British Journal of Pharmacology (1998) 125, 1188–1193
http://www.stockton-press.co.uk/bjp

Functional cross-talk between endothelial muscarinic and α_2-adrenergic receptors in rabbit cerebral arteries

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1 Interactions between two classes of receptors have been observed in several cell lines and preparations. The aim of this work was to assess the impact of simultaneous stimulation of endothelial muscarinic and α_2-adrenergic receptors (α_2-AR) on vascular reactivity.

2 Rabbit middle cerebral arteries were isolated and changes in isometric tension were recorded in the presence of indomethacin.

3 Inhibition of nitric oxide (NO) synthase with N^{ω}-nitro-L-arginine (L-NOARG, 100 μmol l^{-1}) revealed α-AR-dependent contractions. Pre-addition of acetylcholine (ACH, 1 μmol l^{-1}) augmented oxymetazoline (OXY, 10 μmol l^{-1}, α_2-AR agonist)-, but decreased phenylephrine (PE, 10 μmol l^{-1}, α_1-AR agonist)-induced contraction ($P<0.05$). The effects of ACH were endothelium-dependent.

4 Vessels were precontracted with 40 mmol l^{-1} KCl-physiological salt solution (PSS) in the absence of L-NOARG, or PE or OXY in the presence of L-NOARG. In the presence of high external K^+ or PE, ACH induced a potent relaxation ($P<0.05$). In the presence of OXY, however, ACH mediated contraction ($P<0.05$).

5 After pertussis toxin (PTX, inactivator of $G\alpha_{i/o}$ proteins) pre-treatment, α_2-AR-dependent contractions were abolished. Forty mmol l^{-1} KCl-PSS induced contraction was not altered by PTX whereas ACH-induced relaxation was augmented ($P<0.05$).

6 To investigate if endothelin-1 (ET-1) intervened in the endothelium-dependent contractile response to ACH in the presence of OXY-dependent tone, vessels were incubated in the presence of BQ123 (1 μmol l^{-1}), an ET_A receptor antagonist. OXY-mediated tone was not affected by BQ123; however, ACH-induced contraction was reversed to a relaxation ($P<0.05$).

7 These data indicate that activation of endothelial α_2-AR triggers an endothelium-dependent, ET-1 mediated, contraction to ACH. This suggests that activation of α_2-AR affects muscarinic receptor/G protein coupling leading to an opposite biological effect.

Keywords: Cerebral arteries; muscarinic receptors; α_2-adrenergic receptors; endothelium; G proteins; EDHF; endothelin-1

Introduction

Studies using reconstituted receptors and G proteins within phospholipids vesicles have demonstrated the capacity for single receptor subtypes to activate several types of G proteins (Asano *et al*., 1984; Cerione *et al*., 1985, 1986; Kurose *et al*., 1991; Senogles *et al*., 1990). As reconstituted systems are minimalist, these results were viewed with some scepticism as to their pertinence to intact mammalian cell systems, where natural constraints exist. Later, transfection studies with well-defined receptor subtypes unequivocally showed that single receptor subtypes can couple to distinct (or even opposing) distal signalling pathways in different host cell types (Allgeier *et al*., 1994; Herzog *et al*., 1992; Liu & Albert, 1991; Vallar *et al*., 1990). Hence, specificity of signalling through G proteins incorporates complex regulation of effectors in the interactions of hormones with receptors, receptors with G proteins, and G proteins with receptors. Indeed, it has been shown that activation of a second class of receptors could modify the signalling pathway of a first class of receptors within a homogeneous cell population (Milligan, 1993). This is refereed as a functional cross-talk (for review, see Selbie & Hill, 1998). This change in signalling pathway observed in the presence of two hormones activating two classes of receptors has been associated with changes in G protein availability (Milligan, 1993). Similarly, competition at the receptor binding site between multiple hormones and a single receptor that can activate multiple G protein pools (Bylund, 1992) and overexpression of receptors or G proteins (Alblas *et al*., 1993; Cotecchia *et al*., 1990; Jones *et al*., 1991; MacNulty *et al*., 1992) will also modify the selectivity.

