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REVIEW

Localization of α-adrenoceptors: JR Vane Medal Lecture

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This review is based on the JR Vane Medal Lecture presented at the BPS Winter Meeting in December 2011 by J.C. McGrath. A recording of the lecture is included as supporting information. It covers his laboratory's work from 1990 to 2010 on the localization of vascular α_1-adrenoceptors in native tissues, mainly arteries. Main points: (i) α_1-adrenoceptors are present on several cell types in arteries, not only on medial smooth muscle, but also on adventitial, endothelial and nerve cells; (ii) all three receptor subtypes (α_{1A}, α_{1B}, α_{1D}) are capable of binding ligands at the cell surface, strongly indicating that they are capable of function and not merely expressed. (iii) all of these cell types can take up an antagonist ligand into the intracellular compartments to which endocytosing receptors move; (iv) each individual subtype can exist at the cell surface and intracellularly in the absence of the other subtypes. As functional pharmacological experiments show variations in the involvement of the different subtypes in contractions of different arteries, it is concluded that the presence and disposition of α_1-adrenoceptors in arteries is not a simple guide to their involvement in function. Similar locations of the subtypes, even in different cell types, suggest that differences between the distribution of subtypes in model systems do not directly correlate with those in native tissues. This review includes a historical summary of the alternative terms used for adrenoceptors (adrenergic receptors, adrenoreceptors) and the author's views on the use of colours to illustrate different items, given his partial colour-blindness.

Tables of Links

TARGETS
GPCRs
α_{1A}-adrenoceptors
α_{1B}-adrenoceptors
α_{1D}-adrenoceptors
β-adrenoceptors

LIGANDS
Adrenaline
BMY7378
Noradrenaline
Phenylephrine
Prazosin
Rauwolscine
RS100 329

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013b).

Introduction

A catecholamine, noradrenaline, is released from sympathetic nerve endings to act as neurotransmitter, in the brain and almost all peripheral organs. Noradrenaline and another catecholamine, adrenaline, are released from adrenal medullary cells into the bloodstream to act as endocrine hormones. They activate a family of receptor molecules (the adrenoceptors; a note on terminology is included at the end of this article) located on the cells of the target organs to initiate physiological signals that regulate almost all organ systems in the body.

For almost 120 years, the chemical and biological properties of the adrenoceptors and their natural activators have provided a central strand in the interaction between physiology and drug discovery, each informing the other. However, the story is not complete. A gulf remains between our knowledge of the identity and structure of the main chemical players and our understanding of how the receptors operate physiologically. One reason for this is our limited understanding of the localization and distribution of the receptors, in general, in heterogeneous natural tissue, and how these relate to the nervous and hormonal regulation in which the receptors participate. Specifically, we do not have a complete conceptual view of the localization of adrenoceptors on cells within tissues even though we are starting to understand how they behave at the individual molecular and cellular level.

Adrenoceptors were defined initially by pharmacological techniques that exploited the differences between various drugs, hormones or neurotransmitters to mimic or block these actions at the different receptors. After the initial identification of two divisions, α and β, a family of nine adrenoceptors was defined by both functional and genetic means. The α-family was split into two subfamilies α_1 and α_2, each comprising three members, with the α_1 subfamily comprising three members, α_{1A}, α_{1B} and α_{1D}. There was an original convention to make the alphabetical subscript lower case, for example, α_{1a}, for receptors defined genetically, and upper case, for example, α_{1A}, for those defined by functional pharmacology. However, this convention has not been maintained. In this review, the upper case subscript will be employed throughout the text. The focus of this review is on α_1-adrenoceptors.

The theme for the lecture on which this review is based was 'Black Boxes', indicating both the opaque nature of the operation of receptors and the black background ubiquitously employed in fluorescence imaging studies.

Questions pursued

1. Where in blood vessels are α_1-adrenoceptors located; in which tissue layers and on which cell types?
2. Are α_1-adrenoceptors at the cell surface capable of binding ligands? Does this apply to all three subtypes? Does it apply to all cell types?
3. Can vascular smooth muscle cells take up antagonist ligands into the intracellular compartments to which endocytosing receptors move. Does this apply to all three subtypes of adrenoceptor and all cell types expressing the receptors?
4. Are the subtypes dependent upon each other for their cellular disposition? Is there interaction between subtypes in their tissue expression?

Our laboratory has addressed these questions using fluorescent ligands and microscopy, to visualize the location of receptors in relation to tissue cell membrane and sub-cellular structures, particularly in small arteries and particularly for the α_1-adrenoceptors, with excursions into the study of β- and α_2-adrenoceptors. The most surprising and interesting outcome was that receptors were found to be located on many cell types that had not previously been considered targets for adrenoceptor agonists or blockers (McGrath *et al.*, 2005; Daly and McGrath, 2011).

Combining knockouts with pharmacology to overcome poor drug selectivity

We decided in the early 1990s to approach the problem of identifying the subtypes of α-adrenoceptors involved in responses to adrenoceptor agonists and sympathetic nerves, by combining the use of the most 'selective' agonists and antagonists and of knockouts of the receptor subtypes. This arose from earlier studies where the focus was to discriminate α_1 from α_2-adrenoceptors; this pharmacological discrimination became more complex once the existence of subtypes started to emerge from functional and binding properties of both α_1 and α_2 families (McGrath, 1982; McGrath and Wilson, 1988; Brown *et al.*, 1990; Wilson *et al.*, 1991).

We employed mainly arteries in these studies and concentrated mainly on the three α_1-adrenoceptors, for which we were eventually able to use all three single knockouts, all three double knockouts and triple knockout animals. Our first example was actually a functional knockout of an α_2-adrenoceptor generously provided by Dr Lee Limbird (MacMillan *et al.*, 1998), which we used to identify the α_{2A}-adrenoceptor subtypes involved in endothelial vasodilatory responses to noradrenaline (Shafaroudi *et al.*, 2005). We also applied these techniques to prostatic tissue. Benign prostatic hyperplasia is currently the major therapeutic target for α_1-adrenoceptor antagonists, the other being blood vessels in hypertension and heart failure (still used in some countries; Mackenzie *et al.*, 1999; 2000; McGrath *et al.*, 1999a,b). We also applied them to hepatic tissue, which was the only example we identified in which the knockouts showed evidence of substitution of another subtype; when the α_{1B}-adrenoceptor was eliminated, it was 'replaced' by α_{1A}-adrenoceptors (Deighan *et al.*, 2004).

In parallel with this, we employed fluorescent ligands to identify the receptors (Daly *et al.*, 1992; Daly and McGrath, 2003). This allowed us to map their presence and relate this to the responses that we saw in arteries. On the basis of previous literature, using more indirect methods, on the association between autonomic nerves and post-junctional receptors (Hansen *et al.*, 1999), we expected to find that receptors on

smooth muscle would be located preferentially near to the sympathetic nerves. This turned out not to be the case: they were fairly uniformly distributed over the muscle cell population and throughout the depth of the muscle layer, according to our analysis.

In summary, we found three α_1-adrenoceptor subtypes throughout the arterial smooth muscle layers in both large and small arteries but, unexpectedly to us, they were found on the endothelial cells and also on several cell types of the adventitial layer. This heterogeneity of cell types containing the receptors suggested a potential for heterogeneity of physiological responses to catecholamines or adrenoceptor agonists. We pursued this idea using knockouts and selective agonists and antagonists. During the ensuing 20 year period, several other groups also identified adrenoceptor-mediated responses arising from adventitia and endothelium (Filippi *et al.*, 2001; Somoza *et al.*, 2005; de Andrade *et al.*, 2006; Bulloch and Daly, 2014) allowing us to build up a more complex scenario for adrenoceptor action in blood vessels than hitherto existed (McGrath *et al.*, 2005; Daly and McGrath, 2011).

Distribution of α_1-adrenoceptors throughout the vascular wall

Because we were interested in identifying the location of adrenoceptors in relation to noradrenergic nerves, in our first experiments, we combined a fluorescent ligand with the visualization of noradrenaline. Rabbit saphenous arteries, which contract to α_1-adrenoceptor agonists (Dunn *et al.*, 1989) were incubated in the ligand, washed to remove non-bound ligand, then freeze dried and exposed to formaldehyde to convert noradrenaline to a fluorescent compound (Carlsson *et al.*, 1961; Gillespie and McGrath, 1974). The tissue was then sectioned and the ligand and the sites of 'noradrenaline stores' photographed separately on a conventional fluorescence microscope using appropriate excitation/emission wavelengths. The two images were then combined (Figure 1a; McGrath *et al.*, 1996a).

