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M_3-Muscarinic Receptor Mediates Prejunctional Inhibition of Noradrenaline Release and the Relaxation in Cat Femoral Artery

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Abstract—The aim of the present study was to analyse the muscarinic receptors involved in the vasodilation elicited by acetylcholine (ACh) and the carbachol inhibition of electrically-evoked [^{3}H]noradrenaline (NA) release in cat femoral artery. For this purpose, the following receptor antagonists were used, atropine, pirenzepine (M_1-antagonist), AF-DX 116 (M_2-antagonist) and 4-diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP; M_3-antagonist). The order of potency (pA_2 values) of these drugs at postjunctional level was: atropine (9·7) ≥4-DAMP (9·6) > pirenzepine (7·2) > AF-DX 116 (6·0), and at prejunctional level (pIC50 values) was: 4-DAMP (9·3) > atropine (8·5) > AF-DX 116 (7·1) > pirenzepine (5·9). These findings indicate that the muscarinic receptors mediating the vasodilation induced by ACh and the carbachol inhibition of NA release are of the M_3-subtype.

Muscarinic cholinergic receptors comprise different subtypes. With the aid of the M_1-antagonist pirenzepine, it has been possible to classify muscarinic receptors into M_1- and M_2-subtypes (Hammer & Giachetti 1982; Eglen & Whiting 1986). The M_1-subclass was initially found especially in neuronal tissue and the M_2-subtype in peripheral effector organs and also in the central nervous system (Eglen & Whiting 1986; Mei et al 1989). The latter subtype was demonstrated to be non-homogeneous by using antagonists such as methoctramine (Melchiorre et al 1987) and AF-DX 116 (Giachetti et al 1986; Eglen & Whiting 1986; Micheletti et al 1987) which have elevated selectivity for the cardiac muscarinic receptor (termed M_2-receptors), and 4-diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP) (Eglen & Whiting 1986) which has selectivity for muscarinic receptor subtypes present in glandular or smooth muscle preparations (termed M_3-receptors). Therefore, it is widely accepted that those receptors with elevated affinity for methoctramine and AF-DX 116 are classified as M_2 and those showing elevated affinity for 4-DAMP and hexahydrosiladifenidol as M_3 (Doods et al 1987; Mei et al 1989). In blood vessels, the muscarinic receptors present in the endothelium have a marked functional importance, because they are necessary for cholinergic agonist-induced vasodilatation mediated by the release of relaxant substance(s) (endothelium-dependent relaxing factor EDRF) (Furchgott 1983).

The existence of prejunctional muscarinic receptors producing inhibitory modulation of noradrenaline (NA) release has been described in vascular preparations (Vanhoutte & Levy 1980; Duckles & Kennedy 1982; O'Rourke & Vanhoutte 1987), which appear to be of the M_2-subtype (O'Rourke & Vanhoutte 1987; Remie et al 1990). Yet, there are few studies which have analysed the muscarinic receptor subtype involved in NA release inhibition and relaxant responses elicited by cholinergic agents in the same vascular preparation.

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The aim of the present study was to determine in cat femoral arteries the muscarinic receptor subtypes, present in adrenergic nerve terminals and endothelial cells involved in inhibition of NA release and relaxation evoked by carbachol and acetylcholine (ACh), respectively.

Material and Methods

Cats of either sex, 1·5–4 kg, were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.p.) and killed by exsanguination. Thereafter, the femoral arteries were carefully removed and placed in a Petri dish containing Krebs-Henseleit solution at 4°C. In this medium, the arteries were divided into cylindrical segments of 4 mm in length which were used in the reactivity and tritium release experiments.

