Presynaptic muscarinic receptor subtypes involved in the inhibition of acetylcholine and noradrenaline release in bovine cerebral arteries

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Summary. Experiments were performed in bovine cerebral arteries preincubated with [³H]-choline or [³H]-noradrenaline to analyze the presynaptic muscarinic receptors involved in inhibition of acetylcholine and noradrenaline release induced by electrical stimulation (4 Hz, 200 mA, 0.3 ms, 1 min). For this purpose, the actions of several muscarinic receptor antagonists on the [³H] overflow and on the carbacol-induced inhibition of this overflow were assessed. The evoked [³H]-acetylcholine release and [³H]-noradrenaline release were markedly reduced by the presence of tetrodotoxin, Ca²⁺-free medium, and the inhibitor of both choline transport and choline acetyltransferase, AF64A. Chemical sympathetic denervation with 6-hydroxydopamine (6-OHDA) decreased the uptake of [³H]-noradrenaline, and AF64A reduced mainly the uptake of [³H]-choline, but also of [³H]-noradrenaline. Carbachol reduced the evoked [³H]-noradrenaline and [³H]-acetylcholine release; the IC₅₀ values were 0.37 and 0.43 μmol/l, respectively. Atropine and 4-DAMP, but not AF-DX 116, methoctramine or pirenzepine, increased the evoked [³H]-acetylcholine release. However, these muscarinic antagonists failed to modify the evoked [³H]-noradrenaline release. Carbachol inhibited the release of both acetylcholine and noradrenaline. The inhibition was blocked by the antagonists. The rank orders of potency (based on pIC₅₀ values) were, in the case of [³H]-acetylcholine release, atropine > 4-DAMP > AF-DX 116 ≥ pirenzepine ≥ methoctramine, and, in the case of [³H]-noradrenaline release, atropine > 4-DAMP > AF-DX 116 ≥ methoctramine ≥ pirenzepine. These results suggest (1) that the prosynaptic receptors that modulate endogenous acetylcholine release are likely of the M₃ subtype, whilst those involved on the effect of the exogenous agonist carbachol are of M₂ subtype, and (2) that those which inhibit noradrenaline release are probably a mixture of M₂ and M₃ subtypes as well. The autoinhibition of the acetylcholine release was functionally active under our experimental conditions, while noradrenaline release does not appear to be modulated by muscarinic receptors in physiological conditions.

Key words: Bovine cerebral arteries – Presynaptic receptors – Muscarinic receptors – Acetylcholine release – Noradrenaline release

Introduction

Histochemical studies have proved the existence of dense adrenergic and cholinergic networks in cerebral vessels of different animal species; these neurones are in close association with each other (Iwayama et al. 1970; Edvinsson et al. 1972; Amenta et al. 1980). This suggests that the acetylcholine released from cholinergic terminals may modulate the cerebrovascular tone and the adrenergic neurotransmission by reducing noradrenaline release (Edvinsson et al. 1972; Marín and Rivilla 1982).

The existence of presynaptic muscarinic receptors inhibiting noradrenaline release has been demonstrated in different tissues, including blood vessels (Westfall 1977; Vanhoutte and Levy 1980; O'Rourke and Vanhoutte 1987). On the other hand, muscarinic receptors on cholinergic nerve endings (autoreceptors) are known to inhibit acetylcholine release (Muscholl 1979; Starke et al. 1989).

At least three different subtypes of muscarinic receptors have been characterized pharmacologically. Muscarinic receptors that show high affinity for the antagonist pirenzepine are called M₁ receptors (Hammer and Giachetti 1982; Eglen and Whiting 1986; Giachetti et al. 1986; Doods et al. 1987), whereas those with low affinity for this agent are classified into M₂ (named cardiac receptors) and M₃ (named ileal or smooth muscle receptors) (Eglen and Whiting 1986; Giachetti et al. 1986; Doods et al. 1987). The different antagonists now available permit to determine the muscarinic receptor subtype present in the tissues. Thus, AF-DX 116 (11-[(2-[(diethylamino)methyl]-1-piperidinyl)acetyl]-5,11-dihydro-6H-
pyrrole-[2-3-6][1, 4] benzodiazepine-6-one) (Eglen and Whiting 1986; Giachetti et al. 1986; Mei et al. 1989) and methoctramine (Melchiorre 1988; Mei et al. 1989) are selective for \( M_2 \) and 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) (Barlow et al. 1976; Eglen and Whiting 1986) and hexahydrosalidifendinol (Fuder et al. 1985; Mei et al. 1989) for the \( M_3 \) receptors.