Unexpectedly, we found both diffuse and punctate ligand fluorescence on virtually all tissues making up the artery. It was on smooth muscle cells, where we expected to find it, except that it showed no preferential location near to nerves. It was also on endothelial cells, which was surprising, but not entirely unexpected, since functional endothelial α_2-adrenoceptors had been demonstrated on blood vessels much earlier (Miller and Vanhoutte, 1985; Angus *et al.*, 1986). In addition, it was found on cells in the adventitia, which was a complete surprise (McGrath *et al.*, 2005). Binding was specific as it was absent in the presence of the usual very high concentration of phentolamine (10 μM) as routinely used in radioligand binding, but we were rather skeptical of whether we were actually seeing the localization of receptors. Precise localization to a particular part of the cell was not possible due to the damage done by the freeze drying process so we were not surprised that our images did not show binding confined to discrete plasmalemmal membranes, which would also have been our expectation. There seemed to be plenty of fluorescence within the various cell types, which was also not our expectation as we anticipated it to be confined to smooth muscle cells.

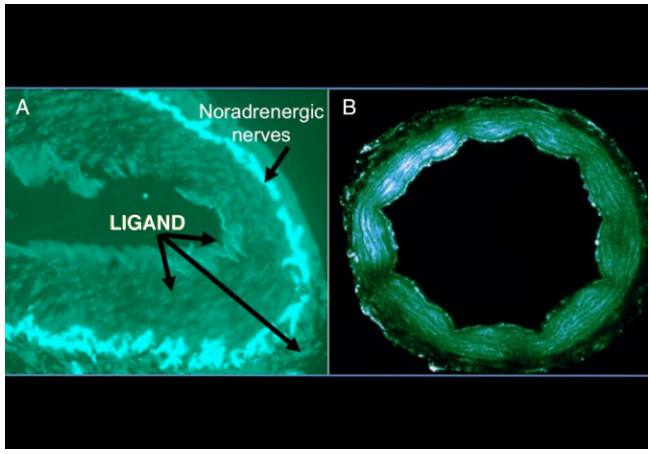

Figure 1

Examples of binding of the α_1-adrenoceptor ligand BODIPY-FL-prazosin (QAPB) to arteries. A: Rabbit saphenous artery. Noradrenaline-derived fluorescence shows the sympathetic noradrenaline-containing nerves (white) at the adventitia-medial border. The green colour, which appears in all parts of the artery, represents the binding of BODIPY-FL-prazosin (QAPB). In the medial smooth muscle layers, this revealed punctate binding orientated in a direction consistent with the arrangement of smooth muscle cells; this was where binding was expected. All of the green colour is ligand-dependent. The ligand-free control had no green at all. Thus, the binding to cells other than smooth muscle was unexpected in this early example of the fluorescent ligand-binding technique. B: Rat third-order mesenteric artery, isolated then fixed under 70 mmHg pressure, fixed with formaldehyde then sectioned and subsequently exposed to QAPB. This shows binding to all smooth muscle cells and most endothelial cells and binding to cells in the adventitia (macrophages and fibroblasts). The image is a collage and the brighter fluorescence at the upper left sector is not significant, reflecting less 'fade' when this part was captured. Courtesy of Jose Maria Gonzalez Granado (PhD Thesis, Autonomous University of Madrid, 2004). This later example, employing confocal microscopy, shows in some detail that there is ligand binding to various cell types and that this is both on the surface and inside cells.

Later, once we had improved on resolution by using gentler fixation, or none at all, and confocal microscopy, we were able to confirm the rather surprising results in Figure 1a. Figure 1b shows a later cross-sectional image where the cellular detail in each layer of a small mesenteric artery is clear, showing the presence of receptors both on the cell surface and at intracellular sites. Figure 2 takes this a stage further, showing the presence of binding throughout the vessel wall. This figure shows 'optical slices' through a 3D reconstruction of the artery so that several layers of the vessel are shown. Binding was intense on the nerve plexus and on some adventitial cells, with lower levels inside the smooth muscle layer.

It was obviously necessary to validate the technique and understand how the ligand accessed various parts of the cells.

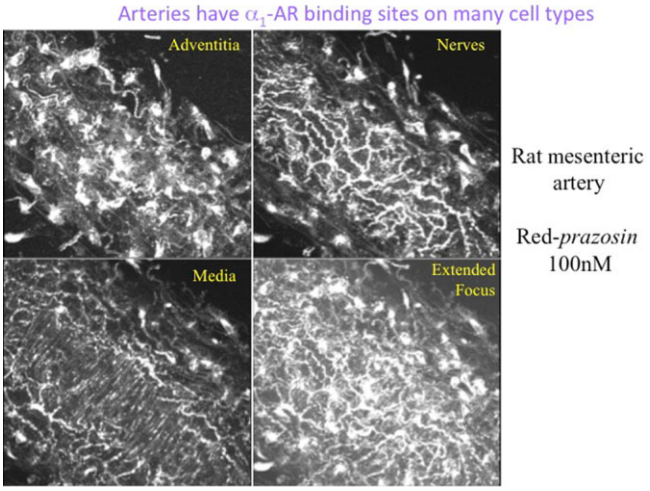

Figure 2

Confocal images of BODIPY-FL-prazosin (QAPB) binding to adventitia, nerve plexus at the adventitial-medial junction and within the media in different focal planes of rat third-order mesenteric artery. Bottom right image is an extended focus showing binding in all layers. Courtesy of Anna Briones and Elisabet Vila.

Validation of fluorescent ligand binding to α-adrenoceptors

First, we had to validate the technique for the main ligand that we used, QAPB (quinazoline piperidine Bodipy). This is marketed by Molecular Probes, Inc. (Eugene, OR, USA) under the name 'Bodipy-prazosin', but we felt that this name was misleading as the fluorescent moiety is substituted for the furan group that makes this particular compound 'prazosin' rather than the many other members of the family such as doxazosin or alfuzosin, which have different substituents at this point. So, it really represents this whole family of antagonists rather than prazosin (Figure 3).

We decided to use a combination of cell culture with recombinant adrenoceptors and studies of tissues, the adrenoceptor pharmacology of which we were familiar, such as small mesenteric arteries and rat anococcygeus muscle. Because these tissues had been suggested to have α_{1A}-adrenoceptors from their functional pharmacology (Mackenzie *et al.*, 2000), we employed cells that we understood had been transfected with α_{1A}-adrenoceptors. However, at a late stage in our investigation, we tested the ligand-binding properties of the receptors using a series of selective α_1-adrenoceptor antagonists and this showed that the receptors had the pharmacological properties of α_{1D}-adrenoceptors. When we traced back the history of the cells, we found that they had been transfected at a time when this clone had been named as α_{1A} but it had subsequently been reclassified as α_{1D} (see Hieble *et al.*, 1995). Fortunately, we discovered this before submitting the data for publication but it must have seemed odd to the readers that we chose this receptor rather than the α_{1A}-adrenoceptors present in our 'real tissue' comparator (Fig. 4).

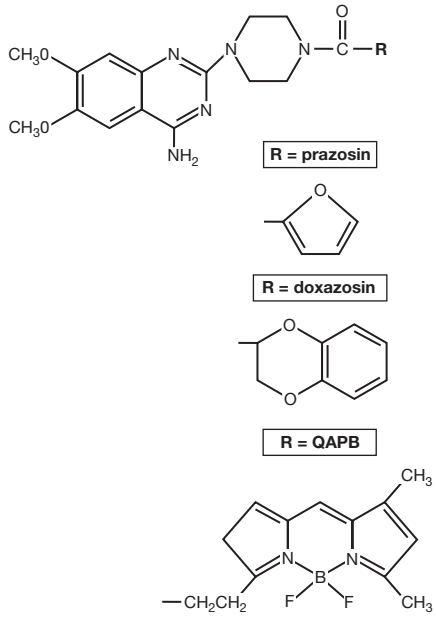

Figure 3

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 488 nm and emits above 515 nm. The compound was obtained from Molecular Probes and is listed in their catalogue as 'BODIPY-FL-prazosin' but because it lacks the furan group that defines prazosin, as opposed to other compounds that 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). Reproduced with permission from Daly *et al.* (1998).

