Vascular Muscarinic Receptors: Pharmacological Characterization in the Bovine Coronary Artery

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ABSTRACT
The goal of this study was to make functional comparisons between muscarinic receptors mediating endothelial-dependent relaxation responses in the rabbit ear artery and receptors mediating endothelial-independent contractile responses. Ring segments of the bovine coronary artery with the endothelium removed proved to be an excellent model for studying the properties of muscarinic receptors mediating vascular smooth muscle contraction. Although endothelial-dependent relaxation responses could be seen with the calcium ionophore A-23187, no relaxation responses to cholinergic agonists were seen in the bovine coronary artery, whether or not the endothelium was present or in the presence or absence of smooth muscle tone. In ring segments of the bovine coronary artery or the rabbit ear artery, the cholinergic agonists, acetylcholine, methacholine and carbachol, proved to be approximately equipotent in evoking contraction or relaxation, respectively. In contrast, the putative M₁ selective agonist McN-A-343 did not produce any effect in either tissue; nor did McN-A-343 have any effect on a perfused rabbit ear artery segment. Measurement of antagonist affinities indicated that the bovine coronary artery muscarinic receptors show low affinity for both pirenzepine (pKᵦ = 6.9) and AF-DX 116 (11-2-[[2-[diethylaminomethyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one) (pKᵦ = 6.3). Pirenzepine affinity was also low in the perfused rabbit ear artery preparation. Values for pirenzepine and AF-DX 116 affinities are quite similar to those reported previously for endothelial-dependent relaxation in the rabbit ear artery, suggesting the conclusion that vascular muscarinic receptors mediating endothelial-dependent relaxation and those mediating contraction have similar properties and can be classified as M₂ receptors based on low affinity for pirenzepine. Furthermore, their low affinity for AF-DX 116 suggests that vascular muscarinic receptors can be distinguished from those in cardiac tissue. Future studies will be necessary using additional muscarinic antagonists to determine whether vascular muscarinic receptors conform to the recently described M₂ subclass.

Although there has been evidence for a number of years suggesting that there may be subtypes of muscarinic receptors (Eglen and Whiting, 1986), it was not until the development of pirenzepine that the concept was widely accepted (Hammer et al., 1980; Hammer and Giachetti, 1982). Thus affinity for pirenzepine is generally considered to define the M₁ (high affinity for pirenzepine) and M₂ (low affinity) subtypes. More recently, development of the cardioselective muscarinic antagonist AF-DX 116 has led to the suggestion that M₂ receptors may be subdivided further into two classes: one with high affinity for AF-DX 116 (cardiac) and one with low affinity (Giachetti et al., 1986; Hammer et al., 1986). Three subtypes of muscarinic receptors have now been proposed; these have been labeled M₁α, M₁β and M₂. They can be differentiated on the basis of affinity for four antagonists: pirenzepine, AF-DX 116, 4-DAMP and dicyclomine (Dooods et al., 1987).

Given the fast moving field of molecular biology, it is not surprising that the existence of muscarinic receptor subtypes has very rapidly been confirmed by cloning and sequencing DNA structures which code the receptor proteins, followed by elucidation of primary structures of the receptor (Bonner et al., 1987; Fukuda et al., 1987; Peralta et al., 1987). Expression of these receptors in cell lines has made it possible to explore the pharmacological properties of each distinct structure, leading to direct correlation between gene and protein structures and pharmacological properties. Porcine cerebral muscarinic receptors (M₁) and porcine cardiac muscarinic receptors (M₂) have been identified by two independent laboratories (Fukuda et al., 1987; Peralta et al., 1987). In another study, four distinct muscarinic receptors have been cloned, three with high affinity for pirenzepine and one with low affinity (Bonner et al., 1987).

Vascular muscarinic effects have also recently gained much attention due to the seminal observation that relaxation responses to cholinergic agonists are mediated by an action on the endothelium to release a vasodilator substance which has
been termed EDRF (Furchgott and Zawadzki, 1980). Recent studies of endothelial-dependent muscarinic receptors in the rabbit ear artery using a functional approach (relaxation response to methacholine) demonstrate a low affinity for pirenzepine, placing these receptors in the M₂ class (Hynes et al., 1986). Use of the cardioselective antagonist AF-DX 116 further demonstrates that the vascular muscarinic receptors responsible for relaxation in the rabbit ear artery are distinct from cardiac M₂ receptors (Duckles et al., 1987).

