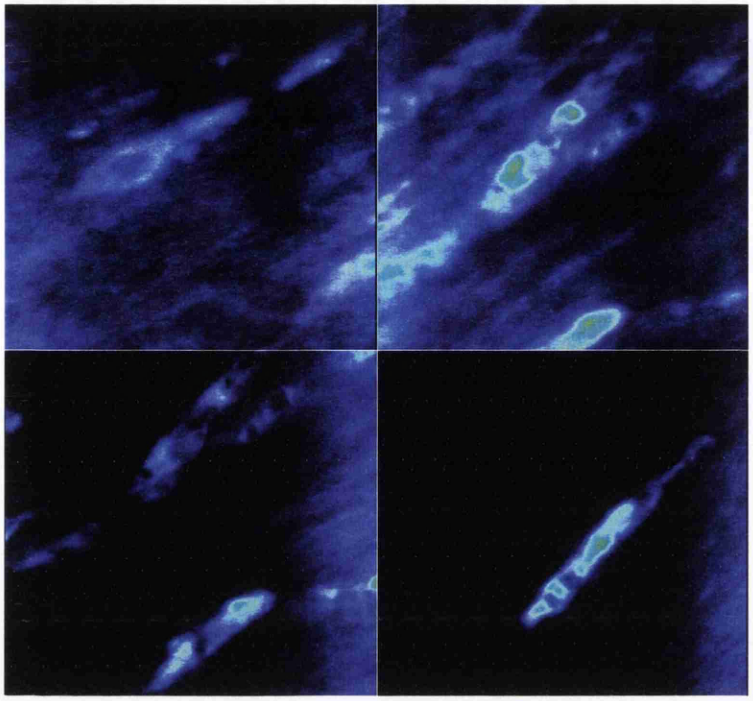
Knockout

Carotid artery

Knockout
Aorta

Figure 3.11 Representative images of cultured VSMCs stained with a cy3 conjugated smooth muscle α-actin monoclonal antibody

a)



b)

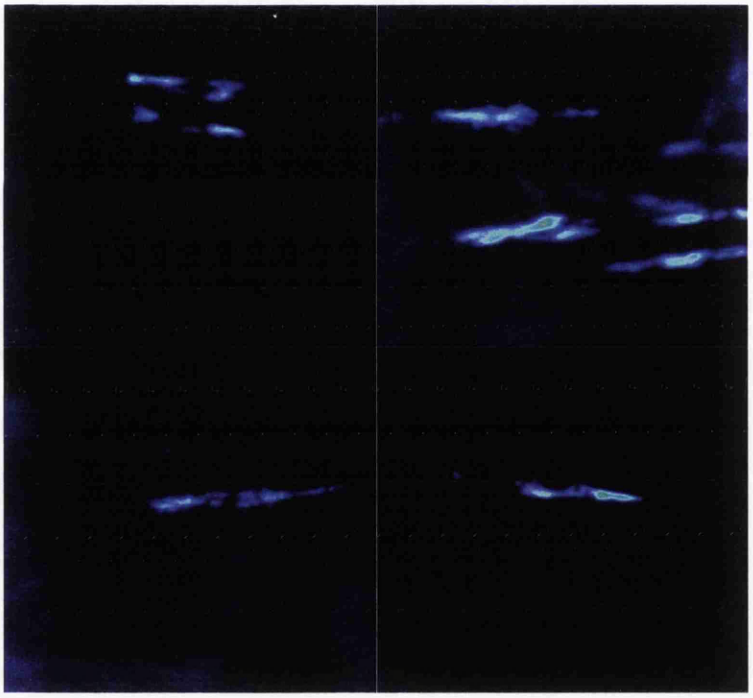
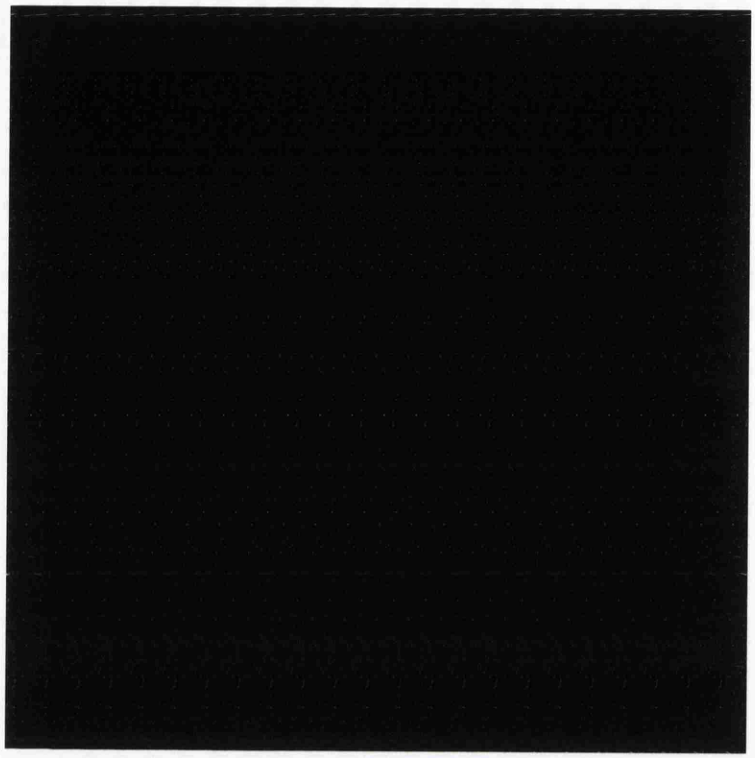
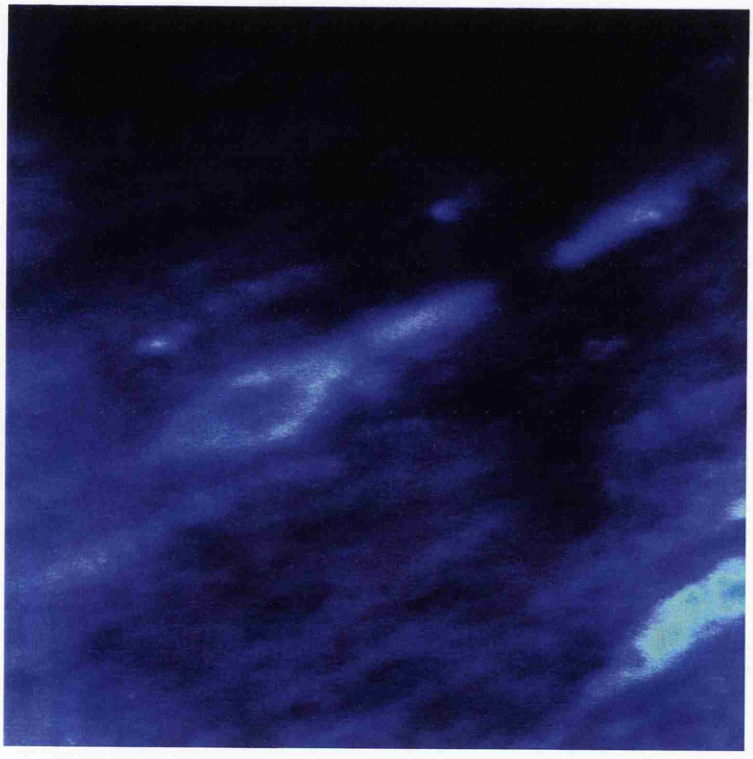
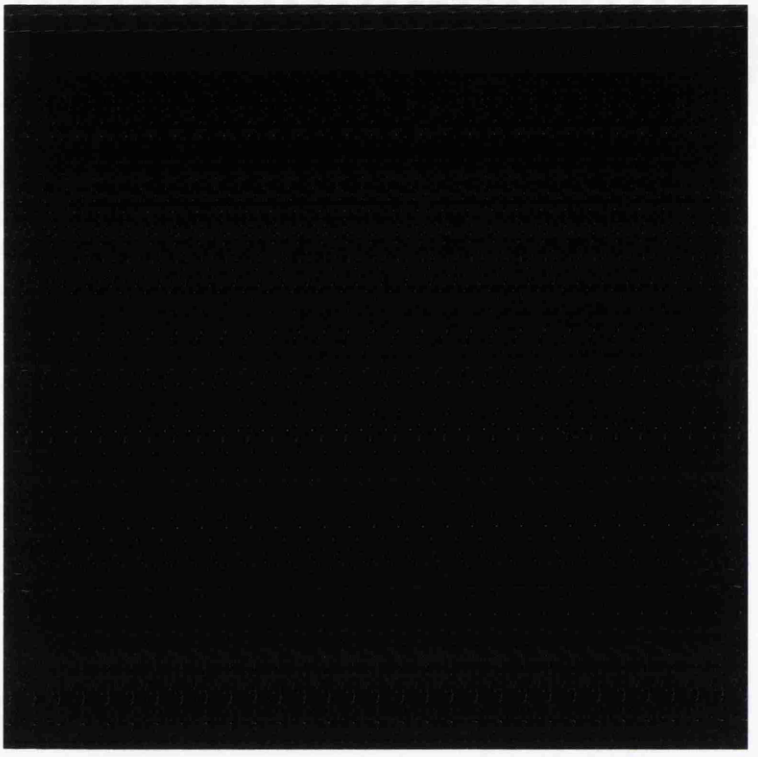


