Release of endothelium-derived hyperpolarizing factor (EDHF) by M₃ receptor stimulation in guinea-pig coronary artery

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1 The muscarinic receptor subtype(s) involved in the release of endothelium-derived hyperpolarizing factor (EDHF) were studied in the guinea-pig coronary artery by recording the membrane potential in the smooth muscle cells with intracellular microelectrodes.

2 Acetylcholine (ACh, pD₂ 6.68) was 10 times more potent than the M₂ agonist, oxotremorine (pD₂ 5.65) and 500 fold more potent than the M₁ agonist, McN-A-343 (pD₂ 3.95) in evoking the EDHF hyperpolarization.

3 The M₃ muscarinic antagonist, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) was the most potent (pA₂ 9.5) in inhibiting the release of EDHF evoked by ACh, being more potent than pirenzepine (pA₂ 6.7), and AFDX-116 (pA₂ 6.1) which preferentially block M₁ and M₂ receptors, respectively.

4 These results suggest that EDHF is released from the endothelium of the guinea-pig coronary artery upon the activation of the muscarinic M₃ receptor subtype.

Keywords: Coronary artery; endothelium; hyperpolarization; EDHF; muscarinic receptors; M₃ receptors

Introduction

Stimulation of muscarinic receptors on the endothelium in most vascular beds produces muscle relaxation upon the release of up to three relaxing agents; endothelium-derived relaxing factor (EDRF), identified as nitric oxide (NO), prostacyclin (PGI₂) and endothelium-derived hyperpolarizing factor (EDHF), all of which are also capable of hyperpolarizing the underlying smooth muscle (Parkington *et al.*, 1993). Pharmacological and radioligand binding studies suggest that the muscarinic receptor involved in the release of both NO and PGI₂ is of the M₃ subtype (see review Eglen & Whiting, 1990, Jaiswal & Malik, 1990; Adeagbo & Triggle, 1993). The muscarinic receptor involved in the release of EDHF is still controversial. It has been proposed that EDHF is released upon M₁ receptor stimulation because atropine, a non-specific agonist, was as effective as pirenzepine at inhibiting the hyperpolarization induced by acetylcholine (ACh) but was 100 times more potent at inhibiting the ACh-induced relaxation in rabbit saphenous artery (Komori & Suzuki, 1987b). These same authors (Komori & Suzuki, 1987a) found that oxotremorine did not evoke membrane hyperpolarization in the rabbit saphenous artery, suggesting that M₂ receptors were not involved in initiating the endothelium-dependent hyperpolarization. In another study, atropine was found to be more potent than pirenzepine at inhibiting the ACh-evoked hyperpolarization in the guinea-pig circumflex coronary artery, suggesting that M₁ receptors were not involved in the endothelium-derived hyperpolarization in this artery (Keef & Bowen, 1989). In the perfused rat mesenteric arterial bed, *p*-F-HHSiD (a M₃ receptor antagonist) was more potent than pirenzepine at inhibiting the EDHF-induced, L-NAME- and indomethacin-insensitive dilatation, suggesting that EDHF release occurs through M₃ receptor stimulation (Adeagbo & Triggle, 1993). However, it has been suggested that *p*-F-HHSiD may not be a suitable probe with which to distinguish between M₃ and M₁ receptors with any degree of confidence (Eglen *et al.*, 1990).

The aim of the present study was to clarify the nature of the muscarinic receptor(s) involved in the release of EDHF. The release of EDHF cannot be studied directly since its chemical identity remains elusive. The hyperpolarization it induces is particularly large in the guinea-pig coronary artery which

makes this a suitable tissue in which to investigate this factor. The component of hyperpolarization evoked by EDHF is clearly distinguishable from the components induced by NO and PGI₂, whereas the relaxations merge into one (Parkington *et al.*, 1995). The relationship between the hyperpolarization and relaxation in guinea-pig coronary artery has been studied in detail (Parkington *et al.*, 1995). NO and PGI₂ can produce relaxation in the absence of hyperpolarization. In contrast, the relaxation evoked by EDHF appears to be elicited through hyperpolarization of the smooth muscle cells. Thus, the hyperpolarization response to EDHF was studied in preference to the relaxation because it was clearly separable and closely associated with relaxation.