Although endothelial-dependent relaxation to cholinergic stimulation is predominant in most blood vessels, there are vascular tissues, such as the canine saphenous vein, that respond to cholinergic agonists with a contractile response also mediated by muscarinic receptors (O'Rourke and Vanhoutte, 1987). In the rabbit ear artery, on the other hand, muscarinic M₂ binding sites appear to be located on the smooth muscle cells, although their functional significance remains in doubt, as no contractile response to muscarinic agonists is seen in this tissue, even when endothelial cells are removed (Hynes et al., 1986). Thus, the muscarinic ligand [³H]QNB labels a population of binding sites with low affinity for pirenzepine, and the number of binding sites is not decreased by elimination of the endothelial cell layer.

Therefore the goal of the present study was to make functional comparisons between muscarinic receptors mediating endothelial-dependent relaxation responses (rabbit ear artery) and receptors mediating endothelial-independent contractile responses using subtype selective antagonists. To accomplish this, we have found that, in the bovine coronary artery, endothelial-dependent relaxation responses can be revealed with the calcium ionophore A-23187, but muscarinic contractile responses are entirely dependent on the smooth muscle, and no relaxation response to acetylcholine can be measured. Thus the bovine coronary artery with endothelium removed is an ideal model for exploring the functional properties of muscarinic receptors mediating vascular smooth muscle contraction and making comparisons to receptors mediating endothelial-dependent relaxation.

Methods

Bovine hearts were obtained fresh from the slaughterhouse and placed in ice-cold Krebs’ solution for transport to the laboratory. Segments of epicardial coronary arteries of 0.5 to 1 mm diameter were isolated and cleansed with the aid of a dissecting microscope, placed in Krebs’ solution at room temperature and cut into ring segments of 2 mm length. Adult New Zealand White rabbits (2–3 kg) of either sex were decapitated. Central ear arteries were removed, placed in Krebs’ solution at room temperature and cut into ring segments of 2 mm length.

Ring segments were mounted in tissue baths for measurement of isometric force. Two pieces of platinum wire were passed through the vessel lumen. One wire was connected to a Gould Statham UC2 Universal transducing cell with microecale accessory for isometric recording of changes in force with a potentiometric recorder. The other wire was attached to a moveable plastic support for adjustment of the vessel lumen. One wire was connected to a Gould Statham UC2 Universal transducing cell with microecale accessory for isometric force. Two pieces of platinum wire were passed through the solution at room temperature and cut into ring segments of 2 mm length.

After equilibration for 60 min at 37°C, the bath solution was replaced. Bovine hearts were obtained fresh from the slaughterhouse and placed in ice-cold Krebs’ solution for transport to the laboratory. Segments of epicardial coronary arteries of 0.5 to 1 mm diameter were isolated and cleansed with the aid of a dissecting microscope, placed in Krebs’ solution at room temperature and cut into ring segments of 2 mm length. Adult New Zealand White rabbits (2–3 kg) of either sex were decapitated. Central ear arteries were removed, placed in Krebs’ solution at room temperature and cut into ring segments of 2 mm length.

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After equilibration for 60 min at 37°C, the bath solution was replaced. Tissues were then stretched to the previously determined optimum resting force of 1.2 g for the bovine coronary artery and 1.0 g for the rabbit ear artery. Concentration response curves were obtained by cumulative addition of cholinergic agonists to the tissue bath. In the case of the rabbit ear artery, vessels were precontracted with norepinephrine, 3 x 10⁻⁷ M, as described previously (Hynes et al., 1986). For some studies of the bovine coronary artery, vessels were precontracted with an EC₅₀ concentration of serotonin (5 x 10⁻⁷–10⁻⁶ M). In some cases, endothelium was removed intentionally by running a doubled length of 5-0 silk through the lumen. The integrity of the endothelial layer was assessed histologically using a silver staining method (Abrol et al., 1984). Except when stated otherwise, the bovine coronary artery was stripped of endothelium using this method.