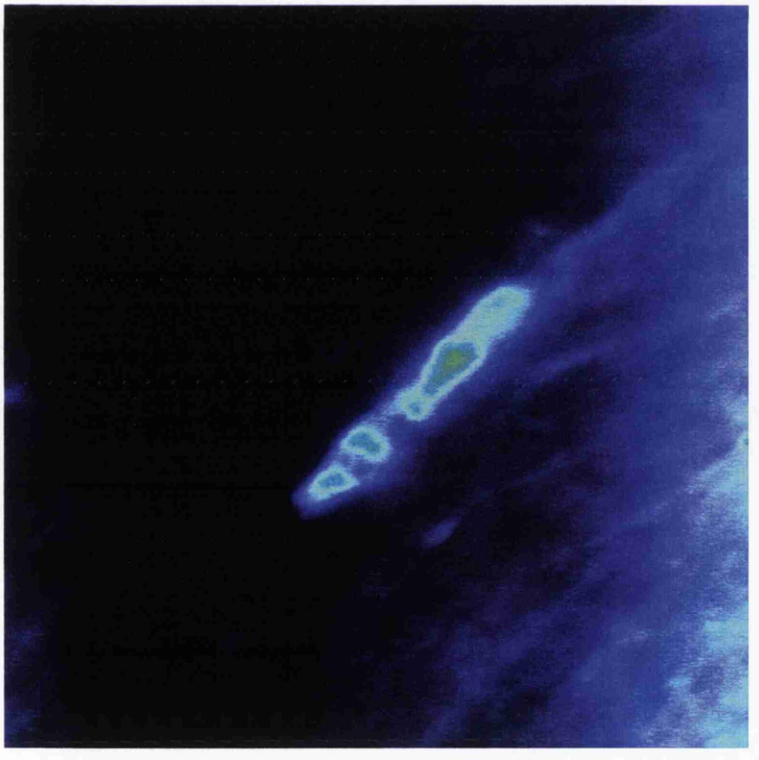
Figure 3.12 Manipulation and expression of recombinant GFP-tagged α_{1b}-AR constructs in mouse α_{1B}-AR knockout thoracic aorta *in situ.* Arterial segments were transfected *in vitro* as described in Methods, and visualised for GFP-associated fluorescence after 5 days in culture post-transfection. Images from both segments were collected under identical conditions using a x40, oil immersion objective. (a) pEGFP/human α_{1b}-AR. (b) pmouse α_{1B}-AR/human α_{1b}-AR. The above 2D images are also presented as movies and 3D reconstructions. They are labelled as follows: a) i, ii, iii and iv and b) i, ii, iii and iv corresponding to the constructs used, as detailed above.