These early studies also showed us an unexpected property of the fluorescent ligand. It fluoresced only when bound and not when free in solution. This allowed us to study binding in live cells in real time and at equilibrium between the free ligand and that bound to the cell. This gives an advantage over conventional ligand binding, which employs 'post-wash' measurement of bound ligand: this means that the cells or tissues are exposed to ligand to allow binding but then have to be washed to remove unbound ligand; it is necessary with radioligands and with compounds that are fluorescent in solution because they would give too much background counts, swamping the bound ligand's measurement. With our method, extracellular non-fluorescent compound is not detected so we can carry out measurements in real time and at equilibrium.

In the first study, we concentrated on quantifying the binding in different visual 'domains'. Most binding was 'diffuse' but some was in 'clusters'. However, both had similar binding characteristics when we quantified the fluorescence at different ligand concentrations and this was similar in blood vessels and in cells containing the recombinant receptors. Image analysis methods had to be devised for quantifying receptor distribution at the subcellular level in isolated cells and tissues (Luo *et al.*, 1998; Shang *et al.*, 2000a,b,c). We then made the critical observation that antagonists could

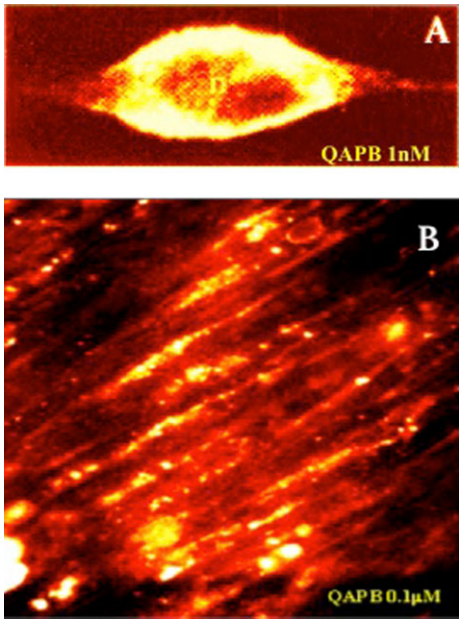

Figure 4

Early QAPB binding experiments. Top: single cell transfected with α_{1D}-adrenoceptors (QAPB 1 nM). Bottom: sheet of smooth muscle of rat anococcygeus muscle (QAPB 10 nM). (Daly *et al.*, 1998) J Pharmacol Exp Ther 1998 286(2): 984–990, permission applied for.

prevent ligand binding in real time (Daly *et al.*, 1998; Mackenzie *et al.*, 2000). By then, we had started to use confocal microscopy, but because the cultured cells were very thin, we could not be certain whether the fluorescence and, therefore, the receptors, were on the surface or inside the cell.

Intracellular binding to adrenoceptors

As our studies progressed on cell culture of different cell types, it became clear when visualizing thicker cells that the clusters were mainly intracellular (Mackenzie *et al.*, 1998; 2000; McGrath *et al.*, 1999a,b). Because by then we were using living cells, this gave us another series of phenomena and questions. It showed that binding could occur to intracellular receptors – but how did the ligand access these? If an antagonist ligand could access them what would this mean for the pharmacology of antagonists? Would the kinetics vary according to whether the antagonist could enter the cell? Would this also vary with the relative surface/intracellular location of different receptors or receptor subtypes? We were in uncharted territory here.

Around that time, various suggestions emerged concerning the differential localization of receptor subtypes that could affect their pharmacology. Our subsequent investigations did not validate many of these concepts, but they gave us hypotheses to test and disprove.

Hirasawa *et al.* (1997) hypothesized that, in COS-7 cells, a relatively greater proportion of α_{1A} receptors were located intracellularly than the proportion of α_{1B} receptors that were located intracellularly. If generally true, this might influence the access to ligands and hence the ability to activate or block the receptors. Hirasawa *et al.* (1997) wrote 'Together, the results showed that a hydrophilic alkylating agent CEC preferentially inactivates α_1-adrenoceptors on the cell surface irrespective of their subtype, and that the subtype-specific subcellular localization rather than the receptor structure is a major determinant for CEC inactivation of α_{1B}-adrenoceptors.' This provided a possible explanation for why CEC could block α_{1B}-adrenoceptors more effectively than α_{1A}-adrenoceptors. However, this was incompatible with the subsequent finding that α_{1D}-adrenoceptors, which are CEC-susceptible, are predominantly intracellular (Chalothorn *et al.*, 2002; Hague *et al.*, 2003). However, with our approach, we could not find evidence of this either. In our hands, in native tissues and in cell cultures, all three α_1-adrenoceptors could be found to be at both plasmalemmal and intracellular sites.

All three human α_1-adrenoceptors can be plasmalemmal or intracellular

When we looked at the distribution of the three human recombinant subtypes in rat-1 fibroblasts, we did not find any major differences between them. Furthermore, we made similar observations in various tissues including human prostatic smooth muscle and blood vessels (McGrath *et al.*, 1999a,b; Mackenzie *et al.*, 2000). We also compared fluorescent ligand binding to intact cells and to membranes and found no differential effects between the three subtypes. The fluorescent ligand QAPB also had similar affinity for all three subtypes, which means that it labels all three subtypes equally (Mackenzie *et al.*, 2000; Fig. 5). We also demonstrated that the recombinant receptors were functional and had appropriate agonist/antagonist pharmacology, using Ca^{2+} signalling (Pediani *et al.*, 2000).

As far as we could see, when we expressed the subtypes in different cell types, we saw similar distributions with a lot of ligand binding in the endoplasmic reticulum, which had various degrees of 'clustered' or 'punctate' nature according to the cell type.

We were also confident that our ligand was getting access to all the receptors because the distribution was similar to that found when we labelled the receptors with GFP and the ligand colocalized with the GFP (Pediani *et al.*, 2005).

We showed that α_{1A}-adrenoceptor agonist/antagonist pharmacology 'worked' at the single cell level with the recombinant receptors, using intracellular calcium as the readout. We even showed that phenylephrine could displace fluorescent antagonist from the cells (these experiments did not have 3D spatial resolution so we could not distinguish whether displacement was both from inside and from on the cell surface; McGrath *et al.*, 1995; Mackenzie *et al.*, 1999; Pediani *et al.*, 2000). This gave some hope that, in future experiments, we might identify receptors activated by the noradrenaline released from nerves and localize them by its displacement of ligand. However, we have not yet succeeded in this because our ligands have an off-rate which is too slow, that is, they dissociate too slowly to be replaced by the transiently high local concentration of neurotransmitter noradrenaline.

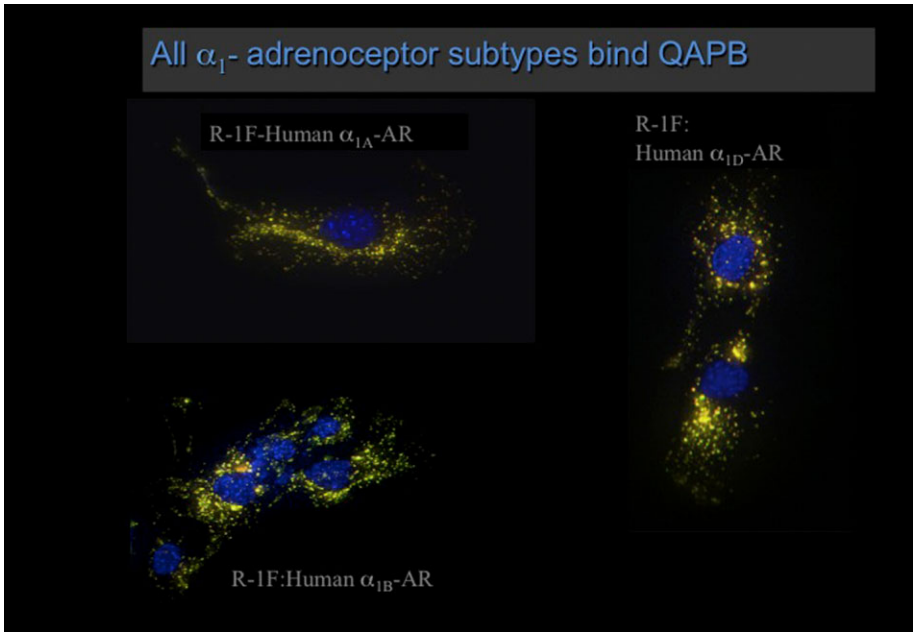

Figure 5
The three recombinant human α_1-adrenoceptors subtypes expressed in rat-1 fibroblasts all bind 10 nM QAPB. Punctate intracellular binding is clear for all subtypes. Courtesy of John Pediani.