Reactivity experiments

For isometric tension recording, each cylindrical segment was set up in an organ bath according to the Nielsen & Owman (1971) method. The organ bath contained 5 mL of Krebs-Henseleit solution at 37°C continuously bubbled with 95% O_2–5% CO_2 (pH 7·4). Two horizontally arranged stainless steel pins, 150 μm diameter, were passed through the lumen of the vascular cylinder. One pin was fixed to the organ bath wall while the other one was connected vertically to a strain gauge for isometric tension recording. The isometric contraction was recorded through a force-displacement transducer (Grass FTO3C) connected to a Grass, model 7D polygraph. The segments were subjected to a tension of 1 g (optimal resting tension), which was readjusted every 15 min during a 120 min equilibration period before the drugs were administered. The vessels were contracted with NA (10^{-5} M) and once the response had reached a stable plateau a cumulative concentration-response curve to ACh was achieved. When the effects of atropine, pirenzepine, AF-

DX 116 and 4-DAMP were tested on the ACh relaxation response, they were added to the bath 20 min before NA.

The endothelium was removed in several segments to test its influence on the ACh response. The absence of endothelium was analysed histologically by the method of Caplan et al (1971).

Tritium release experiments

The cylindrical segments were placed in a nylon net and immersed for 30 min in 10 mL Krebs-Henseleit solution at 37°C continuously gassed with 95% O_2–5% CO_2 (stabilization period). Thereafter, they were incubated for 60 min in 1 mL oxygenated Krebs-Henseleit solution at 37°C containing (±)-[^{3}H]NA (2 μCi mL^{-1}, 2×10^{-7} M, sp. act. 12·8 Ci mmol^{-1}). The arteries were transferred into a superfusion chamber with two parallel platinum electrodes, 0·5 cm apart, connected to a stimulator (Cibertec model CS9, modified to supply the adequate current strength) for field electrical stimulation (200 mA, 0·3 ms, 4 Hz, for 1 min). The arteries were superfused at a rate of 2 mL min^{-1} with oxygenated Krebs-Henseleit solution at 37°C for 100 min during which the basal level of tritium efflux reached steady-state. The superfusate was collected in vials (10 in total) at 30 s intervals. These vials were distributed in the following manner: two before stimulation to determine the basal level of tritium efflux, two during and six after stimulation, covering the period needed to recover the basal levels of tritium efflux. Ready-Protein (Beckman) was added to the vials and the radioactivity measured in a scintillation counter (Beckman LS 2800).

Three electrical stimulation periods (S_1, S_2 and S_3) were applied to the arteries at 30 min intervals. To determine the effect of carbachol on tritium release, carbachol was added 20 min before S_2 and S_3, and when the agonist plus an antagonist was applied (always before S_3), the latter was added 5 min before carbachol). Cocaine (10^{-5} M) and normetanephrine (10^{-5} M) were added to the superfusion fluid after the incubation period to block the neuronal and extraneuronal uptake of [^{3}H]NA.

The stimulation-evoked tritium release was calculated by subtraction of basal tritium release from the evoked release. Thereafter, the ratios S_2/S_1 and S_3/S_1 were calculated to eliminate differences between the arteries; these ratios were not significantly different. The effects of drugs on stimulated efflux were analysed by calculating their effects on these ratios.

Solutions, drugs and statistical evaluation

The composition of Krebs-Henseleit solution (mM) was: NaCl 115, $CaCl_2$ 2·5, KCl 4·6, KH_2PO_4 1·2, $MgSO_4 \cdot 7H_2O$ 1·2, $NaHCO_3$ 25, glucose 11·1, Na_2EDTA 0·01.

Drugs used were: atropine methylbromide, noradrenaline hydrochloride, acetylcholine chloride, carbachol chloride and normetanephrine hydrochloride (Sigma, St Louis, MO, USA), pirenzepine hydrochloride and 11-[(2-[(diethyl-amino)methyl]-1-piperidinyl) acetyl]-5, 11-dihydro-6H-pyrido-[2-3-6][1,4]-benzodiazepine-6-one (AF-DX 116, Boehringer Ingelheim, Germany), 4-diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP, kindly provided by R. B. Barlow, Bristol, UK), (±)-[^{3}H]noradrenaline hydrochloride (New England Nuclear, Boston, MA, USA), cocaine hydrochloride (Depósito de Estupefacientes, Ministerio de Sanidad y Consumo).