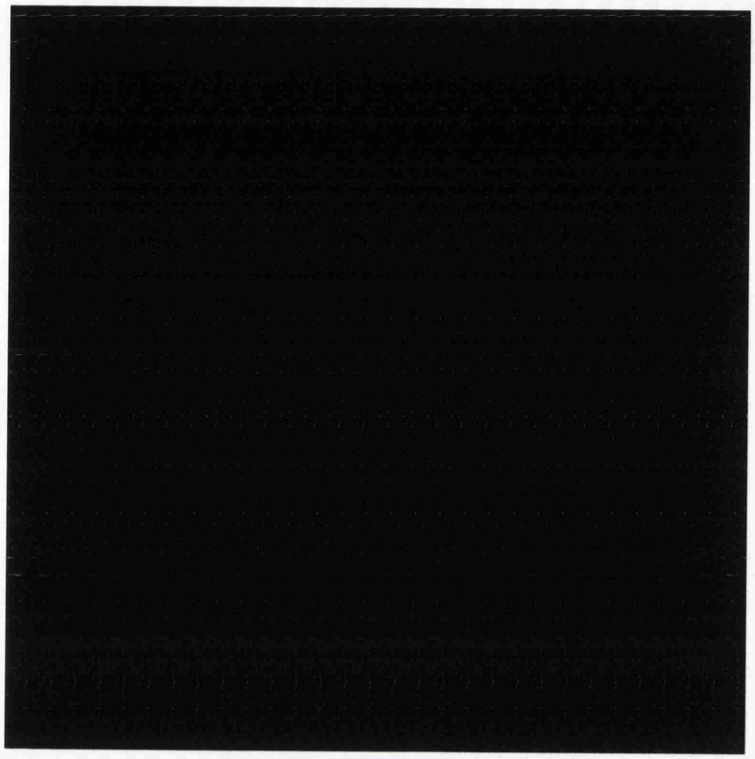


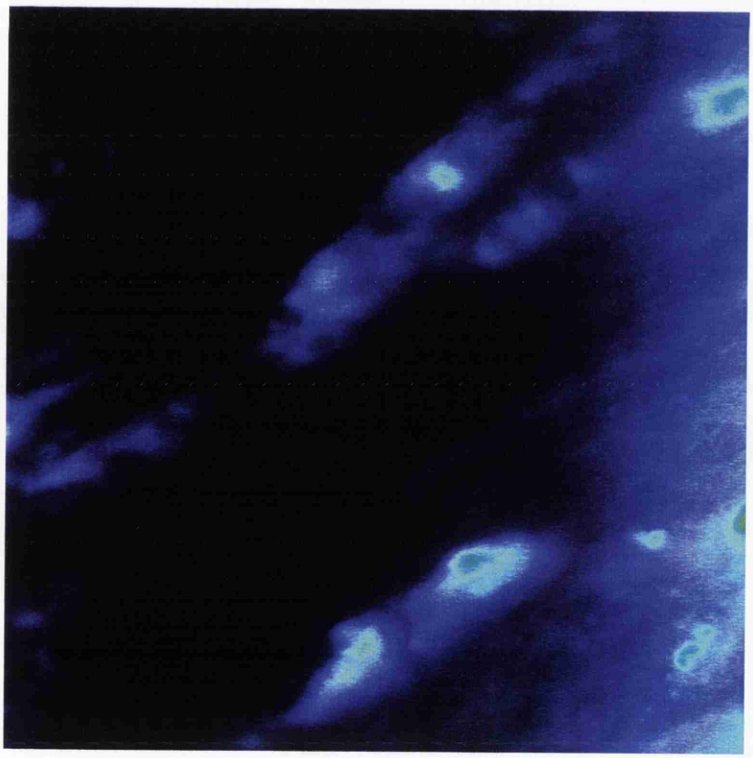
a) i



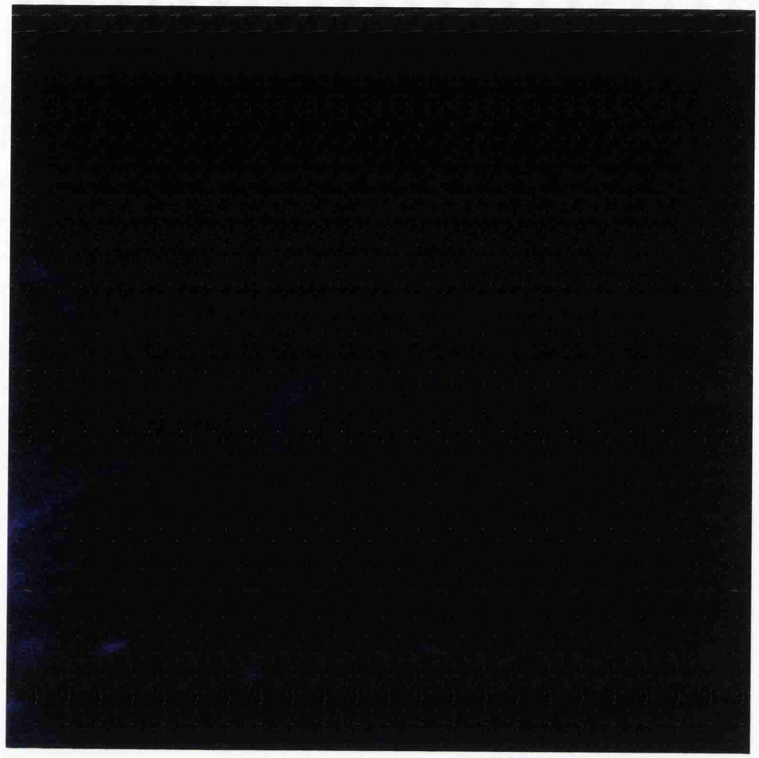


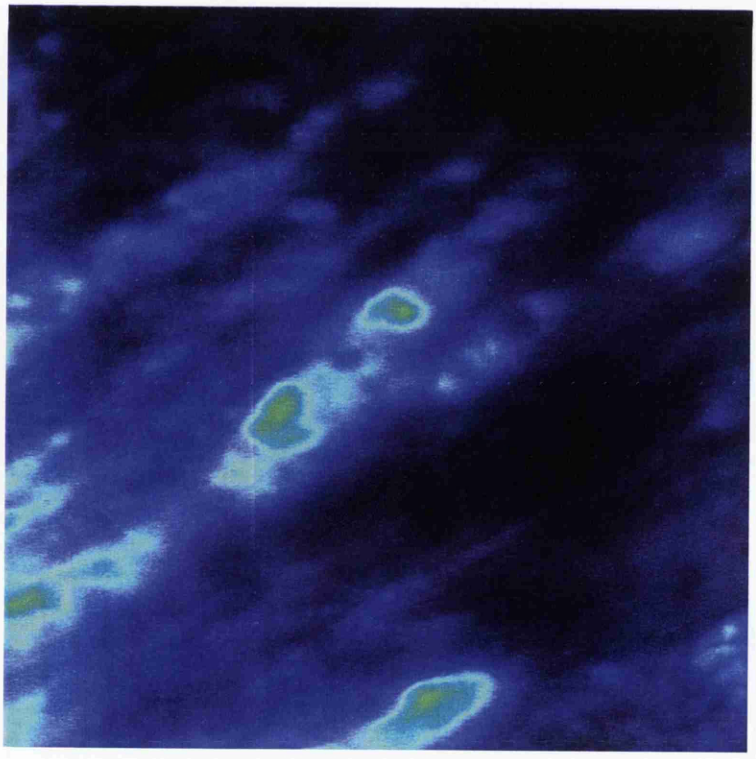
a) ii



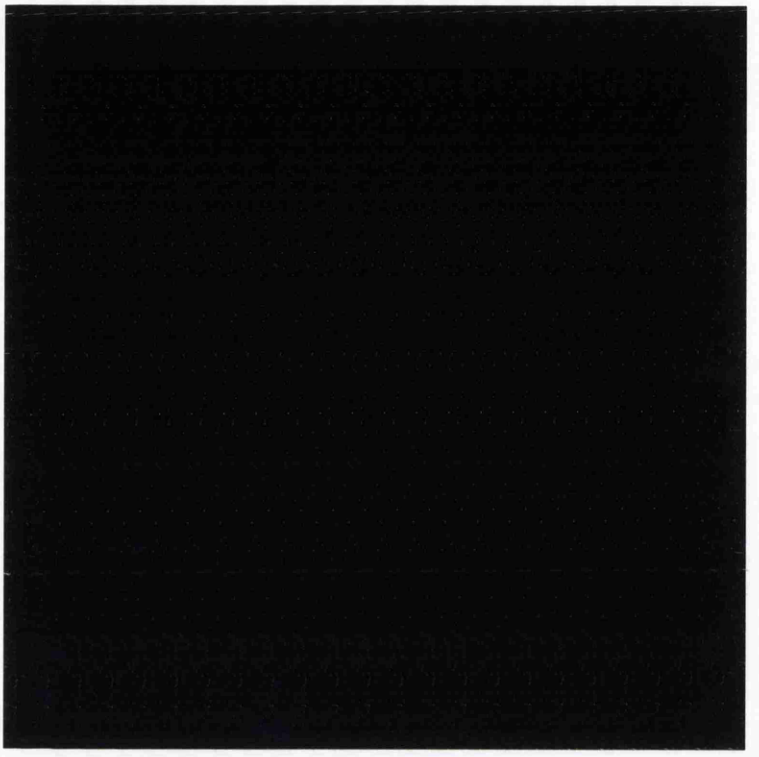