The findings reported in Mackenzie *et al*. (2000) were also seminal to our subsequent work in that they established the selectivity of the key non-fluorescent antagonists RS100 329 (selective against α_{1A}, compared with α_{1B} and α_{1D}-adrenoceptors) and BMY7378 (selective against α_{1D}, compared with α_{1A} and α_{1B}-adrenoceptors). We went on to use both of these antagonists when imaging subtypes and when correlating these data with functional pharmacology.

Ligand is internalized by binding to α_1-adrenoceptors undergoing spontaneous endocytosis

By 2000, we still did not understand how the ligand accessed the intracellular receptors. However, Morris *et al*. (2004) showed that α_{1A}-adrenoceptors spontaneously internalise, which generated a new concept ('constitutive internalization') for the distribution of receptors and placed them in a dynamic context. Later, Stanasila *et al*. (2008) demonstrated this for recombinant α_{1B}-adrenoceptors though the two groups had contrary evidence on which, between α_{1A}-adrenoceptors and α_{1B}-adrenoceptors, internalized to the greater extent.

Meanwhile we had been investigating the hypothesis that the fluorescent ligand gained entry to the cell after binding to the receptor (Pediani *et al*., 2005) but Morris *et al*. (2004) published the idea of constitutive internalization before we published our work, so we confirmed their finding by a different approach. They had dealt only with internalization of the receptors. We were interested in seeing whether the ligand could be taken into the cell attached to the receptor and where it would go inside the cell.

We had developed a hypothesis of how the ligand ended up inside the cell. This was based on observation of the development of fluorescence when cells were exposed to the fluorescent ligand. It could be seen to bind first to the plasmalemmal membrane then to intracellular structures and finally, when the ligand was removed, the process reversed, the intracellular fluorescence disappearing first. Our hypothesis was that the ligand binds to the receptor on the external cell surface, then, when the receptor and its surrounding membrane undergo spontaneous endocytosis, the ligand-receptor complex is internalized; furthermore the binding site would now be on the inside of the endocytic vesicle. The vesicles then move inside the cell and fuse with the endoplasmic reticulum; this explains the punctate nature of the fluorescence and how we could see fluorescent structures moving back and forward inside the cells. Subsequently the structures moved back to the cell surface to undergo exocytosis, allowing the ligand to escape and leaving the receptor ready to operate once more in its extracellular location.

The alternative possibility was that the lipophilic ligand entered the cell by diffusing through the plasmalemmal membrane then found and bound to receptors that were already inside the cell. This requires that the ligand penetrates two membranes: the plasmalemmal membrane, then the membrane of the intracellular organelle since the receptors here are inward facing. Spontaneous endocytosis of membrane-bound receptors provides a much simpler explanation.

The key experiment that proved our hypothesis and disproved 'entry by diffusion' was to show that entry of the

ligand into the cell was absent in β-arrestin-deficient cells. β-arrestin is necessary for endocytosis. The β-arrestin-deficient cells had been transfected with α_{1B}-adrenoceptors labeled with GFP, which were visible both inside the cell and on the surface, so if the ligand could enter the cells it would have bound to these intracellular adrenoceptors. However, the extracellularly applied ligand colocalized with GFP-labelled receptors on the cell surface but did not bind to the GFP-labelled receptors inside the cell. This showed that the ligand could not pass the plasmalemmal membrane unless endocytosis of the receptors took place: endocytosis was its only route into the cell. This idea was confirmed by blocking endocytosis with concanavalin A or hyperosmotic sucrose. Importantly, we also showed that the ligand partially colocalized with β-arrestin in recycling and late endosomes, indicating receptor transit without destruction (Pediani *et al.*, 2005).

We went on to follow the time course of internalisation with the fluorescent ligand, showing that it took approximately an hour for all receptors in the cell to equilibrate with the ligand and a similar time to leave the cell when ligand was removed. This provided an insight into the rate of cycling of the receptors and changed our view of the stability of the plasmalemmal membrane receptor population. This is illustrated in simplified cartoon form in Figure 6.

We could not contribute to the argument about whether the subtypes were differently involved in agonist-induced internalisation because our antagonist ligand, at any concentration that is useful for receptor localisation, blocks responses to even high concentrations of phenylephrine (Pediani *et al.*, 2000). However, by labeling the surface receptors with an antibody that only fluoresces once internalised, colleagues were able to show that both α_{1A}- and α_{1B}-subtypes could be internalised after activation by high concentrations of phenylephrine with only a small difference in time course (Flacco *et al.*, 2013; Perez-Aso *et al.*, 2013; Segura *et al.*, 2013).

Locating adrenoceptors in native tissues

Our work on recombinant receptors and then isolated dissociated cells was merely background for our main interest of locating adrenoceptors in native tissues using fluorescent ligands. In addition to our initial expectation that receptors would be seen on the plasmalemmal membranes, it was of interest to know whether the ligands could penetrate cells in native tissues, as was the case in cell cultures. These issues are completely different in fixed and live cells.

For fixed cells or tissues, the problems are similar to those encountered in the immunohistochemical localization of receptors. The ligand or antibody must be able to penetrate to the binding site inside the tissue, to the plasmalemmal membrane and to the intracellular organelles. This can be achieved by chemical treatment to make membranes porous and/or by thin sectioning to expose the insides of the cells. We could achieve this as shown for example by Figure 1a with freeze drying or Figure 1b with fixation of the *in vitro* pressurized vessel (see also Miquel *et al.*, 2005). However, even our early experiments had shown that in live cells, the QAPB ligand could bind to all receptors, as shown by colocalization with GFP-labelled receptors, so we decided to continue using unfixed 'live *in vitro*' tissues.

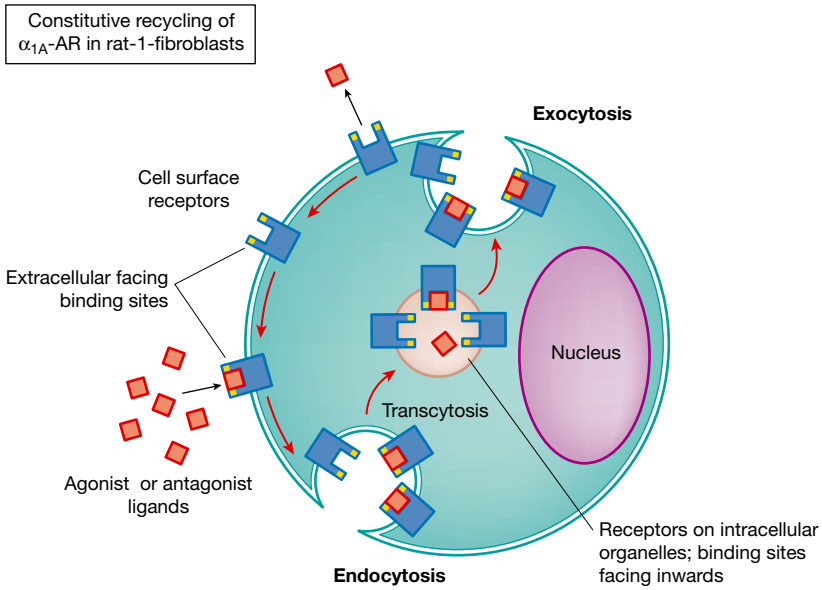

Figure 6

Cartoon of constitutive recycling of α_1-adrenoceptors. Starting from the left, ligands bind to the outward facing recognition site on the receptor and are then taken into the cell by spontaneous endocytosis, becoming trapped inside the intracellular organelles. These organelles then move back to the cell surface where the receptors become re-incorporated into the plasmalemma and the ligands are released back into the extracellular space. This accounts for the punctate nature of the labelling of the intracellular receptors and the reversibility of the binding. Our estimate of the entire cycle is around 1 h and the receptors spend approximately one third of the time at the surface. Principles from Pediani *et al.*, 2005.