The responses to three muscarinic agonists; ACh, (non-specific), McN-A-343, M₁ and oxotremorine, M₂ were studied. The effect of three antagonists; pirenzepine, M₁, AFDX-116, M₂ and 4-DAMP, M₃ on the hyperpolarizations induced by ACh were assessed in guinea-pig coronary artery. Some of these results have been presented in abstract form (Hammarström *et al.*, 1993).

Methods

Preparation

Guinea-pigs of either sex, 250–800 g, were killed by decapitation and their hearts removed. The left descending main coronary artery, from the coronary sinus to its entry into the cardiac muscle underneath the pulmonary artery, was dissected free. Ring segments of artery (1–2 mm in length) were threaded with a pair of wires (40 µm in diameter) and fixed in two supports in a myograph. One support was attached to a micrometer screw and the other to a force transducer (Mulvany & Halpern, 1977). Segments were continuously superfused with physiological saline solution (PSS) (mM): NaCl 120, KCl 5, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1, NaHCO₃ 25, glucose 11, bubbled with O₂ : CO₂, 95% : 5% and maintained at 35°C. The flow of PSS was at a rate of 3.6 ml min⁻¹ in the bath the capacity of which was approximately 0.5 ml. The segments were stretched incrementally until their tension was equivalent to a transmural pressure of 60 mmHg, the mean arterial pressure in guinea-pigs. Membrane potential recordings were made with

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conventional glass intracellular microelectrodes filled with 1 M KCl and having resistances of 80–100 M Ω as described by Parkington *et al.* (1993).

Agonist potency (pD_2)

The peak amplitude (in mV) of the transient component of the hyperpolarization, attributable to EDHF, recorded in response to each agent was plotted against the concentrations of agonist. A sigmoid curve was fitted to the concentration-response curves by a least-squares method in the computer software package InPlot (GraphPad Software Inc.). The concentration of agonist which was effective in producing a response that was 50% of maximal hyperpolarization (EC_{50}) was determined from the curve. The EC_{50} and slope of the concentration-response curve (20–80%) obtained for each preparation were used to generate an averaged curve that was representative of all preparations in each group. pD_2 ($-\log EC_{50}$), has been used as a measure of agonist potency throughout this study. The maximum amplitude of the hyperpolarization for each agonist has been designated V_{max} according to convention.

Antagonist potency (pA_2)

The concentration-response curves to ACh were repeated in the presence of three concentrations of each antagonist. Only 1–2 concentrations of antagonist were tested in each preparation. When two concentrations were applied to the same preparation, the antagonist was washed out and a new control concentration-response curve was established before the second antagonist concentration was applied. Antagonists were allowed to equilibrate for 20 min before repeating the concentration-response curve to ACh. The method of Arunlakshana & Schild (1959) was used to determine pA_2 values. The highest concentration of AFDX-116 caused a depolarization of the smooth muscle (5 ± 1 mV, $n=8$). This made the hyperpolarizations appear larger than in the absence of AFDX-116 due to an increase in the driving force for potassium, to which the conductance increases during the hyperpolarization (Bolton *et al.*, 1984). The peak amplitudes of these hyperpolarizations in the presence of high concentrations of AFDX-116 were therefore corrected for these changes in driving force which involved scaling the data according to the Goldman-Hodgkin-Katz equation for a potassium current to give the value expected with an unchanging membrane potential. In these calculations an equilibrium potential for potassium of -90 mV was assumed (Hirst & Edwards, 1989). These calculations had no significant effect on the pD_2 values.

Statistical analysis of data

The mean $\pm$ standard error of the mean (s.e.mean) of responses are quoted throughout. The n values quoted represent the number of preparations studied. Unpaired t tests were used to test and a significance level of $P < 0.05$ was used throughout.