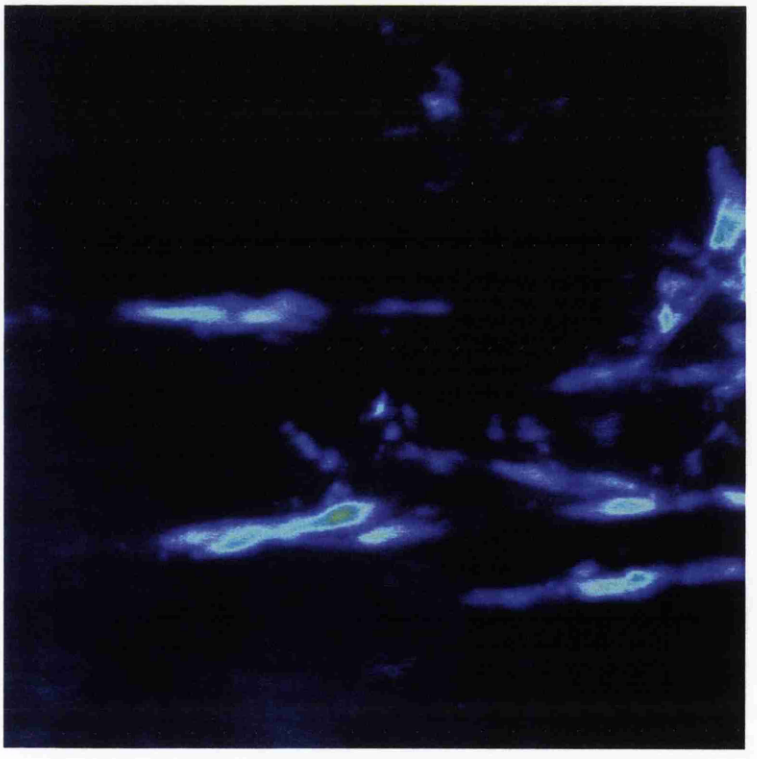
a) iii



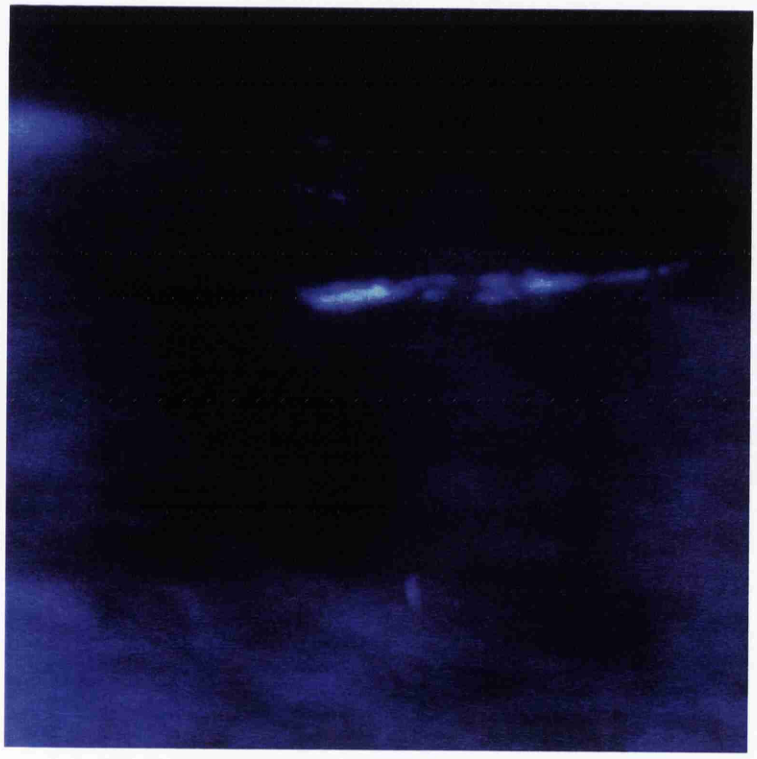


a) iv

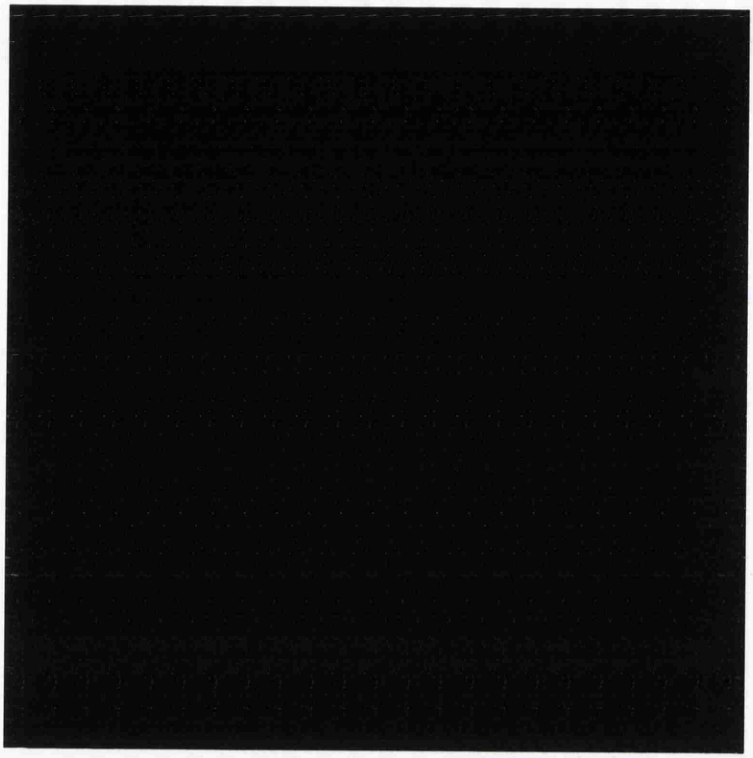


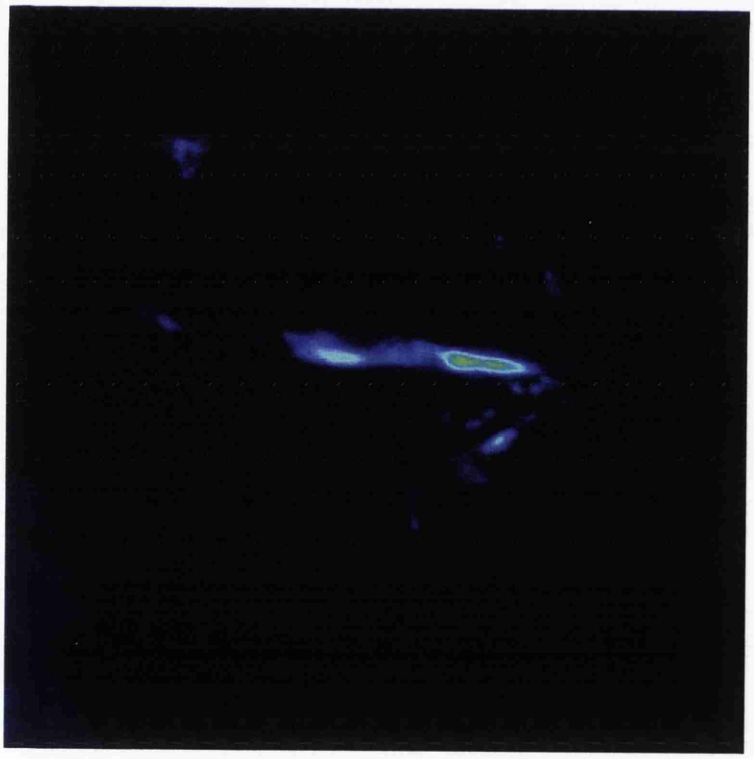


b) i

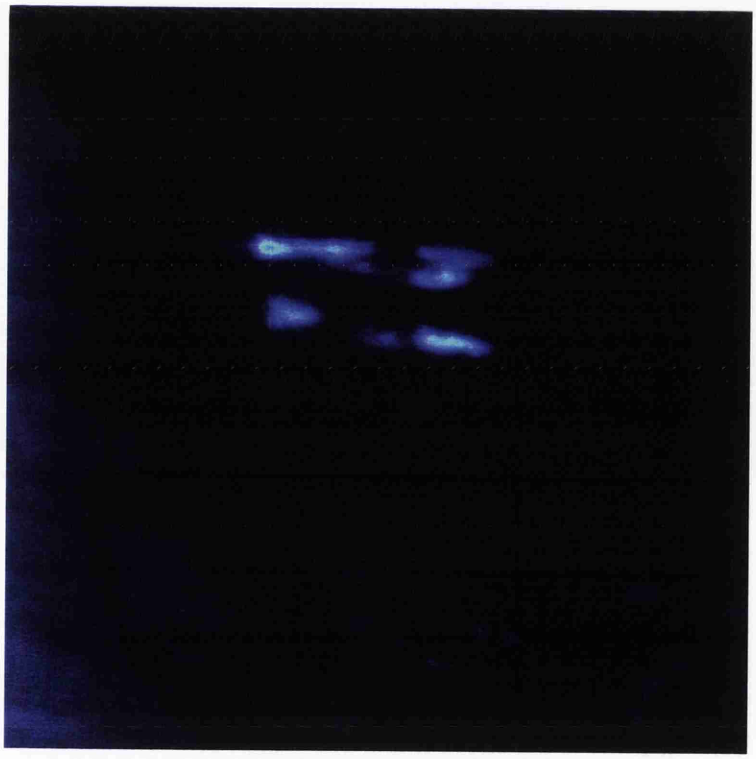


b) ii





b) iii



b) iv

a)