Our basis, since no intracellular binding is seen in the absence of β-arrestin, is that, in live cells, the ligand cannot penetrate the cell unless carried in bound to the receptor. Thus, intracellular relocation in live tissue would happen only if receptors start off at the surface, bind ligands and spontaneously internalize, taking the ligand with them. If we are to use the fluorescent ligand to compare the locations and properties of different subtypes of α_1-adrenoceptor, then we need to know whether each subtype has the appropriate properties, that is, located at the cell surface and able to spontaneously internalize, as shown in the recombinant cells (Pediani *et al.*, 2005). The question was now whether the same would occur in native tissues. There was reason to believe, from the literature, that the subtypes might differ, favouring different ratios of surface to intracellular location (Hirasawa *et al.*, 1997; Chalothorn *et al.*, 2002; Hague *et al.*, 2003) or having different tendencies towards spontaneous internalization (Morris *et al.*, 2004; Stanasila, 2008). However, as discussed earlier, these authors found contradictory results not parallel ones.

α_1-adrenoceptor subtypes in vascular smooth muscle

Our work on vessels from a small artery (mesenteric first order) and a large artery (carotid) from wild-type (WT) mice showed images for smooth muscle cells similar to those that we saw earlier in rat mesenteric third-order arteries (Figure 2) and rat aorta (Miquel *et al.*, 2005).

The next questions were which α_1-adrenoceptor subtypes are present and, if more than one subtype, do they differ in tissue or cellular location and do they handle the ligand differently?

To identify the subtypes, we combined two approaches aimed at isolating subtypes: selective ligands and selective receptor subtype knockout. In each case, we sought tissue location microscopically and quantified this from average fluorescence intensity of selected equivalent areas of smooth muscle. First, we identified the receptors to which the ligands bound using subtype-selective pharmacological agents, to prevent binding selectively; secondly, we used receptor knockout strains, to isolate each subtype physically by eliminating the others; subsequently we then combined the two methods for cross validation.

Elimination of subtype binding by knocking out subtypes genetically

We generated mice with three single knockout, three double knockout and the triple knockout of the α_1-adrenoceptor subtypes. The triple KO showed no QAPB fluorescent ligand binding, acting as an excellent negative control. The fluorescent image in the presence of QAPB was, literally, a 'black box'. Compared with the WT, each single KO showed reduced intensity of fluorescence and this was reduced further in each double KO (Figure 7). This was demonstrated quantitatively in images of the smooth muscle layer (Methven *et al.*, 2009a,b).

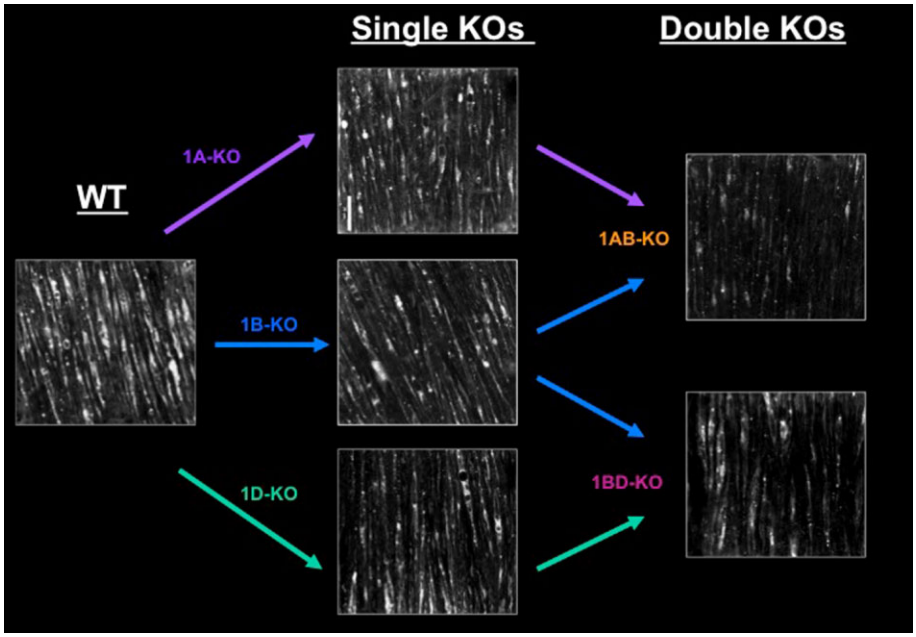

Figure 7

Knocking out subtypes reduces QAPB binding. Images of QAPB binding to smooth muscle layers of first-order mesenteric arteries from WT mouse, the three single knockouts of α_1-adrenoceptor subtypes and the two double knockouts, AB and BD. Note the progressive reduction in fluorescence as first one then two subtypes are eliminated. Based on Methven *et al.*, 2009a,b.

Elimination of subtype binding with antagonists to identify receptor subtypes in smooth muscle of arteries from WT mice

Using recombinant receptors expressed in cell culture, we developed a protocol using selective antagonists to occlude binding of particular subtypes. We would have preferred to have selective antagonists for each of the three subtypes. However, despite trying various putatively selective α_{1B}-adrenoceptor subtype-selective antagonists, we were unable to find one that, in our hands, was selective, using recombinant human α_1-adrenoceptor subtypes. Therefore, we used only an α_{1A}-adrenoceptor selective ligand, RS100 329 and an α_{1D}-adrenoceptor selective ligand, BMY7378. These ligands were chosen to be potent as well as selective as they need to bind more effectively than QAPB at the concentrations employed. We first showed that QAPB binds with similar affinity to all three subtypes of receptor (Mackenzie *et al.*, 2000). Both selective antagonists were then shown to have 100-fold selectivity for their high affinity subtype compared with the other two α_1-adrenoceptor subtypes (Mackenzie *et al.*, 2000). Our criterion for identifying α_{1B}-adrenoceptors was susceptibility of QAPB binding to an α_1-adrenoceptor-selective concentration of prazosin and resistance to the α_2-adrenoceptor antagonist, rauwolscine, coupled with resistance to selective concentrations of RS100 329 and BMY7378.

Having developed the protocol on the known receptor subtypes in culture, we applied it to analyse the unknown receptor subtypes in the smooth muscle of blood vessels. We did this first on smooth muscle cells isolated from prostate and blood vessels (Mackenzie *et al.*, 1998; 1999) then applied it systematically to intact blood vessels.

The results were essentially similar in the two types of artery that we employed: carotid, representing a large conducting artery, and first-order mesenteric, representing a 'resistance' artery. The α_1-adrenoceptor selective antagonist, prazosin, eliminated all fluorescence, whereas equivalent concentrations of the α_2-antagonist, rauwolscine, were ineffective, indicating that all binding was to α_1-adrenoceptors (Fig. 8; Methven *et al.*, 2009a,b; data for the α_{1AD} KO, Methven, unpublished).

Either of the subtype-selective antagonists (RS100 329 and BMY7378.) reduced fluorescence intensity and the two combined had a greater effect than each alone. It was assumed that α_{1B}-adrenoceptors were responsible for the remaining fluorescence that was sensitive to prazosin (Fig. 8).