In some experiments a perfused segment of the rabbit ear artery was studied. The proximal 3 to 4 cm length of the central ear artery was cannulated at both ends with PE tubing. To prevent longitudinal displacement, both cannulas were fixed tightly, and the vessels were perfused with Krebs’ solution at a steady rate of 2.5 ml/min by means of a peristaltic pump. The perfusion pressure was monitored with a Statham P23 Db transducer and potentiometric recorder. After an equilibration period of 60 min, tissues were contracted with methoxamine, 2.5 x 10⁻⁴ M, added to the bath. Relaxation responses were tested by bolus administration of cholinergic agonists into the perfusate in volumes of 10 µl. In one series of experiments, dose-response curves to methacholine were determined before and after administration of pirenzepine (3 x 10⁻⁴ M) added to the bath. Control tissues without addition of pirenzepine were tested to determine the shift in the dose-response curve due to time and repeated exposure to the agonist.

In experiments designed to measure antagonist affinity using ring segments, control methacholine concentration-response curves were first determined. This was done in the presence of norepinephrine (3 x 10⁻⁷ M) in the case of the rabbit ear artery. After completion of the concentration-response curve, antagonists were added to the bath and equilibrated for 30 min. This was followed by a second methacholine concentration-response curve. In each experiment, one tissue was used to determine the control shift in the concentration-response curve. In this case no antagonist was added, but two concentration-response curves were measured. For determination of antagonist affinities, Kᵣ values were calculated as described by Tallarida and Jacob (1979) from the agonist dose-ratio (A'/A) in the absence and presence of an antagonist (B) using the equation:

\[ Kᵣ = B/[A'/A] - 1 \]

Inasmuch as several concentrations of antagonist were used in each experiment, the method of plotting described by Arunlakshana and Schild (1959) was also used to determine pA₂ values. Any shifts in the dose-ratio related to length of time in the baths or tissue fatigue were eliminated by dividing the observed dose-ratio in the presence of antagonist by the control dose-ratio to give the corrected dose-ratio (Purchgott, 1972).

The following drugs were used: acetylcholine chloride, carbachol chloride, methacholine chloride, serotonin creatinine sulfate, norepinephrine bitartrate and A-23187 (Sigma Chemical Co., St. Louis, MO); pirenzepine HCl and AF-DX 116 (courtesy of Drs. G. Trummlitz and W. Reuter, Dr. Karl Thomas GmbH, Bibersach an der Riss, Germany) and McN-A-343 (Research Biochemicals, Inc., Wayland, MA).

Drug solutions were made up in distilled water (except as noted below), and volumes of 50 µl were added to the bath. Stock solutions of A-23187 were made in dimethylsulfoxide. AF-DX 116 stock solutions were made in 0.05 N HCl, and stock solutions of norepinephrine were made in 0.001 N HCl.

Results

The bovine coronary artery responds to muscarinic agonists with a concentration-dependent contraction. Figure 1 shows responses in vessels where the endothelial cell layer has been removed. This is in contrast to the rabbit ear artery where cholinergic agonists produce an endothelial-dependent relaxa-
tion, as reported previously (Hynes et al., 1986). In a series of ring segments of the bovine coronary artery, special care was taken to preserve the endothelial cell layer, and preservation of endothelial cells was also confirmed by light microscopy. The functional status of the endothelial layer was confirmed using the calcium ionophore, A-23187. This substance elicited relaxation responses in the presence of smooth muscle tone induced by serotonin, and these relaxation responses were abolished after endothelial damage (fig. 2). However, in the bovine coronary artery, muscarinic agonists always produced contractile responses whether or not the endothelium was present and either in the presence (n = 4; fig. 3) or absence (n = 10; fig. 1) of smooth muscle tone.