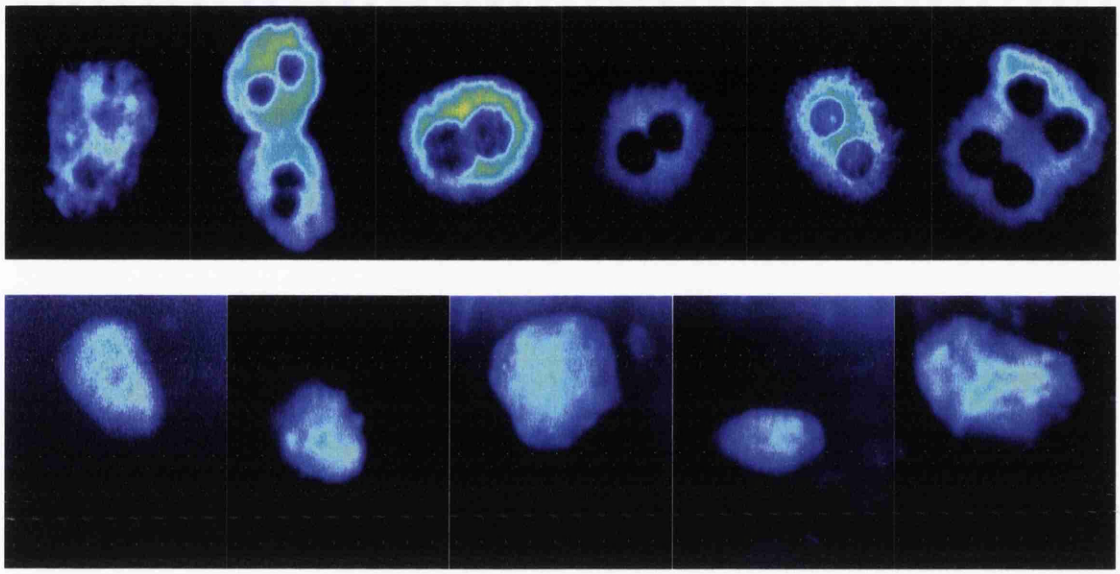
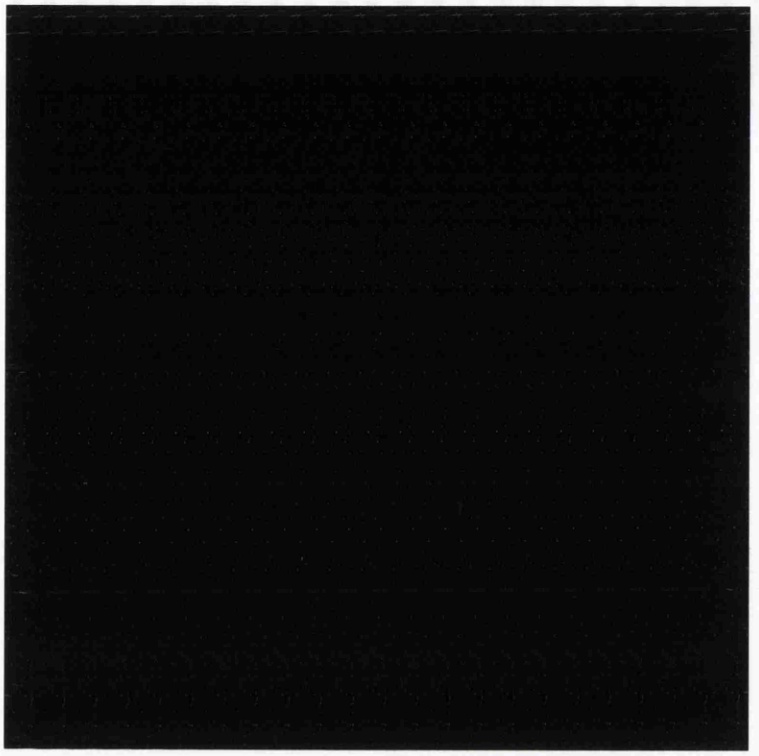
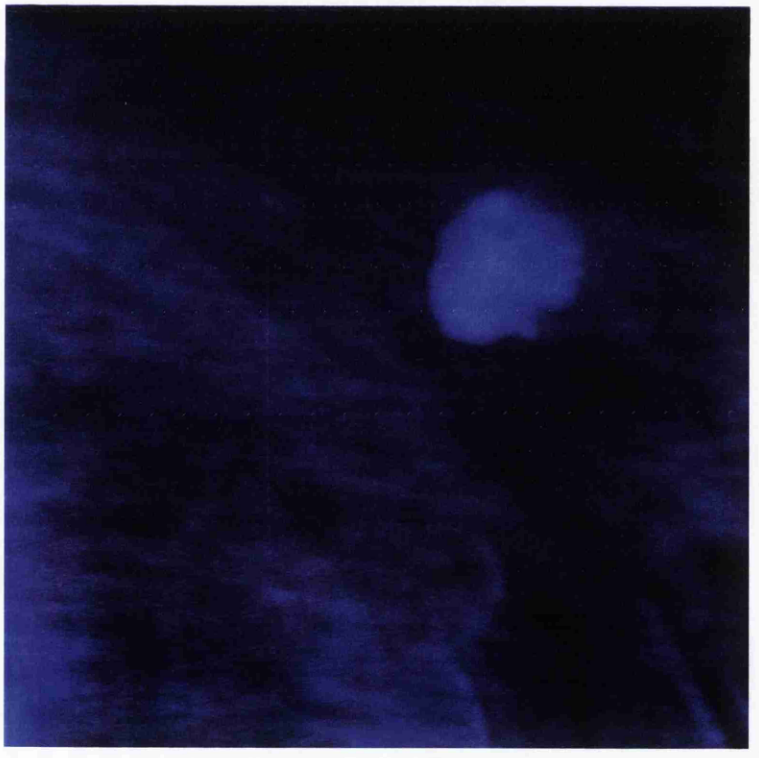
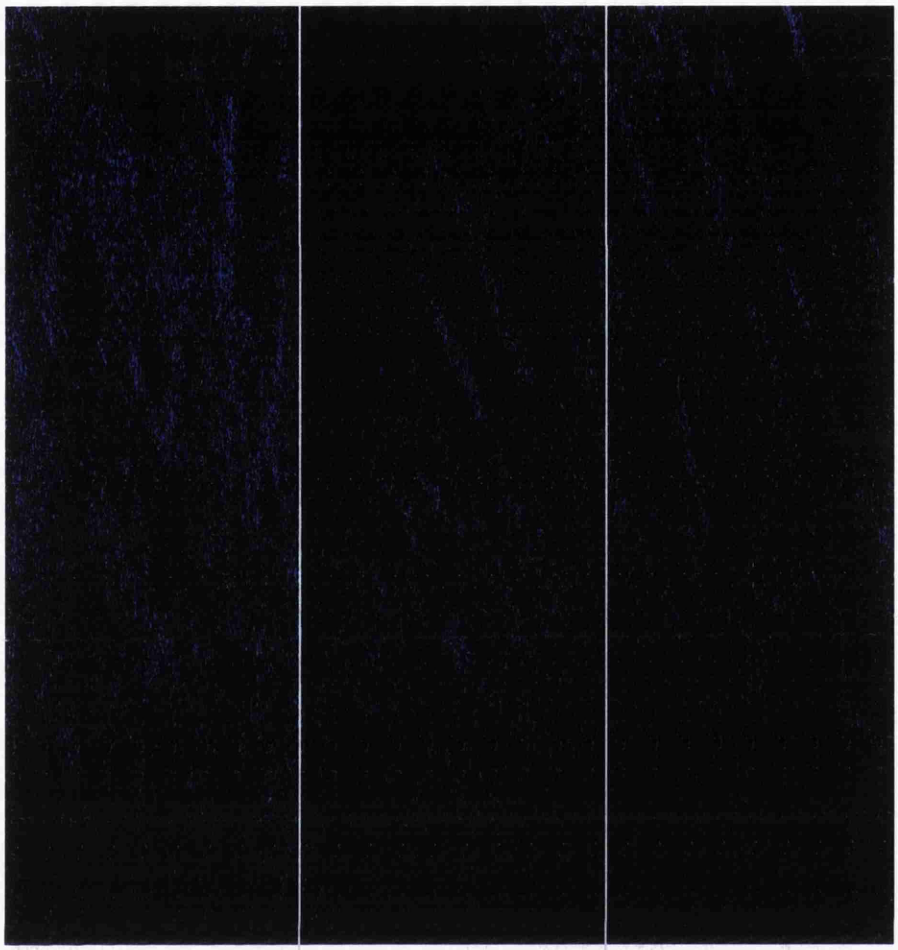


Figure 3.13 (a) GFP-associated fluorescence representing cells transiently expressing pEGFP/human α_{1b}-AR. Arterial segments were transfected *in vitro* as described in Methods, and visualised for GFP-associated fluorescence after 5 days in culture post-transfection. (top panel) Cells found in the surrounding culture medium. (bottom panel) Cells within the transfected vessel wall, situated in the adventita. (b) A movie and 3D reconstruction of a transfected adventital cell within the vessel wall.



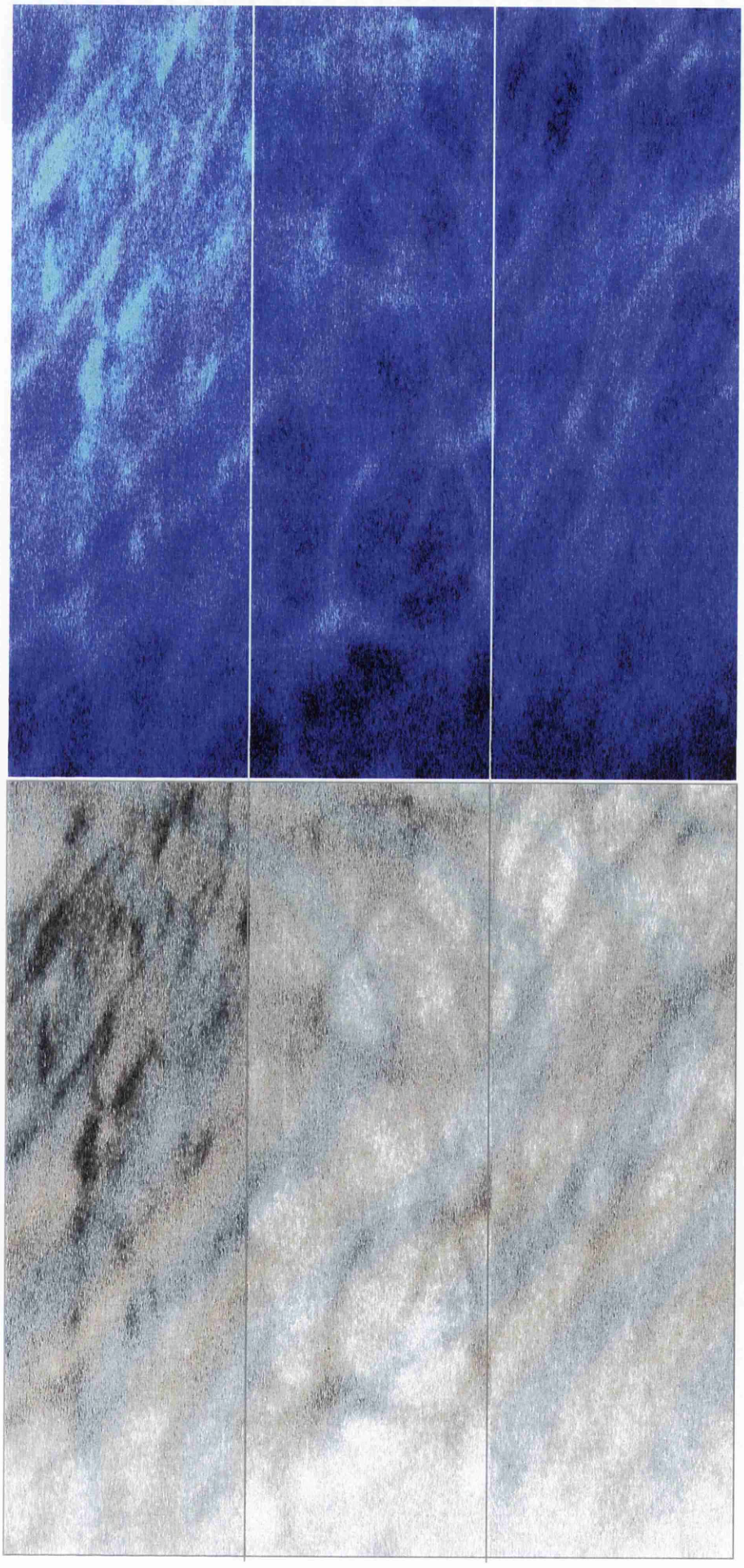
b)