The simplest explanation for these observations is that all three subtypes are present in arterial smooth muscle. There was no detected difference between the antagonist treatments except for overall fluorescence intensity, that is, there was no evidence for differences in distribution between subtypes. This was not necessarily expected from functional data, since previous work, including our own pharmacological assessment of responses to the α_1-agonist phenylephrine (Daly *et al.*, 2002; Deighan *et al.*, 2005) had shown a tendency

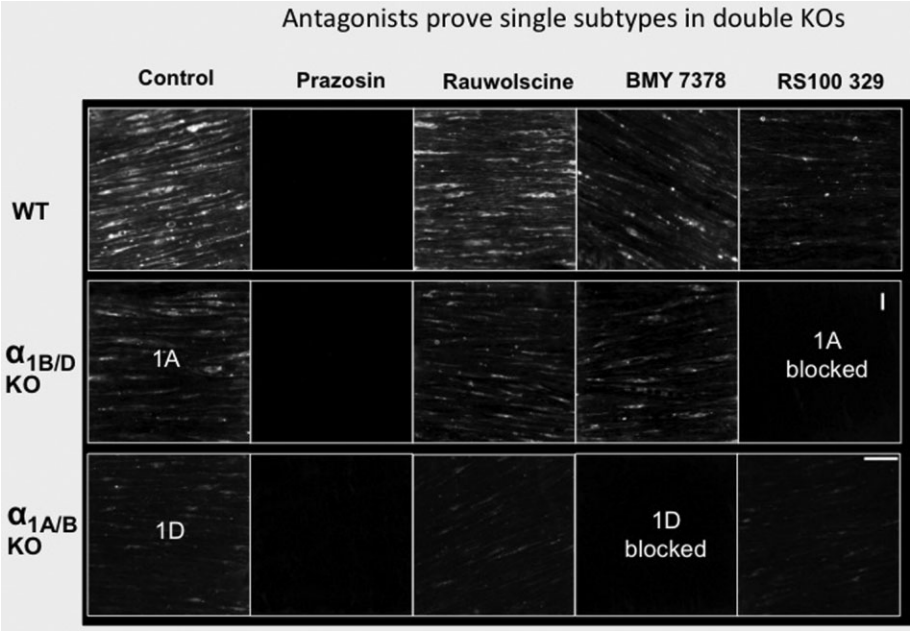

Figure 8

Antagonist drugs identify binding to α_1-adrenoceptor subtypes. Images of QAPB binding to smooth muscle layers of mesenteric arteries from WT mouse and the two double knockouts, BD and AB. Prazosin was effective in blocking all binding and rauwolscine blocked none, validating QAPB binding as α_1- and not α_2-adrenoceptors. The selective α_{1D} antagonist BMY 7378 reduced binding in WT and abolished it in ABKO when only the α_{1D}-adrenoceptor was present. Conversely the α_{1A} antagonist RS100 329 reduced binding in the WT and abolished it in the BD KO when only the α_{1A}-adrenoceptor was present. This validates the selective antagonists and shows that the WT harbours all three subtypes (the α_{1B} being responsible for the prazosin-sensitive but RS and BMY-resistant binding in the WT). Based on Methven *et al.*, 2009a,b.

towards greater involvement of α_{1D}-adrenoceptors in large arteries and of α_{1A}-adrenoceptors in small arteries. However, previous work had indicated that mRNA for all three subtypes could be detected (Hrometz *et al.*, 1999). So we could now confirm this for the protein and show that it was functional up to the point of ligand binding.

α_1-adrenoceptor 'knockouts'

Combining the selective antagonists with the knockouts had the anticipated effects thereby confirming the selectivity of the antagonists, an important finding. In the single or double knockouts, the selective antagonists reduced binding only in the examples where their 'selected' subtype was present, for example, BMY 7378 reduced fluorescence in WT, α_{1A}-adrenoceptor knockout (A KO), α_{1B}-adrenoceptor knockout (B KO) and α_{1A} and α_{1B} receptor knockout (AB KO), but not in α_{1D}-adrenoceptor knockout (D KO) or the other double knockouts (AD KO, BD KO).

The double knockouts provided the opportunity to observe subtype distribution when the subtypes were present individually, as for the recombinant receptors in cell culture, which were shown to have different distributions (Hague *et al.*, 2003). However, we found no differences in the surface or intracellular distribution of ligand binding between cells that expressed the different subtypes. Each was found both on the surface and intracellularly (Fig. 9).

This suggests that the ligand was handled similarly by all subtypes, each of which must be expressed on the cell surface and capable of spontaneous internalization, in order to achieve the distribution at equilibrium that we saw. This indicates that the differential distribution of subtypes seen in model systems does not apply in arterial smooth muscle. Furthermore, internalization of ligand occurred in every native cell type that we investigated: hepatocytes, fibroblasts, endothelial cells, as well as smooth muscle from blood vessels and prostate.

Only one difference in fluorescence distribution was detected visually: in the BD KO, in which only α_{1A}-adrenoceptors were present, fewer cells showed fluorescence (Fig. 10). In other words, the receptor population was more intermittent across the smooth muscle cell population in these animals than when the other two subtypes were expressed alone. This was reinforced by an unpublished observation made earlier: in a mouse strain in which either the α_{1A}-adrenoceptor or the α_{1B}-adrenoceptor was labelled with GFP (Papay *et al.*, 2004; 2006), we had found that α_{1A}-adrenoceptors were expressed intermittently in mesenteric arteries, whereas α_{1B}-adrenoceptors had a faint but more even distribution. We did not publish these data, since we felt that the expression level of both α_{1A}- and α_{1B}-adrenoceptors was too low in arteries to be usefully demonstrated by its fluorescence but, in the light of the intermittent distribution of α_{1A}-adrenoceptors in the BD KO, we now feel that these observations reinforce each other. (In the published work using these α_1-adrenoceptor-GFP fusion mouse strains immunohistochemistry with antibodies to GFP was used to map the α_1-adrenoceptor subtypes in the brain because the GFP fluorescence was insufficient for visualization; Papay *et al.*, 2004; 2006).

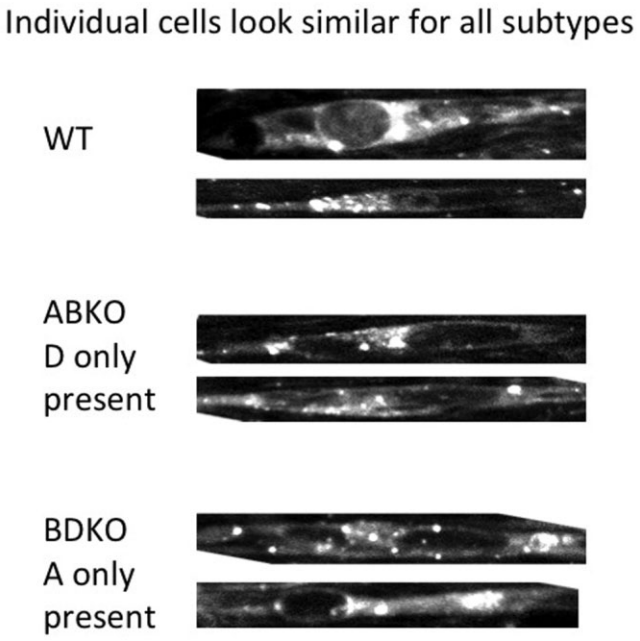

Figure 9
Optical isolation of single smooth muscle cells in mouse carotid arteries shows that QAPB binding looks similar for α_{1A} and α_{1D} subtypes. Based on Methven *et al.*, 2009a,b.

Adrenoceptors on endothelium

The idea that adrenoceptors are present on the endothelium and mediate vasodilator responses is long known (Miller and Vanhoutte, 1985; Angus *et al.*, 1986) but receives little attention in the literature. There is little information about the location of these receptors except for autoradiography, which has rather low resolution (Summers and Molenaar, 1995).

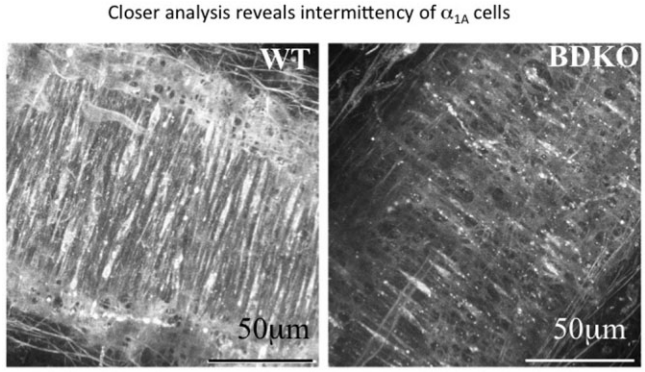

Figure 10
High magnification of smooth muscle in mesenteric arteries from the α_1-BD KO (on right), which has only α_{1A}-adrenoceptors, shows that labelling is intermittent compared with WT (on left). Reproduced from Daly *et al.* (2010).