Solutions

Acetylcholine, oxotremorine, McN-A-343, L-NAME, pirenzepine and 4-DAMP were all dissolved in distilled water, to a stock of 10^{-2} M, and diluted to final concentrations in PSS. Indomethacin (10^{-2} M) was dissolved in 0.1 M Na_2CO_3 and diluted in PSS. Preparations were allowed to equilibrate for at least 20 min with L-NAME before and during the time that any agonists were applied. AFDX-116 was dissolved in dimethyl sulphoxide (DMSO). DMSO, 3 : 1000 dilution, had no effect on either membrane potential or on the amplitude of the hyperpolarizations induced by ACh ($n=2$).

Drugs

The following were used: acetylcholine Cl (Merck) (ACh); oxotremorine sesquifumerate salt; 4-[N(3-chlorophenyl)-

carbamoyloxy]-2-butynyltrimethylammonium chloride (McN-A-343); N^o-nitro-L-arginine-methyl-ester (L-NAME); indomethacin and pirenzepine dihydrochloride (all from Sigma), 11-[(2-[(diethylamino)methyl]-1-piperidinyl)acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AFDX-116, a gift from Boehringer Ingelheim), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, Research Biochemicals Inc.); sodium nitroprusside (Ajax Chemicals); 5-[(E)-(1S,5S,6R,7R)-7-hydroxy-6-[(E)-(3S,4RS)-3-hydroxy-4-methyl-1-octen-6-ynyl]-bicyclo[3.3.0]octan-3-ylidene]-pentanoic acid (iloprost, a gift from Schering, Germany). Drugs were applied using two methods: for prolonged exposure, drugs were added to the superfusate. For short exposures, ACh, oxotremorine and McN-A-343 were applied for 10 s by direct injection into the superfusion line with a 1 s delay between the point of injection and the preparation. The concentration of agonist reached a steady state within 3–4 s (see Parkington *et al.*, 1993). Drugs to be injected close to the preparation were dissolved in PSS. Agonists were injected in 5–50 μ l into the perfusion line. The injection of up to 250 μ l of PSS alone had no effect on membrane potential.

Results

One minute and 10 s application of acetylcholine

The resting membrane potential of intact coronary artery preparations, in the absence of NO and prostaglandin synthesis inhibitors, was -59 ± 1 mV ($n=21$). The hyperpolarizations evoked by including ACh in the superfusate for 0.5–1 min, were biphasic and very long in duration, up to 15–20 min in response to 10^{-5} M ACh. This duration was reduced to 1–2 min in the presence of both L-NAME (2×10^{-5} M) and indomethacin (10^{-6} M). The pD_2 for ACh-induced hyperpolarization in the presence of both blockers was 6.86 ± 0.08 ($n=12$) and the V_{max} was 21 ± 1 mV (Figure 1).

Injection of ACh for 10 s evoked a complex hyperpolarization consisting of a transient component followed by a slower, more prolonged component. The resting membrane potential was depolarized significantly to -46 ± 1 mV ($n=44$) in the presence of L-NAME (2×10^{-5} M) and the onset of the slow component of hyperpolarization was very much delayed (confirming Figure 4 of Parkington *et al.*, 1993). In the presence of both L-NAME and indomethacin (10^{-6} M) the resting membrane potential was -45 ± 1 mV $n=37$ and the slow component of the hyperpolarization was abolished. The amplitude of the transient component which remained, and at-

Figure 1 Neither the pD_2 nor the V_{max} were different when acetylcholine (ACh) was applied for 1 min (●, pD_2 6.86 ± 0.08 , V_{max} 21 ± 1 mV, $n=12$) compared with 10 s (○, pD_2 6.68 ± 0.04 , V_{max} 22 ± 1 mV, $n=38$). Thus, a 10 s injection was used for most experiments throughout this study.

tributable to EDHF, was larger due to the depolarization. At the level of the resting potential in control solution, i.e. after correcting for the change in driving force (see Methods), the peak amplitude of the response in L-NAME and indomethacin was not different from the peak amplitude of the transient component of the response in control solution, similar to previous results (Parkington *et al.*, 1993). The pD₂ (6.68 ± 0.08 , $n = 38$) or the V_{max} (22 ± 1 mV) in response to 10 s exposure to ACh, in the presence of L-NAME alone, were not different when compared with 1 min application in the presence of both L-NAME and indomethacin (Figure 1).