a)

Figure 3.14 (a, and b) Images representative of mouse thoracic aorta before and after 5days in culture, respectively. The laser power used in a) and b) was the same but much greater than that used to detect GFP-associated fluorescence in transfected SMCs.



b)

General Discussion and future research

What is the importance of studying α_1-ARs? α_1-ARs mediate some of the main actions of the natural catecholamines, AD, and NA. They participate in many essential physiological processes, including sympathetic neurotransmission, modulation of hepatic metabolism, and control of vascular tone, cardiac contraction, and the regulation of smooth muscle activity in the genitourinary system. It is now accepted that they also take part in cell growth and proliferation (see general introduction). Since α_1-ARs are heavily involved in normal physiological functions, it is not surprising that defects in such functions can be attributed directly/indirectly to these receptors. As well as understanding their roles in normal physiological functions, it is also of great therapeutic interest to be able to manipulate α_1-ARs, in a subtype-selective manner. Benign prostatic hyperplasia, cardiac hypertrophy and hypertension are examples of pathological conditions treated by drugs acting at α_1-ARs.

Due to the homogeneous structural nature of the three known α_1-AR subtypes, the lack of subtype-selective agents is a persisting problem in this field of research, and will no doubt continue to hinder α_1-AR pharmacologists in the years to come. Until such time as highly discriminative antagonists become available on the drug market, the undesired side effects of non-selective agents will continue to pose a problem. Therefore, it is imperative that a continuous study of the properties of α_1-ARs is carried out, to try to find subtype-specific features that can be modulated with view to drug design.

It is thought that the subcellular distribution of α_1-AR subtypes may point to a new class of pharmacological properties that may distinguish them from each other and other GPCRs (Hirisawa et al., 1997). Therefore it is seems likely that this may hold

as a means for therapeutically targeting individual subtypes. Therefore, an understanding of their distribution in native cell types is of great importance. It is also possible that by refining methods for the localisation of α_1-ARs, new roles will come to light.

The subtype initially chosen for study was the α_{1b}-AR (see general introduction). In chapter 1, fluorescence based methods were used to enable the analysis of the subcellular distribution of recombinant α_{1b}-ARs. Initially, a fluorescent ligand approach was employed to locate mouse and human α_{1b}-ARs stably expressed in live, NCB20 cells. Radioligand binding studies demonstrated the expression of a homogeneous population of α_{1b}-ARs as binding profiles were characteristic of 'α_{1b}-AR' cells. Fluorimetry and calcium imaging were used to demonstrate the functionality of all constructs employed. Thereafter, the main objective was to validate QAPB binding to α_1-ARs in fixed (FLAG-tagged construct) and live (GFP-tagged construct) cells. Coupled with calcium imaging, the GFP-tagged cell line allowed the study of the distribution and active properties of α_{1b}-ARs in response to agonist. Several key observations were made from the study of recombinant α_{1b}-ARs, and are detailed and discussed below:

1. *The distribution of α_{1b}-ARs stably expressed in NCB20 cells was predominantly associated with the plasma membrane. However, in some instances, a predominantly intracellular, non-perinuclear location was observed. Interestingly, receptors were distributed in a similar manner in cells at both high (8000fmol/mg) and lower (4000fmol/mg) receptor densities. In contrast, cells sampled from the same clone, and treated in the same manner, displayed a predominantly intracellular distribution in one yet plasma membrane distribution in another.*

Based on the traditional model of GPCR activation, it is thought that when expressed at high levels, only a fraction of receptors are located at the plasma membrane, thus able to respond to extracellular signals, leaving the excess in an intracellular compartment for recruitment when required. Previous work has indicated that different subtypes of α_1-AR adopt different locations in the same cell (COS-7) (Hirasawa et al., 1997). In contrast, when expressed in rat-1-fibroblasts, two groups have observed a distribution pattern that was similar between subtypes, and associated with both the plasma and nuclear membrane (Hrometz et al., 1999; Mackenzie et al., 2000). The work in this chapter suggests that the distribution of recombinant α_{1b}-ARs stably expressed in NCB20 cells is not as clear-cut as first thought, and suggests that the same subtype is capable of having different distributions within the same host cell line. Such findings have indicated that perhaps the distribution pattern is influenced by the stage each cell is at in their cycle. Therefore caution must be taken when interpreting such data. Further work is required to establish the intracellular location of α_{1b}-ARs in these circumstances. Fluorescent endosomal/lysosomal markers are available and will be used to confirm their intracellular location.

2. *An epitope-tagged (FLAG™) mouse α_{1b}-AR construct was generated with a view to co-localisation studies with QAPB, to assess alterations in QAPB binding post-fixation. When stably expressed in NCB20 cells, the fully functional FLAG-tagged construct had lower affinity than the wildtype receptor for α_1-AR-selective compounds in radioligand binding studies, yet seemed unaffected in confocal analysis on live cells. Unfortunately immunofluorescence studies were not a success*

due to suspected cross-reactivity with cellular proteins and non-specific binding of the FLAG-antibody.

A possible explanation for the higher affinity of QAPB seen in live cells could be a conformational change in the tagged-receptor that permitted high affinity binding in its native environment (live cells), but caused some degree of steric interference in the plasma membrane preparation. It should be noted that QAPB also showed a reduced affinity for wildtype mouse/human α_{1b}-ARs in radioligand binding assays compared to live cell binding. Although not to the same degree, it does suggest that the discrepancy may be due to the binding of QAPB in the radioligand binding protocol, as well as epitope-tagging. Prazosin also had a lower affinity for the FLAG-tagged construct than the wildtype receptor in radioligand binding analysis, confirming that steric interference was likely. Regarding the structure of the α_{1b}-AR, in particular the position of the ligand-binding domain, additions to the N-terminus are likely to cause changes in folding and I am assuming this is what has happened in this case. The tag has restricted access to the ligand-binding domain.

Although the affinity of α_1- selective ligands is reduced slightly in this cell line, it is still within the accepted range (nM) and will be useful in future work.

Expression of this construct in a non-rodent cell line will hopefully reduce the cross-reactivity to cellular proteins and non-specific binding of the FLAG antibody observed during this project.

3. *A fully functional, C-terminal GFP-tagged human α_{1b}-AR construct was stably expressed in NCB20 cells, and demonstrated that such C-terminal fusion proteins do not perturb normal ligand binding, or functionality.*

In contrast to the reduced ligand-binding affinity that resulted from the attachment of an 8amino-acid epitope-tag (FLAG) to the N-terminus of the mouse α_{1b}-AR, addition of >200amino-acids (GFP) to the C-terminus had no adverse effects on prazosin binding affinity. However, regarding QAPB binding, a similar situation was found in this cell line as was observed with the FLAG-tagged construct. Such findings indicate that the reduced affinity of QAPB binding must be attributed to its activity in the radioligand-binding set-up. In the radioligand binding studies, when the affinity of every α_{1b}-AR construct for prazosin was compared with their respective affinities for QAPB, it was obvious that each cell line displayed a much lower affinity for the fluorescently labelled compound. In contrast, the studies in live cells show that QAPB binds to α_{1b}-ARs with the same ability in all cell lines studied. Collectively, these observations indicate that QAPB is more suited to the live cell protocol and for some unexplained reason gives a wide range of binding affinities in the membrane preparation protocol. Such work requires further investigation.