Drug solutions were made in distilled water. Stock solutions (10^{-2} M) of drugs were made in distilled water except AF-DX 116 and NA which were made in 0·05 M HCl and in saline (0·9% NaCl)-ascorbic acid (0·01% w/v) solution, respectively. Stock solutions were kept at −20°C; those corresponding to AF-DX 116 and pirenzepine were protected from the light and were used under sodium vapour light. Appropriate dilutions of these solutions were made in distilled water on the day of the experiment. Results are given as means ± s.e.m. Statistical significance was determined by using Student's *t*-test for paired or unpaired experiments; $P < 0{\cdot}05$ was considered to be significant. pA_2 values were calculated by the method of Arunlakshana & Schild (1959). To analyse the action of antagonists for presynaptic muscarinic receptors in cat femoral arteries the pIC50 values (−log antagonist molar concentration causing 50% inhibition of the carbachol-evoked reduction of stimulated NA release) was calculated by least square regression analysis (Remie et al 1990).

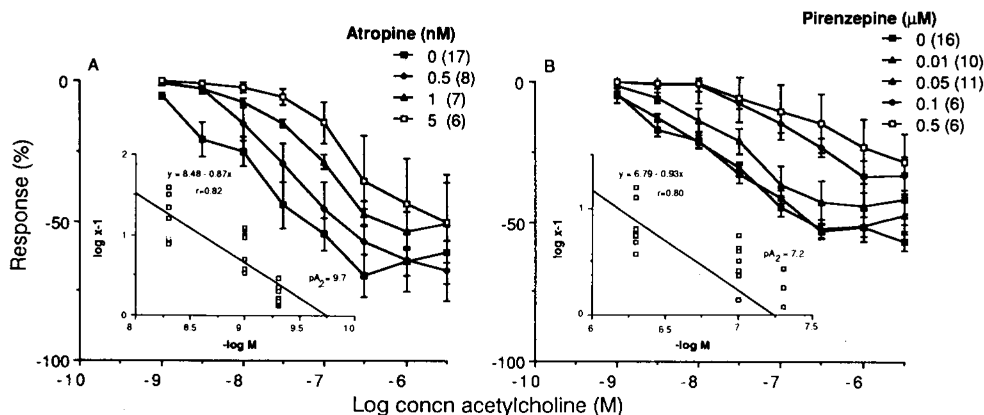

FIG. 1. Effect of atropine (A) and pirenzepine (B) on the concentration-response curve for acetylcholine (ACh) in cat femoral artery segments. Each point represents the mean ± s.e.m. In parentheses, the number of experiments. The insert represents Schild plots for the values obtained with the concentrations of atropine and pirenzepine used. Values are expressed as percentage of the tone elicited by NA (10^{-5} M, 1805 ± 146 mg); r = correlation coefficient of the regression line which is also included in the figure.