Effect of other muscarinic agonists on membrane potential

The hyperpolarizations evoked by oxotremorine and McN-A-343 in control solution were similar in appearance and duration to those induced by ACh (Figure 2a). Due to the good separation between the transient component attributable to EDHF and the slow component in L-NAME alone, only this blocker was used when establishing full concentration-response curves to the agonists. ACh was 10 times more potent (pD₂ 6.68) than oxotremorine (pD₂ 5.65) and 500 times more potent than McN-A-343 (pD₂ 3.95) (Figure 2b). The V_{max} evoked by ACh and oxotremorine were not significantly different. However, the maximum hyperpolarization attributable to EDHF evoked by McN-A-343, up to concentrations of 10^{-3} M, was only half the V_{max} elicited by ACh or oxotremorine (Figure 2b).

Effect of muscarinic antagonists on the EDHF evoked response

The effects of three muscarinic antagonists on the hyperpolarization in response to ACh-evoked release of EDHF from the endothelium were investigated. Three concentrations of each antagonist were applied; pirenzepine ($n = 12$, Figure 3a), AFDX-116 ($n = 11$, Figure 3b) and 4-DAMP ($n = 9$, Figure 3c) all caused concentration-dependent shifts of the hyperpolar-

ization curve in response to ACh to the right. 4-DAMP proved to be the most potent antagonist, pA₂ 9.5, being more potent than pirenzepine, pA₂ 6.8 or AFDX-116, pA₂ 6.1. The V_{max} was progressively reduced by increasing concentrations of pirenzepine (Figure 3a). A reduction of maximal responses by pirenzepine has been observed by others (Garcia-Villalon *et al.*, 1990).

The slope of the Schild plot for each of the antagonists was not different from unity, indicating that they were all acting in a competitive manner (Figure 4).

In addition, the effects of pirenzepine (10^{-6} M) and 4-DAMP (3×10^{-9} M) on the hyperpolarization evoked by longer exposures (0.5–1 min) to ACh were examined. The concentration-response curve in response to 0.5–1 min ex-

Figure 2 Acetylcholine (ACh) oxotremorine and McN-A-343 induced biphasic hyperpolarizations in control solution that were similar in appearance (a). [Responses obtained in separate preparations.] The transient component was isolated by including N^ω-nitro-L-arginine-methyl-ester (L-NAME) in the superfusate and full concentration-response curves were obtained for ACh (○) oxotremorine (●) and McN-A-343 (◇). pD₂ for ACh, 6.68 ± 0.04 ($n = 38$), oxotremorine, 5.65 ± 0.14 ($n = 5$) and McN-A-343, 3.95 ± 0.25 ($n = 5$). The V_{max} for ACh and oxotremorine were not different (22 ± 1 mV, $n = 38$ and 22 ± 2 mV, $n = 5$ respectively), while that of McN-A-343 was significantly less (12 ± 1 mV, $n = 2$).

Figure 3 Full concentration-response curves to acetylcholine (ACh) were obtained in control (○) and in the presence of pirenzepine (3×10^{-7} M ●, 10^{-6} M ◇, and 3×10^{-6} M ◆) (a); AFDX-116 (3×10^{-6} M ●, 10^{-5} M ◇, and 3×10^{-5} M ◆) (b); 4-DAMP (3×10^{-9} M ●, 10^{-8} M ◇, and 3×10^{-8} M ◆) (c). All antagonists shifted the concentration-response curve to the right in a concentration-dependent manner. Data in (b) have been corrected for non-linear summation (see Methods).

posure to ACh was shifted to the right by pirenzepine (Figure 5a(ii)) and 4-DAMP (Figure 5b(ii)) and the shift was not different compared with the shift in response to 10 s applications of ACh (Figure 5a(i),b(i)). The $V_{\max}$ in response to both 10 s and 1 min application of ACh were reduced by pirenzepine (Figure 5a), similar to that described above.