Activation of α_2-AR classically inhibits the activity of a family of adenylyl cyclases by interactions with PTX-sensitive $G_{i/o}$ protein α-subunits (Cotecchia *et al*., 1990; Eason *et al*., 1994; Limbird, 1988). Most (but not all) other physiological signalling pathways linked to α_2-AR, including protein kinase C (Aburto *et al*., 1995; Leprêtre & Mironneau, 1995) and phospholipase C (Cotecchia *et al*., 1990) activation, stimulation of L-type calcium channels (Aburto *et al*., 1995; Asada & Lee, 1992; Leprêtre & Mironneau, 1995; Nebigil & Malik, 1992) and inwardly rectified K^+ currents (Liao & Homcy, 1993), also involve PTX-sensitive G proteins. *In vivo*, and in the absence of tone *in vitro*, occupation of α_2-AR induces an increase in arterial blood pressure and a vasoconstriction, respectively, due to the activation of smooth muscle α_2-AR (Aburto *et al*., 1993; Thorin *et al*., 1997). Like muscarinic receptors, α_2-AR are expressed on the endothelium. Cocks & Angus (1983) demonstrated that α_2-AR agonists induce a relaxation of isolated arteries, an effect confirmed by other groups (Bockman *et al*., 1993; Bryan *et al*, 1995; Ohgushi *et al*., 1993; Thorin *et al*., 1998a,b). Furthermore, and most importantly, α_2-AR-dependent relaxation can be seen only in the presence of arterial tone *in vitro*

In rabbit preconstricted cerebral arteries, activation of muscarinic receptors induces an endothelium-dependent

relaxation (Thorin *et al*., 1997). This relaxation is dependent on the release of at least NO and EDHF. Removal of the endothelium abolishes all muscarinic responses, contrary to α_2-AR agonists that induce tone. Thus, α_2-AR and muscarinic receptors functionally co-exist on the endothelium but not smooth muscle cells in this preparation.

The α_2-AR is a receptor in which the large third cytoplasmic loop and short carboxy terminus resemble those found in the muscarinic M_2-cholinoceptors, which are functionally similar to the α_2-AR (Milligan, 1993). Thus, α_2-AR and muscarinic receptors may represent a good model to study functional interactions and cross-talks between receptors and G proteins as seen in cells overexpressing receptors or G proteins. We hypothesized that activation of one class of receptor would modify the individual biological response of the other. We used rabbit isolated cerebral arteries in which variations of reactivity would attest of a change in receptor function. Our data demonstrate that activation of endothelial α_2-AR reverses the muscarinic response to a contraction.

Methods

Isometric recording of tension of isolated microvessels

Rabbits were anaesthetized with intravenous injection of sodium pentobarbitone (65 mg kg^{-1}) and exsanguinated. The brain was harvested and middle cerebral arteries were dissected-out of the surface of the cortex placed in ice-cold PSS containing indomethacin (10 μmol l^{-1}) and of the following composition (mmol l^{-1}): NaCl 130, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 14.9, EDTA 0.026, glucose 10 and aerated with 12% O_2/5% CO_2/83% N_2 (pH 7.4). Segments of 2 mm long were mounted on 20 μm tungsten wires in microvessels myographs (IMF, University of Vermont, VT, U.S.A.) as previously described (Thorin *et al*., 1997). After 1 h stabilization period, arterial segments were challenged with a 40 mmol l^{-1} KCl PSS; after 15 min washout periods, vessels were stretched again and re-challenged with 40 mmol l^{-1} KCl PSS. This sequence was repeated until a stable contractile response was reached (usually, between 2–3 challenges).