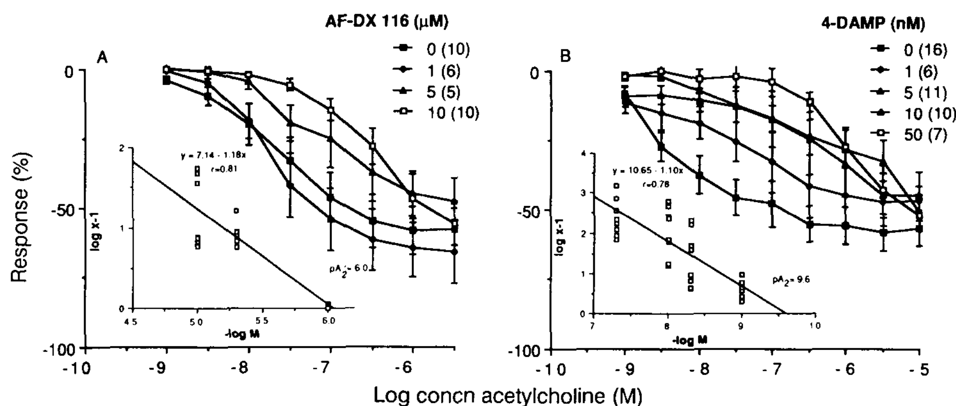

FIG. 2. Effect of AF-DX 116 (A) and 4-DAMP (B) on the concentration-response curve for acetylcholine (ACh) in cat femoral artery segments. Each point represents the mean ± s.e.m. In parentheses the number of experiments. Insert: Schild plots for the values obtained with the concentrations of AF-DX 116 and 4-DAMP used. Expression of results as in Fig. 1.

Results

ACh-induced vasodilator responses

In cat femoral artery segments precontracted with NA (10^{-5} M), ACh induced concentration-dependent vasodilator responses. Endothelium removal abolished this relaxation. The muscarinic antagonists, atropine ($5\times10^{-10}-5\times10^{-9}$ M), pirenzepine ($5\times10^{-8}-10^{-6}$ M) (Fig. 1), AF-DX 116 ($10^{-8}-10^{-7}$ M) and 4-DAMP ($10^{-9}-5\times10^{-8}$ M) (Fig. 2) competitively antagonized the relaxation elicited by ACh; the pA_2 values and the slopes of the Schild plots (which were not significantly different from unity) are shown in Figs 1 and 2.

Atropine and the specific M_3-antagonist 4-DAMP, were equally potent in inhibiting ACh-elicited relaxation ($pA_2=9{\cdot}7$ and 9·6, respectively). Pirenzepine and AF-DX 116 exhibited less affinity for the receptors involved in the relaxant responses; pA_2 values were 7·2 and 6·1, respectively. Therefore, the order of potency was: atropine (9·7) $\geq$ 4-DAMP (9·6) > pirenzepine (7·2) > AF-DX 116 (6·0).

Carbachol-evoked tritium release inhibition

In femoral arteries preincubated with [^{3}H]NA, electrical stimulation induced tritium release. The release obtained in S_2 (95 ± 11) and S_3 (90 ± 15) was less than that observed in S_1 (101 ± 12 counts min^{-1} mg^{-1}) but the ratios S_2/S_1 and S_3/S_1 were not significantly different ($0{\cdot}95\pm0{\cdot}04$ and $0{\cdot}90\pm0{\cdot}04$, respectively). Carbachol (5×10^{-6} M) inhibited about 50% of the tritium secretion elicited by electrical stimulation. This inhibition was reduced in a concentration-dependent way by atropine ($10^{-9}-10^{-8}$ M), pirenzepine ($10^{-7}-5\times10^{-6}$ M), AF-DX 116 (5×10^{-8} and 5×10^{-7} M) and 4-DAMP ($5\times10^{-10}-5\times10^{-9}$ M) with pIC50 values of 8·5, 5·9, 7·1 and 9·3, respectively (Figs 3, 4). The order of potency was: 4-DAMP > atropine > AF-DX 116 > pirenzepine. These