Direct effect of ACh on the smooth muscle

Earlier studies (Bolton *et al.*, 1984; Bény & Brunet, 1987) have shown that cholinergic agonists can have a direct effect on vascular smooth muscle, an action which might contaminate the endothelium-derived hyperpolarizing response observed in the present study. The direct effects of ACh on the smooth

muscle were investigated in 12 preparations of coronary artery denuded of their endothelium. A 1 min application of ACh (10^{-6} – 10^{-4} M) caused only a small depolarization of 3 ± 0.5 mV in 9 of the 12 preparations (data not shown). There was no change in membrane potential in response to ACh in the remaining 3 tissues. To ensure that the removal of the endothelium had not affected the ability of the smooth muscle to hyperpolarize, the smooth muscle was made to hyperpolarize with an endothelium-independent agonist, either sodium nitroprusside (a NO donor) or iloprost (a stable analogue of PGI₂). Thus, the direct effects of ACh on the smooth muscle of the guinea-pig coronary artery appear to be weak, as has been suggested (Parkington *et al.*, 1993), and hence are unlikely to be contaminating the endothelium-derived hyperpolarization described in this study.

Discussion

In this study we have shown that the muscarinic receptor subtype involved in the release of EDHF is likely to be of the M₃ subtype as demonstrated by the ranked potencies of the agonists ACh > oxotremorine >> McN-A-343 and the ranked affinities of the three antagonists, 4-DAMP >> pirenzepine > AFDX-116. These results differ from those reported for rabbit saphenous artery (Komori & Suzuki, 1987b), but are in agreement with observations in the rat mesenteric bed (Adeagbo & Triggle, 1993) and in the guinea-pig circumflex coronary artery (Keef & Bowen, 1989). However, only in the rat mesenteric vascular bed has an antagonist potency, pA_2 , been determined. The M₃ antagonist, *p*-F-HHSD, had a pA_2 8.47 in the rat perfused mesenteric arterial bed whereas pirenzepine in the same concentration-range had no effect. The pA_2 value observed for *p*-F-HHSD is somewhat higher than that obtained by other workers for an M₃ receptor involvement (pA_2 7.7–7.9 in rat aorta, Eglen & Whiting, 1990; pA_2 7.18 in rabbit aorta, Jaiswal *et al.*, 1991).

The high pD_2 values for ACh obtained in the present study are very similar to the potency profile obtained by other workers for an M₃ receptor involvement in the endothelium-dependent relaxation evoked by ACh in rabbit aorta, bovine coronary artery and in the guinea-pig circumflex coronary artery (Choo *et al.*, 1986; Keef & Bowen, 1989; Brunner *et al.*, 1991). In contrast, McN-A-343, an M₁ agonist, had a low potency in the rat aorta (pD_2 4.2, Eglen & Whiting, 1990). Its potency was similarly low in the present study (pD_2 3.95), and it elicited a hyperpolarization the amplitude of which was only half of that evoked by ACh or oxotremorine. This may be explained by a partial agonists action of McN-A-343 in the guinea-pig coronary artery. Oxotremorine was 10 times less potent than ACh (present study) and has not been shown to evoke hyperpolarizations in rabbit saphenous artery (Komori & Suzuki, 1987a). Oxotremorine is more selective for M₂ receptors in the brain (Potter *et al.*, 1984).