The endothelium was removed mechanically by gentle rubbing with a human hair (Thorin *et al*., 1997). The effectiveness of endothelium removal was confirmed by the absence of dilatation to acetylcholine (ACH, 1 μmol l^{-1}) in arteries preconstricted with 40 mmol l^{-1} KCl PSS. To prepare K^+-rich solutions, equimolar amounts of NaCl were replaced with KCl.

In a series of experiments ($n=6$), arterial rings were pretreated with PTX (100 ng ml^{-1}, inactivator $G_{i/o}$ proteins) for 16 h at 4°C. Untreated segments were run in parallel.

ADP-ribosylation assay using PTX

PTX acts readily on G proteins by catalysing the transfer of [^{32}P]-ADP-ribose from [α-^{32}P]-NAD to a cysteine in the α-subunits (absent in α_s). The modified proteins are visualized by autoradiography after separation of polypeptides on SDS gels.

After overnight incubation at 4°C with PTX, membranes were prepared as previously described (Shreeve *et al*., 1996). Ten μl of H_2O and 10 μl of a solution containing 8 mg ml^{-1} of dimyristoylphosphatidylcholine to 5 μg of proteins diluted in 40 ml of a solution containing (mmol l^{-1}) Na-HEPES 20, dithiothreitol 1, EDTA 1, and Lubrol (0.025%) at pH 8.0. Then 5 μl of Tris-HCl (1 mmol l^{-1}) and 100 ng of PTX were added to the sample and the reaction (1 h at 30°C) was started by adding 20 μl of NAD mix [Tris-HCl (20 mmol l^{-1}, pH 8.0, $MgCl_2$ (12 mmol l^{-1}), EDTA (2 mmol l^{-1}), dithiothreitol (6 mmol l^{-1}), thymidine (40 mmol l^{-1}), ATP (2 mmol l^{-1}), NAD (4 μmol l^{-1}), and [α-^{32}P]-NAD (10^6 c.p.m.)]. The reaction was terminated by adding 20 μl of 10% SDS/1 mmol l^{-1} dithiothreitol. Samples were then separated on an SDS gel as previously described (Shreeve *et al*., 1996) and visualized by autoradiography.

Chemicals

The drugs used were: acetylcholine, ATP, EDTA, dithiothreitol, thymidine, indomethacin, HEPES, NAD, N^{ω}-nitro-L-arginine, oxymetazoline, pertussis toxin, phenylephrine, sodium nitroprusside (Sigma), BQ123 (American Peptide Company). For reactivity studies, all drugs were dissolved in PSS except for indomethacin which was dissolved in ethanol; final concentrations of ethanol in the bath were 0.1% (vol/vol). Solutions were prepared freshly every day and kept on ice.

[α-^{32}P]-NAD was purchased from Amersham.

Statistical analysis

Results are expressed as means ± s.e.mean. In all experiments, n equals the number of rabbits. Vasoconstrictions are expressed as per cent of the maximal response (E_{max}) obtained in the presence of 127 mmol l^{-1} KCl PSS at the end of each individual experiment; vasorelaxations are expressed as the per cent inhibition of the preconstricting tone. Statistical differences between means were determined by analysis of variance followed by a Scheffé's F-test. In appropriate conditions, as unpaired Student's t-test was applied. A probability value <0.05 was accepted as significant for differences between groups of data. All experimental procedures were approved by an ethical committee on animal care and performed in accordance with 'Guide to the Care and Use of Experimental Animals' (Canadian Council on Animal Care publication No. [ISBN] 0-9190087-18-3, Ottawa, Canada, 1993).

Results

In intact vessels ($n=8$), OXY (10 pmol l^{-1} to 30 μmol l^{-1}) induced contraction after nitric oxide (NO) synthase inhibition by L-NOARG. In these conditions, pre-addition of ACH (1 μmol l^{-1}) potentiated α_2-AR-dependent contractions (Figure 1). Removal of the endothelium ($n=6$) abolished the facilitatory effect of ACH on α_2-AR agonist-induced contraction (Figure 1). In the presence of an intact endothelium ($n=8$), ACH (1 μmol l^{-1}) decreased PE-induced contraction (Figure 2); this effect was endothelium-dependent (data not shown).