4. *α_{1b}-ARs have a basal level of movement between the plasma membrane and a non-perinuclear, intracellular compartment when stably expressed in NCB20 cells. This movement ceased upon Ca^{++} release from intracellular stores in response to agonist stimulation and resumed activity once Ca^{++} levels returned to base line. During prolonged (35minutes) agonist exposure, there was an obvious overall shift in the density of α_{1b}-ARs residing at the plasma membrane to the intracellular compartment.*

Several interesting observations were made during this experiment, although arriving at logical explanations was perplexing. What surprised me the most was the extent of basal movement of these receptors back and forth from inside the cell to the

membrane. In response to agonist, the balance was shifted and receptors moved from the plasma membrane to inside the cell, presumably to prevent over-stimulation. Interestingly, during the very short-lived period of Ca^{++} release, this movement ground to halt, and did not resume until Ca^{++} levels had returned to baseline. A possible explanation for this could be the cell prioritising its metabolic energy for the release of Ca^{++}. This is the first report to my knowledge of such an observation, and will require further investigation.

5. *In NCB20 cells stably expressing the GFP-tagged construct, QAPB bound to the same areas within the cell as those associated with low GFP fluorescence, representing the location of the tagged α_{1b}-ARs. This was however difficult to interpret as QAPB binding caused a change in the shape of the cell. Rapid vesicular movement within the cell indicated that QAPB enters 'α_1-AR' cells via an endocytic pathway in the absence of agonist. QAPB binding to α_1-ARs did not elicit an intracellular Ca^{++} response.*

Preliminary work done by Dr John Pediani has shown that QAPB binding is blocked in the presence of sucrose/concanavalin-A, inhibitors of clathrin-mediated endocytosis (Pediani, manuscript in preparation). The indications that QAPB gains access to intracellular sites via receptor-mediated endocytosis has brought to light matters that were not previously foreseen. Does this happen because QAPB is non-selective between each of the three α_1-AR subtypes? Once it binds to α_1-ARs on the surface of the cell, and is internalised, what is the fate of the ligand-receptor complex? Does it also gain access by diffusion through the membrane, due to its structurally lipophilic nature? Is the path of endocytosis seen in QAPB binding to α_1-ARs the same as that taken by agonist? Since only agonist stimulation is capable of

mediating an intracellular response, it is unlikley that they are identical. A similar observation has been observed with antagonist binding to the cholecystokinin receptor, where Roettger et al. (1997) have shown that an antagonist is capable of mediating its internalisation in the absence of both receptor phosphorylation and second messenger signalling. Another report regarding the μ-opioid receptor has indicated that the signal for internalisation is apparently independent of second messenger production (Segredo et al., 1997). Further work is required to establish the route taken by QAPB in these cells.

The reason for studying mouse α_{1b}-ARs was to assist our interpretation of data from the α_{1B}- knockout mouse model we were using at that time. Human α_{1b}-ARs were also compared alongside so that ultimately, findings could be related to clinical issues.

Thus far, interpretation of observations made in transgenic mouse models has been hampered due to the lack of knowledge available regarding the distribution of α_1-ARs in the normal mouse. However, recent work has shown that in some cases, simple comparisons between α_{1B}-knockout and wildtype mice can allow specific functional roles to be attributed to the subtype in question (Daly et al, in press), or perhaps indicate subtype redundancy, which has been demonstrated for example, in the liver (C. Deighan, PhD thesis). In fact, the use of this transgenic mouse model has greatly assisted our general understanding of the role of the α_{1B}-AR in this organ, as is emphasised below.

The methods developed using recombinant cell lines were then used to examine the properties of native receptors on isolated hepatocytes. The main objective was to assess the subcellular distribution of native α_{1B}-ARs in wildtype cells, hypothesise as

to their functional location, and to consider the possibility of the upregulation of another α_1-AR subtype in knockout cells. Further to this, intriguing findings from preliminary radioligand binding experiments (C. Deighan, PhD thesis) on intact liver plasma membranes initiated further study at a subcellular level. A series of interesting observations were made in preliminary experiments done in isolated hepatocytes. Detailed below are key points relating to these findings and intended future research:

1. *α_1-AR subtype expression in hepatocytes is heterogeneous and displays a unique form of plasticity: During prolonged culture, an increase in α_1-AR density was observed. In both wildtype and knockout cells these sites were sensitive to RS100329 (α_{1A}) and BMY7378 (α_{1D}).*

   Cultured hepatocytes are capable of expressing all 3 subtypes of α_1-AR, depending on their extracellular environment. Since this is likely to be a direct mitogenic effect of the culture media, it does not represent the natural disposition of these receptors. In view of the fact that our aim was to define α_1-AR expression *in vivo*, the flexible nature of this cell type is not ideal. However, such alterations are of interest, and further work will be carried out. A combination of the two subtype-selective antagonists will demonstrate a role for the α_{1B}-AR subtype in these cultured cells. The contribution of specific mitogenic factors in the culture media will also be assessed as will cell plating density.

2. *α_1-AR expression undergoes ontogenic modulation during the life cycle of the mouse. In the wildtype, there is a predominantly α_{1B}-AR population which shows a decrease in density between 3 and 4 months, likely to represent post-maturational*

decline, as seen in the rat. In the 3month-old knockout, only a small population of α_1-ARs exists compared to the substantial α_{1B}-AR population in the wildtype. The knockout mouse shows up-regulation of an α_{1A}-AR population between 3 and 4months likely to be a compensatory mechanism to replace the α_{1B}-AR subtype. By 4months, a population of similar α_1-AR density is reached in both strains, yet they belong to different subtypes.

It is well documented in the literature that α_1-AR subtype expression in the intact liver varies between species. However, this is the first report to date of such an interchange within the same species. This is of great interest to us as it suggests subtype redundancy in an organ where α_1-AR subtypes are thought to play an important role. Interestingly, in the 3month knockout, a β-AR or even a non-adrenergic mechanism seems likely to compensate for the lack of α_1-ARs seen at this age. At a specific time point between 3 and 4months, a replacement population of α_{1A}-ARs becomes established. Such findings have led us to suspect that α_1-AR subtype expression may undergo alterations during the life cycle of the mouse. It is hypothesised that the α_{1B}-AR is replaced by the α_{1A}-AR subtype in the mature animal. Further studies using 1year-old mice will establish this.

3. *In both wildtype and knockout cultured hepatocytes, α_1-ARs are distributed throughout the cytoplasm of the cell. Minimal expression was associated with the plasma membrane.*

If this represents a similar distribution of the different subtypes found by radioligand binding in the two strains then there would be no subtype-dependent differences in location. However, since prolonged culture increased the density of α_1-ARs (α_{1A} and

α_{1D}), and α_{1B}-AR expression is likely to be downregulated (Gao et al., 1996), it is possible that QAPB binds to similar populations (α_{1A} and α_{1D}) in the two strains explaining the similar intracellular binding pattern. Further work is required to establish the natural disposition of α_1-AR subtypes in hepatocytes.

The multifunctional protein, gC1-qR has been reported to bind to the C-terminus of the α_{1B}-AR, in rat liver, retaining it in an intracellular location and mediating its down-regulation (Xu et al., 1999; Hirasawa et al., 2001). Immunoprecipitation of the gC1-qR-α_1-AR complex from wildtype and knockout liver will test the specificity of this protein. There is a possibility that it is liver-specific, in which case, it may be capable of binding to the α_{1A}-AR in the knockout. Once the specificity of gC1-qR has been established, further distribution analysis will be permitted. Due to the unforeseen alterations in α_1-AR subtype expression during prolonged culture of hepatocytes, studies regarding the natural distribution of these receptors will require to be carried almost immediately post-isolation.

Using techniques developed in the previous chapters, the work in the final chapter represents the study of the subcellular distribution and characterisation of native α_1-ARs in VSMCs, both freshly dissociated and in culture.