According to receptor theory, the dissociation constant for a competitive antagonist acting on an identical drug receptor should be the same regardless of the agonist or preparation used (Kenakin, 1984). Pirenzepine distinguishes between M₁ (pA_2 8.1) and M₃ (pA_2 6.8) receptor subtypes (Hulme *et al.*, 1990). The low affinity for pirenzepine in our study (pD_2 6.8) indicates that the subtype involved in the release of EDHF is not M₁. The potency of AFDX-116 (present study) was also low and within the range of other results published for the endothelium-dependent relaxation (Duckles *et al.*, 1987). The high affinity for 4-DAMP and the low affinity for AFDX-116 are inconsistent with the characteristics of the M₂ receptor identified in other anatomical locations (Eglen & Whiting, 1990). Although there is some variability in the values determined for antagonists using different tissues and techniques, the pA_2 values observed here for pirenzepine, AFDX-116 and 4-DAMP are similar to those found in other arteries (Eglen & Whiting, 1985; Hynes *et al.*, 1986; Choo *et al.*, 1986; Duckles, 1988). In addition, since the Schild slopes for all three an-

Figure 4 Log (dose-ratio – 1) were plotted against the concentration of antagonist 4-DAMP (○), pirenzepine (●), and AFDX-116 (◇) (Arunlakshana & Schild, 1959). 4-DAMP was the most potent (pA_2 9.5) antagonist in inhibiting the release of EDHF while pirenzepine (pA_2 6.8) was slightly more potent than AFDX (pA_2 6.1). The slopes of the Schild plots were not different from unity, (pirenzepine 1.1 ± 0.2 , AFDX-116 0.9 ± 0.1 and 4-DAMP 0.9 ± 0.4) indicating that the antagonism of all three antagonists was of a competitive nature.

Figure 5 The effects of (a) 10^{-6} M pirenzepine (●) and (b) 3×10^{-9} M 4-DAMP (◆) on the hyperpolarizations evoked by 10 s applications (i) were compared with those in response to 0.5–1 min exposures (ii) (control ○ and ◇). The shift of the concentration-response curves caused by the antagonists was not different when longer exposure times were used (dose-ratio 6.8 ± 0.8 ($n=4$) for 1 min compared to 9.0 ± 1.5 ($n=4$) for 10 s and dose-ratio 10.4 ± 3.7 ($n=5$) for 0.5 min compared to 8.4 ± 1.4 ($n=6$) for 10 s).

tagonists did not differ from unity, it is possible to rule out the presence of a heterogeneous receptor population (Kenakin, 1987).

With the recent finding of another two muscarinic subtypes, M₄ and M₅, the possibility that one of these subtypes may be involved in the release of EDHF has to be considered. M₄ receptors in the rabbit lung and NG-108-15 cells (Lazareno *et al.*, 1990) as well as those found for cloned m4 and m5 receptors (Buckley *et al.*, 1989) are pharmacologically distinct from the M₃ receptor subtype. Pirenzepine has been shown to bind to M₄ receptors with relatively high affinity (Peralta *et al.*, 1987; Dörje *et al.*, 1991), and the affinity of AFDX-116 for M₄ receptors was found to be higher than the affinity for M₃ receptors (Dörje *et al.*, 1991). M₅ receptors have so far been identified only in neuronal tissue and their function has yet to be established.

The relationship between the hyperpolarization and relaxation in this tissue has been studied previously in our laboratory (Parkington *et al.*, 1995). The hyperpolarization and relaxation attributable to EDHF appeared to be closely linked. The hyperpolarization always preceded the relaxation. In addition, no relaxation was observed under conditions in which the hyperpolarization was abolished. In contrast, both endogenous and exogenous NO and PGI₂ could both hyperpo-

larize and relax arterial smooth muscle; however, the hyperpolarization was not essential for the relaxation in response to either of these two agents (Parkington *et al.*, 1995). Thus, EDRF/NO and PGI₂ induce relaxation via the second messengers cyclic GMP and cyclic AMP, respectively.

In conclusion, the possibility of independent mechanisms of release of EDHF versus NO and PGI₂ was exciting since it could provide a basis for preferential therapeutic control of endothelium-dependent relaxation. However, the present results demonstrate that the M₃ receptor mediates the release of EDHF. Thus, it is likely that all three endothelium-derived vasodilators are released through stimulation of the same muscarinic receptor subtype in most species and vessels studied to date.

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