Vessels were precontracted with 40 mmol l^{-1} KCl-PSS in the absence of L-NOARG ($39\pm5\%E_{max}$, $n=22$), or PE (10 μmol l^{-1}; $35\pm6\%E_{max}$, $n=9$) or OXY (10 μmol l^{-1}; $11\pm2\%E_{max}$, $n=8$) in the presence of L-NOARG. Either in the presence of high external K^+ ($n=10$) or PE ($n=5$), ACH (1 μmol l^{-1}) induced a potent relaxation ($84\pm3\%$ and $85\pm7\%$, respectively). In the presence of OXY ($n=8$), however, ACH mediated contraction ($143\pm10\%$ of the precontracting tone, $P<0.05$).

After PTX pre-treatment ($n=6$), α_2-AR-dependent contractions were abolished. PE-induced contraction was not affected (34 ± 5 and $44\pm4\%$ E_{max} in the absence and in the presence of

PTX). Forty mmol l^{-1} KCl-PSS induced a potent contraction representing 28 ± 3 and $30 \pm 6\% E_{max}$ in the absence and in the presence of PTX, respectively; addition of ACH induced a relaxation ($68 \pm 3\%$) that was augmented ($P < 0.05$) by PTX pre-treatment ($90 \pm 6\%$).

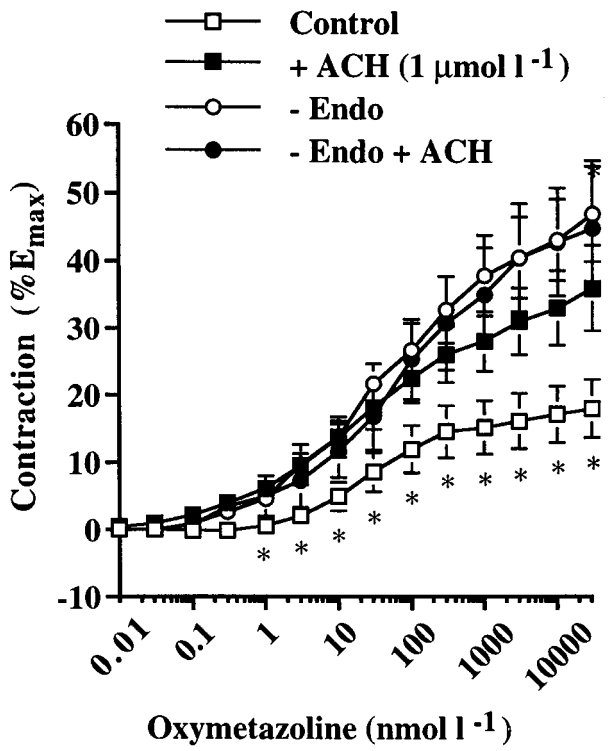

Figure 1 Effect of cumulative addition of oxymetazoline on vascular tone in the absence (Control) or in the presence of acetylcholine (1 μmol l^{-1}). Experiments were performed in the presence or in the absence of the endothelium (−Endo). The physiological salt solution contained L-NOARG (100 μmol l^{-1}) and indomethacin (10 μmol l^{-1}). *$P < 0.05$ compared to other groups. Results are expressed as means ± s.e.mean ($n = 8$, with endothelium; $n = 6$, without endothelium).

As shown by Figure 3, PTX pre-treatment prevented PTX-induced ADP-ribosylation of G proteins confirming that $G_{i/o}$ proteins were inactivated by PTX pre-treatment at 4°C.