We first visualized α_{1A}-adrenoceptors on the endothelium inadvertently. We were seeking to locate α_2-adrenoceptors on aortic endothelium in a study of noradrenaline-induced relaxation of mouse aorta. We used the fluorescent ligand QAPB on the basis that it has affinity for α_2-adrenoceptors, albeit at higher concentrations than for α_1-adrenoceptors, and in the mistaken belief that there would be no α_1-adrenoceptors on the endothelium (Shafaroudi *et al.*, 2005). In the event, we found a great deal of binding of QAPB that was blocked by prazosin. We could not identify the endothelial binding site as a single α_1-adrenoceptor subtype but did eliminate it by using aortae from α_{1B}-KO mice (in lieu of an α_{1B}-adrenoceptor antagonist) together with the same α_{1A}- and α_{1D}-adrenoceptor antagonists that we had employed to analyse smooth muscle receptors. In this preparation, we revealed binding to a α_2-adrenoceptors that was blocked by rauwolscine and by genetic elimination of the α_{2A}-adrenoceptor.

We followed this up in more detail and the majority of endothelial cells showed surface binding of QAPB (Figure 11). This reinforces the work of several groups who have shown α_1-adrenoceptor-mediated endothelium derived vasodilatation in the rat mesenteric bed and carotid (Filippi *et al.*, 2001; de Andrade *et al.*, 2006). Thus, the aortic endothelium possesses all three α_1-adrenoceptor subtypes as well as, at least, the α_{2A}-adrenoceptor. All seem to be involved in the release of endothelial relaxant factors.

Note on colour for comparing localization of different things: α-and β-adrenoceptors

In the course of working with fluorescent ligands, it is inevitable that one will seek to compare localization of different cell types or multiple ligands, using specific indicators. Doing this properly requires quantitative image analysis, which is beyond the scope of this review (but see (Daly *et al.*, 2012). To illustrate the conclusions in a simple way, different colours are applied to the images of the different indicators; in modern microscopes, the signal is an intensity level and has no actual colour, so any colours can be chosen.

I am not well equipped for that since, like around 8% of males, I am colour-blind. I use a green laser pointer because the dots from the red ones are not visible to me. [Please bear this in mind next time you choose a pointer for a presentation.] Like most of those impaired in this way, irrespective of their precise colour pathology, my difficulty is with red/green colour pairs. I can see red and green when they are well separated but mixtures of these colours are very difficult to distinguish. A 50/50 mix of red and green provides yellow so there are already three colours to deal with. Irritatingly, the default position of many microscopes is red-green. So, I believe we should follow the advice of Clifford Saper, former Editor-in Chief of the *Journal of Comparative Neurology*, when

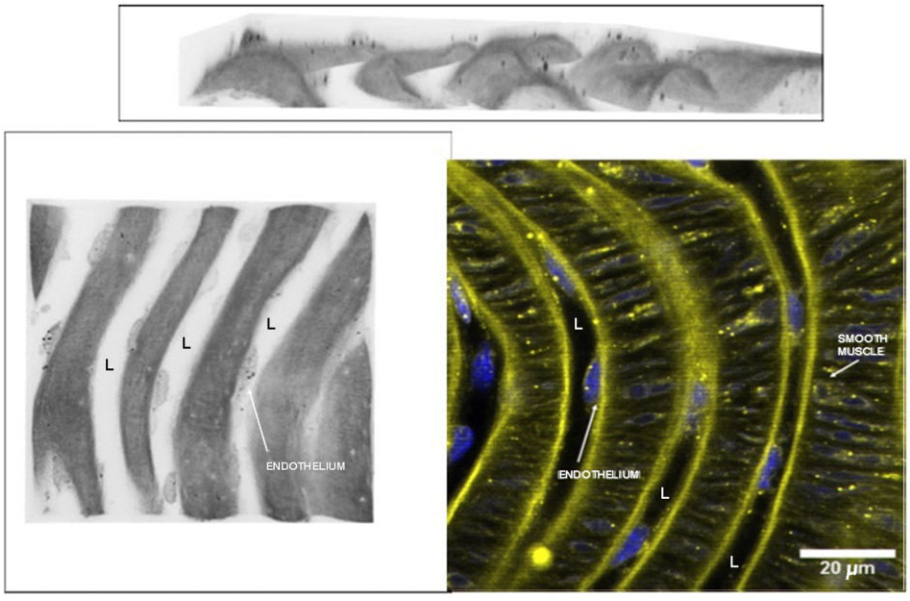

Figure 11

The coloured image in right hand lower panel shows endothelial cells on WT mouse carotid artery attached to folds in the internal elastic lamina (the parallel yellow lines). On the other side of the folds are the cigar-shaped smooth muscle cells: the optical section slices through the folds in the luminal surface showing alternately, lumen (L), endothelium, lamina, smooth muscle, lamina, endothelium, lumen (L), which is then repeated through several folds. There is diffuse binding of QAPB over the surface of endothelial cells and bright yellow spots also near the surface. Cell nuclei are labelled blue, which makes it obvious where the endothelial cells are, and QAPB binding is yellow. The black and white images are 3D reconstructions as if the luminal surface is viewed from inside the lumen (L) at right angles (left) or at an oblique angle (top). These are negative images where the ligand appears as black dots. The endothelial cells appear as objects outside the smooth folds of the intimal elastic lamina (dark grey) which, in contrast to the coloured section, covers up the smooth muscle layer. Images courtesy of Laura Methven.

presenting such images. He recommends magenta and green rather than red/green. Another possibility is blue/yellow (Saper, 2007). For both magenta/green and blue/yellow, the 50/50 mix is white, which is easy to detect against the usually black background.

An example of a vascular image showing the separate localization of α- and β-adrenoceptors is shown in Figure 12 (from Daly *et al.*, 2010). This is from mesenteric arteries from the BD KO mouse where the α_{1A}-adrenoceptors have an intermittent distribution among the smooth muscle cells. This allows us to see that β-adrenoceptors were located in many cells that did not contain α_{1A}-adrenoceptors and *vice versa*. This is very interesting from the point of view of understanding how α- and β-adrenoceptors interact since this would be quite different according to whether they are in the same cell or not. This example shows very limited colocalization, that is, where the two indicators are found in the same pixels of the image at high enough levels to produce the intermediate colour. A few yellow cells in the red/green image and white cells in the other two colour combinations show the small proportion where colocalization could be claimed.

This is a good place to end this review of the evidence for localization of adrenoceptors since the pictures in Figure 12 illustrate the remarkable and, as yet, unexplained, finding that α_{1A}- and β-adrenoceptors populate different cells in mesenteric arteries. This is all the more remarkable because α_{1A}-adrenoceptors are the ones that are the least disputably involved in neurovascular transmission, yet they are present in only a minority of smooth muscle cells. The presence of intercellular gap junctions is essential for the coordinated function of the smooth muscle syncytium.

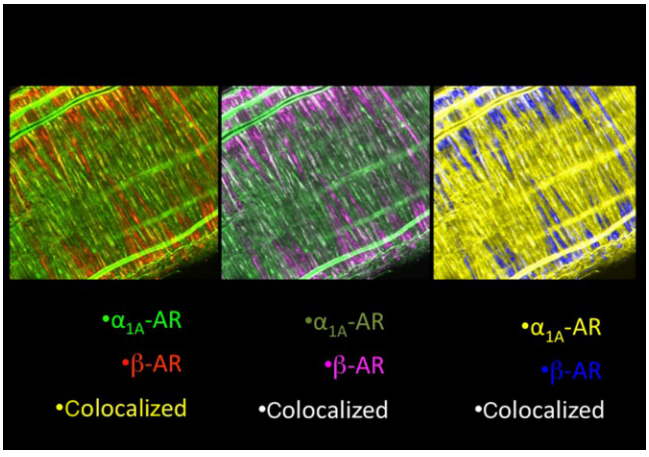

Figure 12

Effect of colours chosen for comparing the distribution of ligands for α_1-adrenoceptors (QAPB) and β-adrenoceptors (TMR-CGP12177) (Baker *et al.*, 2003). Smooth muscle layers of mesenteric arteries from the α_1-BD KO, which has only α_{1A}-adrenoceptors (as in Figure 10) that are not present in all cells. From left to right, the colour pairs are for α-(QAPB)/β-(TMR), respectively, the conventional green/red (left), the recommended green/magenta (centre) and yellow/blue (right). In the left image, colocalization shows up as yellow and in the other images as white. The colour-blind author finds it easiest to see the distinction between the two ligands in the yellow/blue image but the colocalization is clearer in the magenta/green image. Modified from Daly *et al.* (2010). The conclusion of this illustration is very straightforward: in this example, α- and β-adrenoceptors are present in different cells with only a few cells where the two receptor types are present.