In transgenic mouse models, it is likely that the 'knocked out' gene can, at a single cell level, be replaced with labelled recombinant versions. The work in chapter 3 demonstrates how, under the control of a viral or mouse α_{1B}-AR-specific promoter, GFP-tagged α_{1b}-ARs can be expressed in knockout aortic SMCs *in situ*. Once optimised, such manipulation should allow for expression mimicking that found *in vivo*. In terms of drug action, subtype distribution indicated from such studies will be

informative. Detailed below are the key findings from the duration of this project regarding α_1-AR expression in VSMCs:

1. *QAPB bound specifically, and with high affinity, to α_1-ARs present on freshly dissociated (carotid artery) and cultured (carotid artery and thoracic aorta) VSMCs. In freshly dissociated cells, QAPB binding was predominantly intracellular; this was consistent in both wildtype and knockout cells. In cultured VSMCs, QAPB binding varied considerably between individual cells, in both strains, and was influenced by the cellular morphology.*

   Freshly dissociated VSMCs, are likely to represent the natural disposition of α_1-ARs seen *in vivo*. The intracellular distribution observed in both wildtype and knockout cells, indicated that, in their natural environment, α_1-ARs are perhaps situated inside the cell and recruited to the membrane for agonist activation. Alternatively, the endogenous/exogenous ligand may gain access via a transporter, or endocytic mechanism. Such considerations regarding subtype-specific distribution are extremely important with a view to prospective therapeutic intervention.

   Reports in the literature, combined with studies in this project, indicate that the subcellular distribution of α_1-AR subtypes in cultured VSMCs is likely to be indicative of intrinsic properties of subpopulations of SMCs within the vessel wall. In spindle-shaped cells, representative of the contractile phenotype, α_1-ARs are associated predominantly with the plasma membrane, and hence likely to be involved in contraction. A greater proliferative activity was also observed in cells of such morphology. Large, 'fried-egg' shaped cells, displayed clustered QAPB binding in a perinuclear orientation and a rather quiescent growth rate. Such features indicate

that perhaps these cells perhaps contribute differently in vascular function. Therefore cultured cells are not likely to represent an *in vivo* situation.

2. *RS100329 (α_{1A}-AR-selective) was able to reduce the specific binding of QAPB in some of the cells analysed, from both aorta and carotid artery. BMY7378 (α_{1D}-AR-selective) was able to block the specific binding of QAPB in the majority of knockout cells studied, from both strains, and from both vessels. Less of an effect was observed in the wildtype. In knockout VSMCs (aortic and carotid artery), a predominantly α_{1D}AR population was expressed, whereas a mixture of all three is likely in the wildtype carotid artery.*

   A more substantial study, using the ArrayScan™ system is required to provide quantitative data regarding α_1-AR subtype expression at the single VSMC level in these vessels.

3. *Observations were made that wildtype carotid artery VSMCs appeared to grow at a much slower rate that knockout cells.*

   This may be explained by the presence of 'quiescent' shaped cells in the wildtype culture, or is perhaps a feature intrinsic to knockout cells. This requires further investigation. Reports in the literature suggest that subpopulations of VSMCs have different metabolic and growth rates along the length of a given blood vessel (see review Hungerford and Little, 1999)

4. *Using the transfection reagent Tfx™-50, it was possible to express GFP-tagged α_{1b}-ARs in knockout aortic SMCs in situ under the control of both a viral and mouse α_{1B}-AR-specific promoter.*

Further investigation is required to establish if only certain areas in the vessel wall are able to express α_{1b}-ARs under the control of the promoter specific to the mouse α_{1B}-AR gene. Once transfection conditions have been optimised, it will be interesting to compare the distribution of α_{1b}-ARs expressed in aortic segments under the control of the two promoter systems. Will they be expressed uniformly, or will they appear in specific regions within the vessel wall? As suggested by preliminary studies in chapter 3, adventitial cells are capable of expressing α_{1b}-ARs. Are they competent to express the α_{1b}-AR when under the control of the mouse α_{1B}-specific promoter?

I was surprised to find that it was relatively easier to transfect cells within the vessel wall than to transfect single cultured SMCs, where the reagent had visibly toxic effects. Since blood vessel segments are more robust than single cells, I assumed that the need for diffusion of the liposome-DNA complex through the vessel layers before reaching the smooth muscle, had allowed a more gentle approach, and perhaps less potency compared with the exposed surfaces of cultured cells.

Until recently, typical GPCRs were thought to reside solely at the plasma membrane. However, findings contained in this thesis suggest otherwise. Up to this point, however, it is still unclear whether subtypes of α_1-ARs have essentially a specific functional location, irrespective of cell type, or whether their distribution is cell-specific? It is also evident from the work in this thesis that during culture native cells undergo alterations that obscure their true phenotype whilst in their natural environment. These findings emphasise the need for a subtype-expression-system that mimics an *in vivo* environment.

Results from the confocal studies on VSMCs have also exemplified the problems encountered with α_1-AR characterisation of blood vessels at present. At the single cell level, the possibility of all three subtypes being expressed in the wildtype carotid artery was indicated yet only one subtype predominated in the functional response (C. Deighan, PhD thesis). Such findings demonstrate the necessity for functional analysis. Although information gained from single cell work regarding distribution is invaluable, ultimately, the α_1-AR subtype(s) that mediate the functional response of individual vessels is the key to drug intervention.

The lack of correlation between expression and functional response is best illustrated by the α_{1B}-AR. The α_{1B}-AR is expressed throughout the vasculature (Piascik et al., 1997), although several groups have demonstrated a minimal role for this subtype in mediating contraction (see general introduction). To add to this frustration, no 'fool-proof' antagonists are currently available for this subtype, distinguishing it from α_{1A}- and α_{1D}-ARs. How may this be overcome?

The use of transgenic mouse models has the advantage of simplifying the situation by removing individual subtypes from the equation, but they may also have their limitations. The lack of a specific subtype from conception onwards may lead, in some cases, to compensatory alternative mechanisms that will not allow specific functions to be attributed to a particular α_1-AR subtype. In addition to up-regulation of remaining subtypes, it is possible that decreases in responses known to functionally antagonise the subtype of α_1-AR in question may occur. An example of this has been reported in the β_1/β_2-double knockout, where cardiac muscarinic receptor density was reduced, a counterbalancing reduction in a receptor that is known to functionally antagonise stimulatory β-ARs (Roher et al., 1999).

The development of a transgenic mouse model, in which all α_{1B}-competent cells/tissues express a GFP-tagged version of this receptor subtype, holds great promise for this field of research. Our laboratory has been awarded an MRC innovation grant to generate such a model. In collaboration with Professor John Mullins, University of Edinburgh medical school (generating mouse model), and Dr Dianne Perez, The Cleveland Clinic, Ohio (designed construct), we hope to be able to assess the competency of any given tissue/cell to express the α_{1B}-AR. This model has the potential to provide invaluable information that has thus far been restricted due to frustrating technical limitations.

When the GFP-tagged α_{1B}-AR mouse model becomes available, non-invasive agonist stimulation studies of smooth muscle in the intact vessel will hopefully be enabled.

In addition, an α_{1D}-AR knockout mouse has been generated (Tsujimoto), and it is hoped that a colony will be established in our laboratory in the coming months. This subtype plays a large part in mediating contraction of several blood vessels. Therefore, it will be interesting to see what compensatory mechanisms 'kick-in' in its absence. Comparison of data from the two α_1-AR subtype knockout mice will, no doubt, be intriguing.

The methods developed and adapted during this project will hopefully enable further study of internalisation of α_{1b}-ARs, and hopefully we will be able to extend this to native cells and α_{1B}-ARs *in situ*. Considerations for such work will include the rate at which native receptors internalise. Presumably diffusion of drugs through the vessel layers will also be rate limiting. From reports in the literature, it appears that the rate and extent to which the $\alpha_{1B/b}$-AR is internalised, in native and recombinant cells, is cell-dependent (see general introduction).

A final set of aims of this project was to demonstrate α_1-AR distribution within the walls of human small resistance arteries, to extend this to the subcellular level and determine whether receptor cycling could be demonstrated in the intact tissue in response to agonists. Although this final question still remains unanswered, the project did make considerable scientific progress, including the ability to manipulate the α_{1b}-AR population and express GFP-tagged constructs in blood vessel segments.

The biggest hurdle in this project was the development of the FLAG-tagged construct. Being an amateur in the field of molecular biology, and having to employ unfamiliar techniques in a pharmacology laboratory, was both time consuming and challenging. However, generation of the construct was a success, and although it did not contribute significantly to the final outcomes of this thesis, it gave the most personal satisfaction.

I realise now, in hindsight, that the task in hand was technically challenging, but it encouraged me to deal with the situation in a systematic and logical manner. The vast number of techniques and practical abilities required by this project has broadened my outlook as a prospective research scientist.

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