To eliminate possible confounding effects of L-NOARG on the interaction between muscarinic and α_2-AR, experiments were performed in the presence of 40 mmol l^{-1} KCl-PSS to induce tone ($51 \pm 5\% E_{max}$, $n = 13$) in the absence of L-NOARG. In these conditions, addition of OXY (10 nmol l^{-1} to 30 μmol l^{-1}) induced a contraction (Figure 4). The maximal contraction ($11.6 \pm 3.9\% E_{max}$) was similar in amplitude to that obtained in the presence of L-NOARG alone ($14.4 \pm 4.3\% E_{max}$, Figure 1). In the presence of high external K^+, ACH (1 μmol l^{-1}) induced a stable relaxation ($71 \pm 4\%$) that lasted for 30 min. Subsequent addition of OXY-induced contractions were augmented ($P < 0.05$) compared to the response obtained in the absence of ACH (Figure 4). Addition of PE (10 nmol l^{-1} to 30 μmol l^{-1}) in the presence of ACH, however, had no contractile effect (data not shown). In another series of experiments and in the presence of 40 mmol l^{-1} KCl-PSS ($59 \pm 4\% E_{max}$, $n = 12$), ACH was replaced by sodium nitroprusside (SNP, 3 μmol l^{-1}); SNP induced a stable relaxation ($61 \pm 5\%$) that lasted for 30 min. OXY, however, had no contractile effect (Figure 4).

To investigate if ET-1 intervened in the endothelium-dependent contractile response to ACH in the presence of OXY-dependent tone, vessels were incubated with BQ123 (1 μmol l^{-1}), an ET_A receptor antagonist ($n = 6$), 20 min before precontraction with high external K^+. After addition of ACH, OXY-induced contraction was decreased ($P < 0.05$) by BQ123 (Figure 4).

In the presence of L-NOARG ($n = 5$), the precontracting tone induced by OXY (10 μmol l^{-1}; $11 \pm 2\% E_{max}$) was not affected by BQ123 ($9 \pm 2\% E_{max}$). However, ACH-induced contraction ($43 \pm 3\% E_{max}$) was reversed to a relaxation ($63 \pm 23\%$, $P < 0.05$) in the presence of ET_A receptor antagonist.

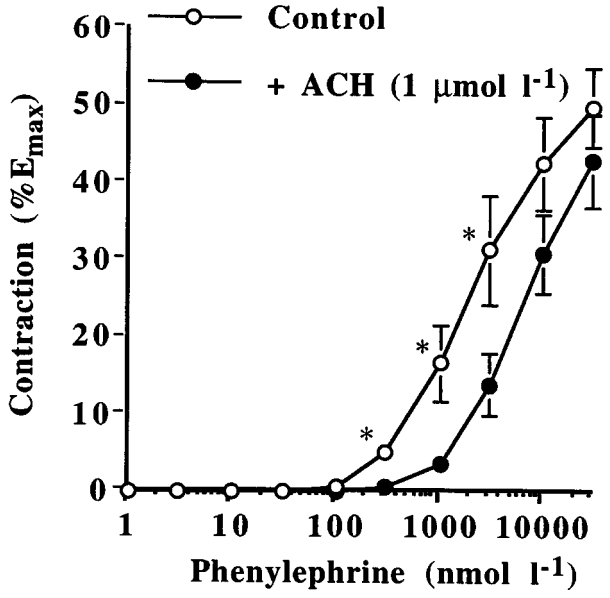

Figure 2 Effect of cumulative addition of phenylephrine on vascular tone in the absence (Control) or in the presence of acetylcholine (1 μmol l^{-1}). Experiments were performed in the presence of L-NOARG (100 μmol l^{-1}) and indomethacin (10 μmol l^{-1}). *$P < 0.05$ compared to ACH. Results are expressed as means ± s.e.mean ($n = 8$).