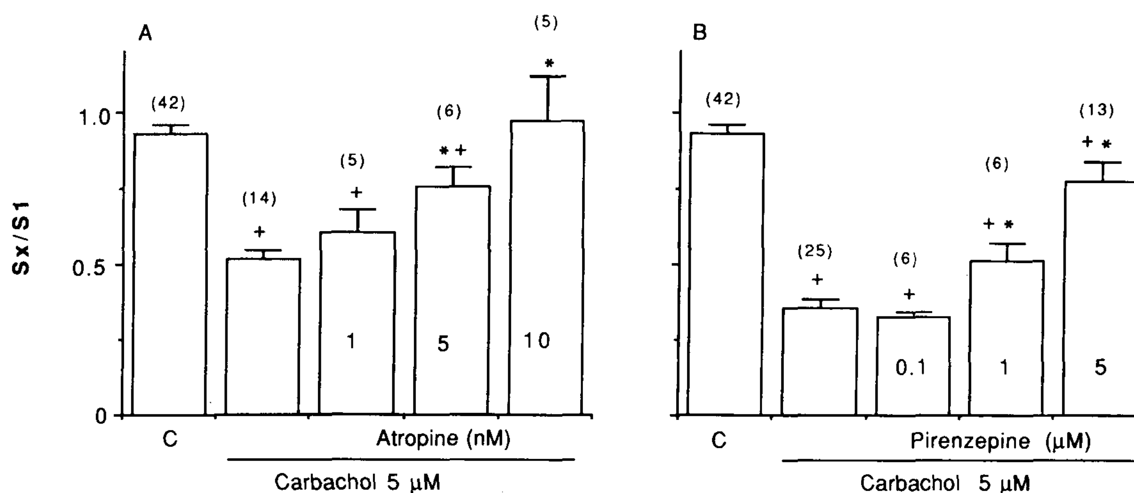

FIG. 3. Effect of atropine (A) and pirenzepine (B) on the inhibition produced by carbachol on the electrically (4 Hz, 0·3 ms, 200 mA for 1 min)-induced tritium efflux from cat femoral arteries preincubated with [^{3}H]NA. The ratios of the tritium efflux induced by three stimulation periods S_x (S_2 or S_3) and S_1 separated by an interval of 30 min are shown. Carbachol was administered 20 min before S_2 and S_3, and the antagonists 5 min before carbachol, always before S_3. The number of experiments is shown in parentheses. The columns and vertical bars represent mean ± s.e.m. * $P<0{\cdot}05$ and † $P<0{\cdot}001$ in comparison with carbachol and control, respectively.

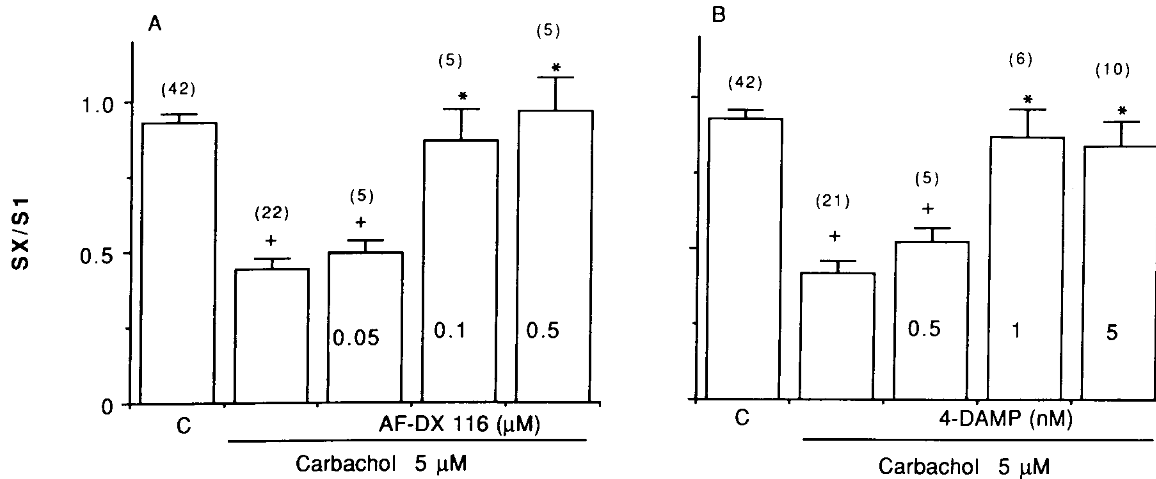

FIG. 4. Effect of carbachol and its inhibition by AF-DX 116 (A) and 4-DAMP (B) on the electrically-induced tritium release from cat femoral arteries preincubated with [^{3}H]NA. The symbols and experimental design as in Fig. 3. $^*P < 0{\cdot}05$, $^+P < 0{\cdot}001$.

concentrations of muscarinic antagonists did not by themselves modify the basal or stimulated tritium release.