Comparisons were made of the relative affinities of a series of cholinergic agonists: acetylcholine, carbachol, methacholine and McN-A-343 in the bovine coronary artery (contraction) and the rabbit ear artery (relaxation) (fig. 1). In both tissues acetylcholine and methacholine were approximately equipotent, with carbachol having the same, or slightly less, potency. McN-A-343 had no effect in either tissue (table 1). In three perfused ear artery segments, McN-A-343 also failed to cause relaxation responses, even though these tissues did completely relax to acetylcholine.

Pirenzepine competitively antagonized muscarinic contractile responses to methacholine in the bovine coronary artery (fig. 4). Schild plot of the data reveals a straight line with a slope of 0.853 and a pA2 of 7.1 (r = 0.976). AF-DX 116 also competitively antagonized contractile responses to methacholine of the bovine coronary artery (fig. 5). In the case of AF-DX 116 the Schild plot slope was 1.2 and pA2 was 6.08 (r = 0.879). In a small series of experiments, affinity of pirenzepine for muscarinic receptors was tested in a perfused rabbit ear artery preparation. Pirenzepine caused a parallel shift in the dose-response curve, and no evidence for a differential effect of

<table>
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<tr>
<th>Agonist</th>
<th>Acetylcholine</th>
<th>Carbachol</th>
<th>Methacholine</th>
<th>McN-A-343</th>
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<tr>
<td>Bovine</td>
<td>6.1 x 10^-7</td>
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<td>5.8 x 10^-7</td>
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<td>Coronary</td>
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<td>Artery</td>
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<tr>
<td>Ear Artery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
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<td>7.0 x 10^-7</td>
<td>1.2</td>
</tr>
<tr>
<td>McN-A-343</td>
<td>NR</td>
<td>NR</td>
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* Based on assigning a value of 1.0 to the potency of acetylcholine.
BOVINE CORONARY ARTERY: PIRENZEPINE

![Graph: Concentration-response curves for Methacholine with Pirenzepine](image)

**Fig. 4.** Effect of pirenzepine on concentration-response curves to methacholine in ring segments of the bovine coronary artery stripped of endothelium. The upper graph illustrates one experiment, with a control tissue and effects of three concentrations of pirenzepine (3 × 10⁻⁷, 3 × 10⁻⁶ and 3 × 10⁻⁵ M). Control #1 represents the mean of control curves in all four tissues; control #2 shows the second curve in the control tissue. The lower graph illustrates a Schild plot for all values obtained with the three concentrations of pirenzepine (n = 15). The log of the dose ratio-1 is plotted as a function of the log of the pirenzepine concentration.

pirenzepine depending on agonist dose could be seen. Using a single pirenzepine concentration (3 × 10⁻⁶ M) a pK<sub>A</sub> value of 6.3 ± 0.12 (n = 6) was determined.

As shown in table 2, comparison of antagonist affinities (as pK<sub>A</sub> values) for the bovine coronary artery and the rabbit ear artery show close correlation for both pirenzepine and AF-DX 116 in these two tissues. This suggests that muscarinic receptors mediating endothelial-dependent relaxation and muscarinic receptors mediating contraction have similar properties, showing low affinity for both pirenzepine and AF-DX 116.

**Discussion**

The bovine coronary artery, as has been described for other vascular smooth muscle preparations (O'Rourke and Vanhoutte, 1987), is a useful tissue for functional studies of muscarinic receptors mediating vascular smooth muscle contraction. We have shown that, in this preparation, although endothelium-mediated relaxation can be induced using a calcium ionophore, the predominant muscarinic response is contraction which is not endothelium dependent. Whether this is due to a lack of cholinergic receptors on the endothelial cells, an inability to induce release of EDRF upon muscarinic receptor activation or an overwhelming smooth muscle-mediated contraction remains to be established. Nevertheless, in this tissue muscarinic receptors producing contraction predominate. Bovine coronary arteries are not unique in having a predominantly contractile response to muscarinic receptor activation. This is also true of pig coronary arteries (Beny et al., 1986; Graser et al., 1986, 1987), although canine coronary arteries do relax to acetylcholine under the same conditions (Beny et al., 1986).