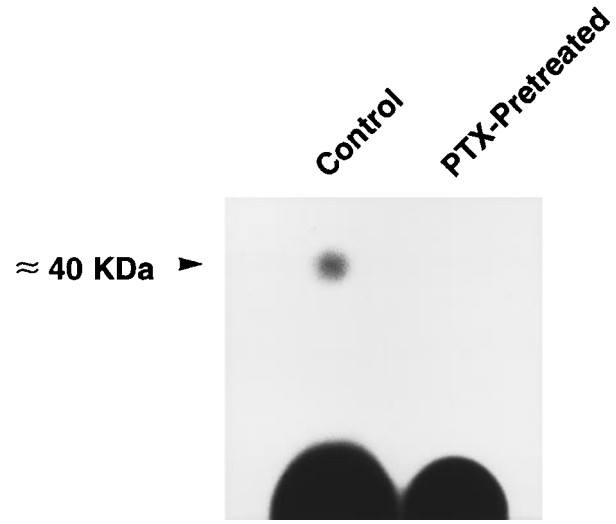

Figure 3 Autoradiogram showing ADP-ribosylation of $G_{i/o}$ proteins (≈42 KDa) by PTX in membranes of control but not PTX pre-treated rabbit middle cerebral arteries. G proteins were separated by 10% SDS–PAGE.

Discussion

The present study was designed to demonstrate the existence and investigate the mechanisms of endothelial receptor cross-talk. α_2-AR and muscarinic receptors were chosen as a model of investigation because of their structural and functional similarities (Milligan, 1993). The results demonstrate that endothelial muscarinic-dependent responses can be inverted by simultaneous α_2-AR activation. Whereas α_2-AR-dependent responses were abolished by PTX pre-treatment, ACH-induced relaxation was augmented suggesting that $G\alpha_{i/o}$ proteins exert a negative effect on muscarinic receptor coupling. The facilitatory effect of ACH on α_2-AR-mediated contraction is endothelium-dependent and associated with a muscarinic receptor-dependent release of ET-1. This latter results demonstrate that muscarinic receptors couple at least to two intracellular pathways leading to two opposite biological effects.

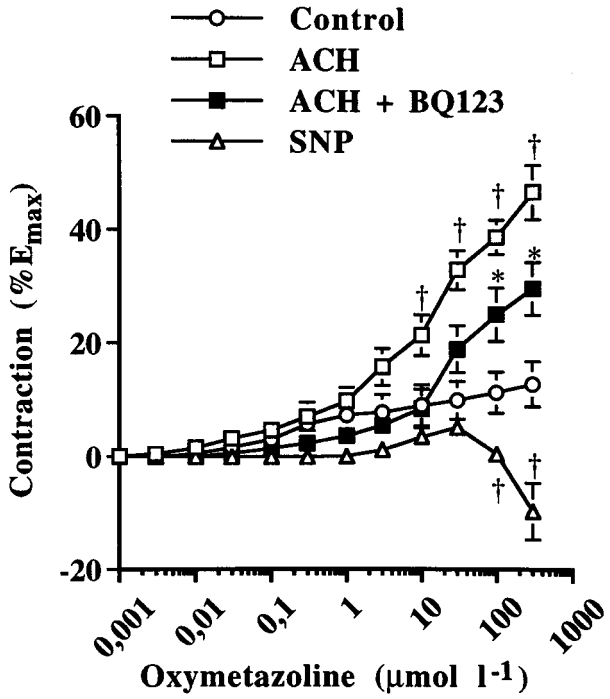

Figure 4 Vascular responses to oxymetazoline of vessels precontracted with 40 mmol l^{-1} KCl-PSS (Control) or after addition of acetylcholine (ACH, 1 μmol l^{-1}, $n=17$) either alone or after 20 min pretreatment with BQ123 (1 μmol l^{-1}, $n=6$), or after addition of sodium nitroprusside (SNP, 3 μmol l^{-1}, $n=12$). Experiments were performed in the presence of indomethacin (10 μmol l^{-1}). *$P<0.05$ *versus* ACH + BQ123; †$P<0.05$ *versus* all groups. Results are expressed as means ± s.e.mean.