Discussion

Postjunctional muscarinic receptors

In the present study, we have analysed the muscarinic receptor subtype involved in ACh-induced relaxation in cat femoral arteries using different antagonists. ACh induced vasodilatory responses in these vessels, as previously observed in other vascular preparations (Edvinsson et al 1977; Furchgott 1983; Eglen & Whiting 1985; O'Rourke & Vanhoutte 1987; Tsukahara et al 1989). The ACh-evoked response was competitively antagonized by atropine with a pA_2 value of 9·7, which is similar to that found in other vessels (9–10·4) (Edvinsson et al 1977; Duckles et al 1987; Van Charldorp & Van Zwieten 1989) indicating that muscarinic receptors are involved in the ACh response. Furthermore, the fact that the relaxation caused by this agent was abolished by endothelium removal, suggests the existence of these receptors in endothelial cells of femoral artery (Furchgott 1983). The existence of muscarinic receptors on the endothelium of human cerebral arteries has been demonstrated by an autoradiographic method using [^{3}H]propylbenzilylcholine mustard, the highest density of these receptors being localized on the luminal surface (Tsukahara et al 1989). However, other authors did not find muscarinic receptors in endothelial cells (Stephenson & Summers 1987).

To analyse the muscarinic receptor subtypes involved in ACh responses, pirenzepine, AF-DX 116 and 4-DAMP, specific antagonists of M_1-, M_2- and M_3-receptors, respectively, were used (Hammer & Giachetti 1982; Eglen & Whiting 1986; Mei et al 1989). The affinity (pA_2) of pirenzepine for the muscarinic receptors involved in ACh-induced relaxation was 7·2. This value is lower than those reported for M_1-receptors ($>8{\cdot}0$) in rat superior cervical ganglion (Brown et al 1980), guinea-pig enteric plexus (North et al 1985) and saphenous vein (O'Rourke & Vanhoutte 1987), higher than its affinity (pA_2 or pK_B) for M_2-receptors (5·6 to 7·0 depending on the species and tissue used) (Eltze 1988; Eglen et al 1989; Fuder et al 1989; Van Charldorp & Van Zwieten 1989) and within the range for M_3-receptors (6·4 to 7·3) (Bognar et al 1989; Fuder et al 1989; Mei et al 1989; Van Charldorp & Van Zwieten 1989). Furthermore, pirenzepine has been found to exhibit intermediate affinity in different vessels in which M_2- or M_3-receptors are present [7·6 rabbit aorta and dog femoral artery (Eglen & Whiting 1985); 6·9 bovine coronary artery (Duckles 1988); 7·3 pig coronary artery (Van Charldorp & Van Zwieten 1989)], which are similar to pA_2 values obtained in cat femoral artery. All these results indicate that the M_1-receptors do not mediate ACh-induced relaxation in femoral arteries.

The ACh responses were inhibited by AF-DX 116 with lower affinity (pA_2 6·1) than pirenzepine. The reported affinities (pA_2 or pK_B) of this agent at M_2-receptors are higher (6·8–7·3) (Giachetti et al 1986; Eltze 1988; Van Charldorp & Van Zwieten 1989; Deighton et al 1990) than at M_1-(6·1, Goyal 1989) and M_3-receptors present in different tissues (6·0–6·4) (Barlow & Shepherd 1986; Micheletti et al 1987; Duckles 1988). These results suggest that M_2-receptors do not mediate the vasodilation elicited by ACh in cat femoral arteries, as reported in rabbit ear artery (Duckles et al 1987) and canine saphenous veins (O'Rourke & Vanhoutte 1987). In pig basilar artery, however, methacholine-induced contraction is mediated by M_2-receptors (pA_2, 7·5 for AF-DX 116) (Van Charldorp & Van Zwieten 1989).