Relative potencies of agonists have frequently been used to infer differences among muscarinic receptors (Goyal and Ratnan, 1978; Eglen and Whiting, 1986). However, differences in receptor reserve among tissues can profoundly alter agonist potencies (Kenakin, 1984), reducing the reliability of this method. For example, although it has been proposed that McN-A-343 is an agonist selective for M₁ receptors, it has also been shown that the affinity of this compound for muscarinic receptors may not differ between tissues with low and high affinity for pirenzepine, suggesting that this agonist is not truly selective (Eglen et al., 1987). Nevertheless, it is most notable that relative potencies of agonists are approximately the same in both the preparations we studied (fig. 1 and table 1). In both the bovine coronary artery and the rabbit ear artery, approximately equal potency of acetylcholine, methacholine and carbacol has been noted. The purported M₂ selective agonist, McN-A-343, produced no effect in either preparation. These findings support the tentative conclusion that muscarinic re-
ceptrons in both tissues are of the M₂ subtype, but can by no means be considered definitive by themselves, for the reasons cited above. Our findings agree in a general way with those reported previously in canine femoral artery rings, demonstrating approximately equal potency of acetylcholine and carbachol and much greater potency than McN-A-343 (Rubanyi et al., 1987). Our results do not agree with those reported previously for endothelium-dependent relaxation of the rabbit thoracic aorta, where acetylcholine was 10-fold more potent than carbachol and 3-fold more potent than metacholine (Purchgott and Cherry, 1984). Reasons for this discrepancy are not clear.

As an alternative approach to defining receptor subtypes, use of antagonist affinities provides a more definitive answer than use of agonist relative potencies. Thus the concept of M₁ and M₂ receptor subtypes rests at this time primarily on affinity for pirenzepine (Brown et al., 1980; Hammer et al., 1980; Hammer and Giachetti, 1982). Studies with the cardiac selective antagonist AF-DX 116 suggest that M₂ receptors need to be subdivided further (Hammer et al., 1986), and three classes of muscarinic receptors have recently been proposed based on both functional and radioligand binding data (Doods et al., 1987).

As we have shown previously for the rabbit ear artery (Hynes et al., 1986; Dukkies et al., 1987), muscarinic receptors in the bovine coronary artery also show low affinity for pirenzepine and low affinity for AF-DX 116. The close similarity in antagonist affinities between muscarinic receptors in the bovine coronary artery and those of the rabbit ear artery (table 2) suggests that these receptors belong in the same subclass of M₂ receptors and are distinct from cardiac muscarinic receptors. This finding of similarity between muscarinic receptors on smooth muscle cells mediating contraction and muscarinic receptors mediating endothelium-dependent relaxation further supports the conclusion of our previous work comparing a functional approach with radioligand binding studies in the rabbit ear artery (Hynes et al., 1986). Because muscarinic binding sites in rabbit ear artery homogenates appeared to represent smooth muscle cell sites, we concluded in that previous study that muscarinic receptors on both endothelium and smooth muscle had similar properties. This conclusion is reinforced by the present study, which relies entirely on a functional approach to compare muscarinic receptors mediating vascular relaxation and contraction.

Given the present data, it is not possible to define clearly the subtype of muscarinic receptor in these vascular tissues based on the recent classification proposed by Doods et al. (1987). Thus further studies using the antagonist dicyclomine and 4-DAMP will have to be carried out to determine if vascular muscarinic receptors can fit into the M₂ category, which has intermediate affinity for pirenzepine and low affinity for AF-DX 116. Although the values for pirenzepine affinity which we have found in both vascular tissues are close to the intermediate affinity seen with radioligand binding studies in the submandibular gland (Doods et al., 1987), values for AF-DX 116 affinity found in the present study do differ somewhat from that found for the submandibular gland. It remains to be seen how closely vascular muscarinic receptors can fit in the classification proposed by Doods et al. (1987).