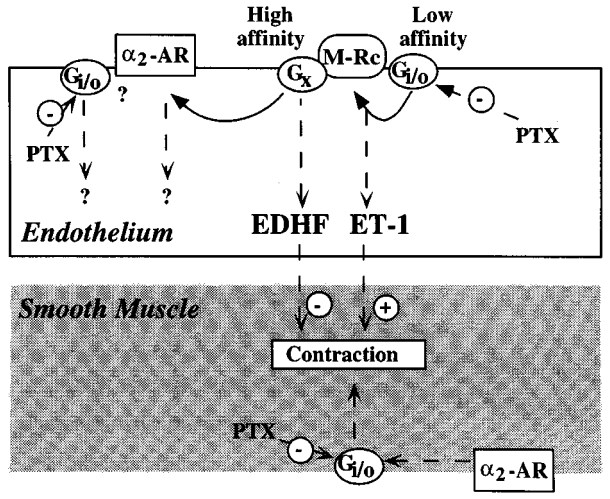

Figure 5 Schematic representation of the proposed model describing the interaction between endothelial muscarinic and α_2-adrenergic receptors.

ACH induces an endothelium-dependent relaxation that is dependent on the release of at least two factors, NO and an EDHF (Thorin *et al.*, 1997). We previously reported that the indomethacin/L-NOARG-resistant relaxation to ACH was prevented by high K^+ in this preparation (Thorin *et al.*, 1997).

α_2-AR stimulation induces contraction after NO synthase inhibition demonstrating the profound NO-dependent inhibitory effect on this contractile response. NO, however, is not the only inhibitory endothelium-derived factor since OXY-induced contractions were further potentiated by endothelial denudation; the near maximal contraction induced by OXY represented 50% of the E_{max} in the absence of endothelium (Figure 2) compared to 15% in the presence of endothelium and L-NOARG (Figure 1). EDHF may also intervene in this effect by preventing OXY-induced contraction as suggested by the contractile response obtained in depolarized condition and in the presence of normal background NO.

Addition of ACH prior OXY challenges potentiated contractions induced by the α_2-AR agonist (Figure 1). This endothelium-dependent effect was selective for the α_2-AR-dependent response since ACH significantly shifted to the right the dose-response curve to PE (Figure 2). These experiments demonstrate the existence of a functional interaction between endothelial muscarinic and α_2-AR.

The endothelial α_2-AR is most likely of the A subtype (Thorin *et al.*, 1997). However, the muscarinic receptor subtype involved remains unknown. Several studies suggest that endothelial M_1 (Komori & Suzuki, 1987; Orphanos & Catravas, 1989), M_2 (Hynes *et al.*, 1986) and M_3 (Duckles & Garcia-Villalon, 1990; Jaiswal *et al.*, 1991) receptors are involved in the effects of ACH in rabbit isolated arteries. ACH-mediated relaxation was PTX-insensitive indicating that M_2 receptors were not involved (Goyal, 1989). Garcia-Villalon and co-workers (1991) reported that M_3 receptors were responsible for ACH-induced relaxation in rabbit cerebral arteries, and this subtype has been shown to stimulate both NO and EDHF release (Wu *et al.*, 1997).

A possible interaction between α_2-AR and muscarinic receptors at the G protein level was investigated. The absence of responsiveness of α_2-AR after PTX treatment confirms the functional coupling of these receptors to $G\alpha_{i/o}$ proteins (see references in Introduction). The effects of PTX on the muscarinic response were more surprising; ACH-induced relaxation was augmented indicating that in normal conditions, $G\alpha_{i/o}$ proteins exert an inhibitory effect on muscarinic receptor coupling. The simplest hypothesis is that a single subtype of endothelial muscarinic receptors couples at least to two different families of G proteins. It has been shown that ACH-mediated endothelium-dependent contraction (from baseline after NO synthase inhibition) or relaxation (in the presence of PE and indomethacin) of aortic rings of spontaneously hypertensive rats was mediated by activation of M_3 receptors (Boulanger *et al.*, 1994). It is however possible that two subtypes of endothelial muscarinic receptors couple to two different G proteins; in the present study, one subtype (M_2?) may couple to $G\alpha_{i/o}$ proteins to induce contraction. PTX would thus favour the relaxant response due to stimulation of