4-DAMP was more potent than pirenzepine and AF-DX 116 at inhibiting ACh-induced relaxation, (pA_2, 9·6, i.e. 315- and 3980-fold higher than values obtained with pirenzepine and AF-DX 116, respectively) indicating that this subtype of muscarinic receptor may mediate this response. The affinities (pA_2 or pK_B) of 4-DAMP at M_3-receptors is about 9 (Batink et al 1987; Roffel et al 1988; Fuder et al 1989; Van Charldorp & Van Zwieten 1989; Eglen & Whiting 1990). Nevertheless, the potency of this antagonist was about 10-fold less at the level of the M_2-receptor, determined by functional (8·0 at prejunctional site in the rabbit iris (Fuder et al 1989); 7·9 guinea-pig atria (Barlow & Shepherd 1986); 7·7 rat left atria (Batink et al 1987); 7·5–7·9 see the review (Mei et al 1989)) and by binding studies (Doods et al 1987; Batink et al 1987;

Roffel et al 1988). The 4-DAMP selectivity for M_1-receptors has been determined by binding experiments in several preparations. The reported results indicate that this antagonist does not markedly discriminate between M_1- and M_3-receptors because the affinities (pK_i values) are similar (M_1-receptors: 8·7 (Doods et al 1987); 9·0 (Michel & Whiting 1988); M_3-receptors: 9 (Michel & Whiting 1988); 8·5 (Batink et al 1987; Doods et al 1987)). These data together with the high potency values of 4-DAMP and the low potency values of both pirenzepine and AF-DX 116, for inhibition of ACh-induced relaxation, permits us to conclude that the receptors involved are of the M_3-subtype.

Prejunctional muscarinic receptors

Our experiments show that electrical stimulation-induced tritium release in femoral arteries was reduced by the muscarinic agonist carbachol; this effect was antagonized by the non-specific blocker atropine. Such a finding suggests the existence of prejunctional muscarinic receptors in these arteries, as described for other vascular preparations (Edvinsson et al 1977; Vanhoutte & Levy 1980; Duckles & Kennedy 1982; O'Rourke & Vanhoutte 1987).

To compare the potencies of muscarinic antagonists in inhibiting the action of carbachol we have used the pIC50 values reported by other authors employing a similar experimental approach (Remie et al 1990). The order of potency was: 4-DAMP (9·3) > atropine (8·5) > AF-DX 116 (7·1) > pirenzepine (5·9). The elevated potency of 4-DAMP (> 9) compared with the low potency of pirenzepine indicates that the receptors involved in the carbachol response are of the M_3-subtype.

Methacholine inhibits the electrically-evoked endogenous NA release mediated by prejunctional M_2-receptors in the rat portal vein; the rank order of potency (pIC50 values) of different antagonists to block this methacholine action was: 4-DAMP (8·5) > AF-DX 116 (8·0) > pirenzepine (7·0) (Remie et al 1990). Similar prejunctional M_2-receptors modulating NA release were obtained in rabbit iris (Bognar et al 1989) and canine saphenous vein (O'Rourke & Vanhoutte 1987).

The muscarinic antagonist concentrations employed in this study failed to modify either the basal or the stimulated tritium release. This suggests that the prejunctional activity of M_2-receptors in resting conditions is low, and their contribution to the modulation of NA release in this situation is minimal.

In conclusion, the pre- and postjunctional muscarinic receptors on cat femoral arteries involved in NA release inhibition by carbachol and the vasodilation induced by ACh appear to be of the M_3-subtype.

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