Current understanding of the relationship between the presence of α_1-adrenoceptor subtypes and physiological function in blood vessels

Over many years, estimates of the presence of the three α_1-adrenoceptor subtypes in blood vessels have been made by a variety of means (mRNA, Western blot and immunohistochemistry for the protein) and attempts made to correlate this with roles for the individual subtypes in vascular regulation (Piascik *et al.*, 1997; Yang *et al.*, 1997; McCune *et al.*, 2000; Chalothorn *et al.*, 2002). The usual assumption has been that these receptors are expressed on vascular smooth muscle (though mRNA detection and Western blot do not localize this) and that they will mediate vasoconstriction. In general, the presence of all three subtypes has been predicted whatever the method used. Functional responses, usually smooth muscle constriction to catecholamines or agonist surrogates, have been analysed pharmacologically and a diversity of results has been found, normally explained as 'mainly' through one subtype, with possible contributions from others.

The advances from our work are that we can demonstrate.

1. Receptors are present on several cell types in arteries, not only smooth muscle cells, but also adventitial cells of several types, nerves and probably Schwann cells, and endothelial cells.
2. All three receptor subtypes are capable of binding ligands at the cell surface, strongly indicating that they are capable of function and not merely present.
3. All three receptor subtypes can carry the antagonist ligand into the intracellular compartments to which endocytosing receptors move.
4. All of these arterial cell types that express the α_1-adrenoceptors can carry the antagonist ligand into the intracellular compartments to which endocytosed receptors move.
5. Each individual subtype is capable of existing at the cell surface and intracellularly in the absence of the other subtypes and does not require an association with another subtype as has been suggested in some heterologous expression systems.

Unquestionably, several differences between the subtypes have been observed in model systems in relation to spontaneous or agonist-induced internalization or receptor disposition. Yet, the quantitative aspects have not been consistent between laboratories. Our work shows more similarities than differences between the subtypes in cellular disposition and receptor mobility in several native cell types in the arterial wall. Internalization of the ligand occurred in adventitial fibroblasts, hepatocytes and vascular endothelial cells as well as in smooth muscle of blood vessels and prostate. We suggest

that such properties are held in common among the subtypes and, although they may be capable of being differentially regulated, this does not seem to occur in the cell types that we have investigated.

It was a notable feature that when the intensity of the fluorescent ligand was quantified in smooth muscle cells, the 'amount' and hence the density of receptors seemed to be consistent for each subtype and independent of the others. For example, the level of fluorescence to which each selective antagonist reduced the total was very similar to the level found when the high affinity receptor for that antagonist was eliminated genetically, for example, WT vascular smooth muscle cells after BMY7378, and those from an α_{1D}-KO had similar fluorescence levels. This contrasted with liver where the WT mouse has only α_{1B}-adrenoceptors but the α_{1B}-KO expresses α_{1A}-adrenoceptors, which are not detectable until a mature adult stage of 4 months old, indicating a delayed compensation (Deighan *et al.*, 2004). In contrast to liver, vascular smooth muscle does not exhibit up-regulation of α_1-adrenoceptor subtypes in response to the absence of other subtypes. All our experiments have been conducted in 4 month old (mature) mice, unlike many other studies in the literature.

The next question is whether the presence of a subtype is a predictor of its role in vascular function. Our functional data concur with the literature that α_{1D}-adrenoceptors are 'dominant' in the carotid artery, whereas α_{1A}-adrenoceptors play a more 'minor' role; conversely, α_{1A}-adrenoceptors dominate in the mesenteric artery, and α_{1D} receptors play a more 'minor' role. None of this functional evidence correlates directly with the demonstrable expression *per se* of all three subtypes in each artery, that is, there is no evidence of 'more' receptors of the subtype that plays the 'major' role. The inescapable conclusion is that the presence of a subtype in an artery is not a predictor of its contractile function *per se*. There are presumably other regulatory factors that determine the contribution of the various subtypes.

Furthermore, the presence of all three subtypes on cell types other than smooth muscle serves to complicate matters further, since we cannot yet predict the 'functional' roles of the subtypes in these cells and, therefore, how they might interfere with the contractile function of the smooth muscle, or indeed, any other functions in the vessel wall.

So much remains to be done.

Note on terminology for receptors activated by the catecholamines, adrenaline and noradrenaline

The official approach to receptor terminology, laid out by IUPHAR (International Union of Basic and Clinical Pharmacology), states that 'The receptor should be named after the endogenous agonist, or the appropriate collective term when a family of related substances may interact with the receptor.' (Vanhoutte *et al.*, 1996). On this basis, the term should be either 'noradrenaline receptor', reflecting the predominant endogenous agonist or 'adrenaline receptor', reflecting history and the gene symbol (ADRA1A, ADRB2 and so on). Alternatively, and in line with the official definition, 'catecholamine receptor' recognizes both endogenous agonists. Indeed, 'adrenaline receptor' was employed by many distinguished pharmacologists up until the early 1960s (e.g. Dale, 1943; Ing, 1943; Tickner, 1951; Fleckenstein, 1952; McDougal and West, 1953; Stafford, 1963).

So, how did it all get so confused that three alternative names (adrenoceptor, adrenoreceptor, adrenergic receptor) are now used, none of them corresponding to these logical names?

The concept of receptors *per se* is usually attributed to J.N. Langley, who suggested that nerves operated to contract muscle through a receptive substance, 'some substance which is not the actual contractile molecule though capable of acting upon it' (Langley, 1905; 1907).

The next development of a naming system for receptors came when Ahlquist (1948) established the idea that there was more than one type of receptor for adrenaline/noradrenaline. He hypothesized that there were two receptors, which he termed α- and β- adrenotropic receptors. 'Adrenotropic', with a suffix more correctly used for 'substances releasing other substances' was never employed again, even by Ahlquist, who employed all the subsequent variations in his later papers. However, the idea of multiple receptor types was taken up critically by Furchgott (1959). He suggested that two receptor types were inadequate and that at least four receptors were needed to explain existing data on the responses to exogenous catecholamines.

Going with Ahlquist's methodology based on relative potency of different agonists, and Greek prefixes, Furchgott suggested α, β, γ, δ; but this did not survive either. What did catch on were his suggestions for the nomenclature for the receptor family, which he based broadly on previous terms for physiological phenomena. Dale had introduced the idea that autonomic sympathetic nerves should be called 'adrenergic', meaning 'works by release of adrenaline' (Dale, 1935) and Langley had coined 'receptive substances' (see above). Furchgott now suggested two terms for the receptors, viz. 'adrenergic receptors' (from Dale's 'adrenergic nerves') and 'adrenoceptive sites' (from Langley's 'receptive substances'). The latter mutated into 'adrenoreceptors' or 'adrenoceptors'. The usage has remained confused ever since.

This journal, the *BJP*, has used 'adrenoceptor' fairly consistently since 1968 and the IUPHAR nomenclature committee uses this term in defining this receptor family from the 1990s up to the time of writing (Bylund *et al.*, 1994; Hieble *et al.*, 1995; Alexander *et al.*, 2013a,b). This is the term I have used in this review. Nevertheless, many authors use the terms 'adrenergic receptors' or 'adrenoreceptors'. Fortunately, most search engines now accept 'adrenergic receptors', 'adrenoceptors' and 'adrenoreceptors' as pseudonyms, so that there seems to be little point in trying to endorse one term against another at this stage. However, it is confusing for students and people entering the field as well as inconsistent with the IUPHAR standard.

Conflict of interest

No conflicts of interest.

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