a second endothelial subtype (M_3?) by ACH. It is difficult, however, to reconcile the data obtained with OXY with the theory of two different subtypes of muscarinic receptors. ACH, by stimulating M_2 receptors, should mimic the effect of OXY on M_3 coupling. It is thus most likely that one muscarinic receptor subtype is responsible for both endothelium-dependent responses to ACH as seen in the spontaneously hypertensive rat aorta (Boulanger *et al*., 1994).

The effect of BQ123 suggest that activation of muscarinic receptors, in the presence of an α_2-AR agonist, triggers contraction and relaxation simultaneously. The contraction is ET-1-dependent and overwhelms the EDHF-dependent response. Thus, activation of α_2-AR partially diverted muscarinic receptor coupling towards an intracellular pathway associated with ET-1 release. In the view of the facilitatory effect of PTX on ACH-induced relaxation, it is tempting to speculate that a change in G protein coupling is responsible for this effect. There is, however, an alternative explanation. We recently showed that endothelium-derived NO could inhibit the α_2-AR-dependent relaxant pathway in mice isolated mesenteric arteries (Thorin *et al*., 1998b). It is therefore possible that activation of α_2-AR stimulates the release of an endothelium-derived factor and/or an intracellular pathway that partially inhibits the muscarinic receptor-dependent relaxation. However, neither NO nor EDHF were involved in the cross-talk between α_2-AR and muscarinic receptors because this interaction was present in the presence of normal background NO and in depolarized conditions (Figure 4).

The physiological and pharmacological significance of these results are important. As recently reviewed by Selbie & Hill (1998), cross-talks between G protein-coupled receptors in physiologically relevant systems have been known for several years. These interactions, however, are usually synergistic, meaning that activation of one class of receptors augments responses to a second class of receptors. In this work, however, activation of α_2-AR inverted the muscarinic-dependent response. This is the first demonstration of such an effect in isolated vessels. However, changes in receptor coupling may intervene in pathological conditions. Lerman and coworkers (1995) demonstrated that ACH augmented circulating ET-1 levels in patients with coronary artery disease, a response not observed in healthy patients. These data indicate that atherosclerosis is associated with changes in receptor function and/or coupling. The exact molecular mechanisms associated with these changes remain to be elucidated.

In conclusion, these data suggest that there is a functional interaction between muscarinic receptors and α_2-AR possibly at the level of the G proteins. The hypothesis is schematically presented in Figure 5: α_2-AR activation is associated with recruitment of $G_{i/o}$ proteins, PTX sensitive, and possibly a second class of G proteins (G_x), PTX insensitive. Activated endothelial muscarinic receptors couple to G_x proteins inducing relaxation: we propose that this pool of G_x proteins is shared with endothelial α_2-AR. This creates competition, altering the coupling of the muscarinic receptor that is diverted towards $G_{i/o}$ proteins involved in the contractile response to ACH *via* the release of ET-1. This suggests that muscarinic receptors can couple at least to two different classes of G proteins linked to two different intracellular pathways and leading to opposite biological responses. However, we can neither demonstrate that the same muscarinic receptor can couple to these two different G proteins simultaneously nor that the same or two different muscarinic receptor subtypes are involved in this process.

This work was supported by the Montreal Heart Institute Research Foundation, the Heart & Stroke Foundation of Quebec, the Fonds de la Recherche en Santé du Québec and the Medical Research Council of Canada.

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(Received May 5, 1998
Revised August 24, 1998
Accepted September 1, 1998)