An effort to summarize current literature to compare antagonist affinity values found by others for vascular tissue does not yield simple answers. Thus a recent study using radioligand binding techniques yields pirenzepine pKᵢ values of 6.8 and 6.97 for the rabbit and dog aorta, respectively (Yamanaka et al., 1986). These values are close to those we have found and can be considered intermediate pirenzepine affinities. However, in the same study much higher pirenzepine affinities were found in the bovine aorta ranging from 7.65 to 8.06, depending on whether media or intima was homogenized. On the other hand, pirenzepine affinity for muscarinic binding sites in homogenates of bovine basilar arteries yielded pirenzepine pKᵢ values of 6.14, with a small population of sites (16%) having higher pirenzepine affinity (Vanderheyden et al., 1986). In homogenates of the bovine splenic vein pirenzepine displaces [³H]QNB binding with a pKᵢ of 6.4, supporting an M₂ classification for these vascular binding sites (Brunner and Kukovetz, 1986). Functional studies are sometimes confounded by the simultaneous activity of muscarinic receptors mediating contraction and relaxation. This may be true, for example, of the rabbit aorta. Much higher pirenzepine affinities (7.8 and 7.5) have been reported for relaxation responses in the rabbit aorta and canine femoral artery, respectively (Eglen and Whiting, 1985).

It has been suggested that muscarinic receptors involved in responses to acetylcholine may be different in the presence of luminal flow, based on potency of the agonists, acetylcholine, carbachol and McN-A-343, and affinities for atropine and pirenzepine (Rubanyi et al., 1987). Thus effects of agents on relaxation of the femoral artery ring and on bioassayed EDRF released from a perfused femoral artery segment have been interpreted to suggest that two distinct muscarinic receptors may be involved in responses of the femoral artery to acetylcholine. For these reasons we studied the perfused rabbit ear artery, but could find no evidence for participation of M₂ receptors in this preparation. Thus McN-A-343 had no effect on this preparation, and values for pirenzepine affinity are in the low range.

Although it has been inferred from a multitude of functional studies that vascular muscarinic receptors mediating relaxation are localized to endothelial cells (Purchgott, 1984), this precept has not been definitively proven. Recent reports using an autoradiographic approach demonstrate a lack of muscarinic binding sites associated with endothelial cells in a variety of blood vessels from several different species (Summers et al., 1987; Stephenson and Summers, 1987). Although [¹²⁵I]substance P binding could be demonstrated over endothelial cells, using either [³H]QNB or [¹²⁵I]QNB no binding sites were localized over endothelial cells, whereas specific muscarinic ligand binding was seen over the smooth muscle cells. These findings were interpreted to suggest that endothelial-dependent cholinergic responses are mediated by muscarinic receptors on smooth muscle cells, with the endothelium playing a permissive effect, possibly involving a mediator communicating from the smooth muscle to the endothelial cell to initiate release of EDRF, which then in turn initiates smooth muscle relaxation (Summers et al., 1987). Although this scheme may seem unduly complex, it is interesting that in previous studies using homogenates of the rabbit ear artery we were unable to demonstrate

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<th>Antagonist</th>
<th>Rabbit Ear Artery</th>
<th>Bovine Coronary Artery</th>
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<tr>
<td>Pirenzepine</td>
<td>6.71 ± 0.07*</td>
<td>6.67 ± 0.05 (15)</td>
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<tr>
<td>AF-DX 116</td>
<td>5.86 ± 0.04*</td>
<td>6.32 ± 0.06 (19)</td>
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* Dukkies et al., 1987.
any muscarinic binding sites on the endothelium, although binding sites were associated with smooth muscle cells (Hyennes et al., 1986). Furthermore, it has been observed that cultured endothelial cells do not have muscarinic binding sites (Peach et al., 1985), although this observation could be due to lability of muscarinic receptors under tissue culture conditions. These intimations of an inadequacy in the prevalent concept which places muscarinic receptors on endothelial cells themselves adds further interest to the close correlation found in the present study between the properties of endothelium-dependent and endothelium-independent muscarinic receptors. However, whether or not endothelium-dependent muscarinic receptors prove to be localized to the endothelium itself, the finding that vascular muscarinic receptors can be differentiated from both M1 and cardiac M2 receptors and the promise of further definition of vascular muscarinic receptor subtypes continues to add to our understanding of the heterogeneity of the cardiovascular system.

Acknowledgments

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References