The postsynaptic muscarinic receptors have been more extensively studied than those present at presynaptic level, which are involved in the modulation of noradrenaline and acetylcholine release. The \( M_2 \) receptor is the most frequently reported subtype implicated in the release of noradrenaline [i.e., rat portal vein, Remie et al. (1990), rabbit iris, Bognar et al. (1989) and canine saphenous vein, O'Rourke and Vanhoutte (1987)], whereas the \( M_2 \) (rat heart, Bognar et al. (1990a) and guinea-pig iris, Bognar et al. (1990b)] and \( M_3 \) receptors [guinea-pig ileum (Dammann et al. 1989) and rat striatum (De Boer et al. 1990)] are involved in the case of acetylcholine release.

The present study was undertaken to determine in bovine cerebral arteries the muscarinic receptor subtype involved in acetylcholine and noradrenaline release by using carbachol as muscarinic agonist and different selective muscarinic antagonists. To our knowledge this is the first study of this type carried out on brain vessels.

### Methods

**Experimental design and superfusion studies.** The bovine cerebral arteries used in the present study were branches of middle cerebral arteries. The bovine brain was obtained from the abattoir and transported to the laboratory in Krebs-Henseleit solution of 4°C. After isolation, the vessels were placed in a Petri dish containing Krebs-Henseleit solution of 4°C, divided into segments of 5 mm in length, pooled and carefully cleaned of traces of blood and adherent tissues. Then, they were set up in a nylon net and immersed for 30 min in 10 ml of Krebs-Henseleit solution of 37°C continuously gassed with 95% O2-5% CO2 mixture (equilibrium period). Thereafter, they were incubated for 60 min in 1 ml of oxygenated Krebs-Henseleit solution at 37°C containing 95% O2-5% CO2 mixture (equilibrium period). Thereafter, they were incubated for 60 min in 1 ml of oxygenated Krebs-Henseleit solution at 37°C containing 95% O2-5% CO2 mixture (equilibrium period). Thereafter, they were incubated for 60 min in 1 ml of oxygenated Krebs-Henseleit solution at 37°C containing 95% O2-5% CO2 mixture (equilibrium period). Thereafter, they were incubated for 60 min in 1 ml of oxygenated Krebs-Henseleit solution at 37°C containing 95% O2-5% CO2 mixture (equilibrium period).

The amount of 3H overflow induced by electrical stimulation, as well as the net 3H overflow evoked by S2 and S1 were calculated (S2/S1) in control situation and in the presence of the drugs used, in order to eliminate differences among the arteries. For the same reason, the ratio between the basal 3H efflux before the second period of stimulation (S2) and the first one (S1) was also determined. In order to analyze the effect of Ca2+-free medium on the evoked 3H overflow, the arteries were incubated in a solution without Ca2+ from 20 min prior to S2.

The amount of 3H overflow induced by electrical stimulation, as well as that present in the tissue was expressed in pg/mg. The composition of Krebs-Henseleit solution was as follows (mmol/l): NaCl 115, CaCl2 2.5, KCl 4.6, KH2PO4 1.2, MgSO4 7H2O 1.2, NaHCO3 25, glucose 11.1, Na2 EDTA 0.03 (to prevent the oxidation of unstable substances). The composition of Ca2+-free medium was similar except that CaCl2 and Na2EDTA were omitted, while 1 mmol/l EGTA was added.

**Ethylcholine mustard aziridinium ion (AF64A).** AF64A was used to analyze its effect on [3H]-choline uptake and on evoked 3H overflow. This agent is a potent and irreversible inhibitor of the high-affinity choline transport and depressor of choline acetyltransferase, producing a cholinergic denervation (Rylett and Colhoun 1980; Sandberg et al. 1984; Estrada et al. 1988). AF64A was freshly prepared from acetylcholine mustard hydrochloride (acetyl-AF-64) by alkaline hydrolysis. A 10 mmol/l aqueous solution of this mustard was treated with NaOH (1 mol/l) at pH 11.3–11.7 for 30 min, and thereafter adjusted to 7.4 with HCl (1 mmol/l). The final concentration of AF64A was adjusted to 200 μmol/l; cerebral arteries were then incubated in this continuously oxygenated solution for 3 h at 37°C. Afterward, the arteries were immersed in normal Krebs-Henseleit solution.

**Drugs, solutions and statistical evaluation.** Drugs used were: atropine sulphate, acetylcholine chloride, tetrodotoxin, 6-hydroxydopamine and carbachol chloride (Sigma, St, Louis, Mo, USA), pirenzepine hydrochloride and AF-DX 116 (Boehringer Ingelheim, FGR), methoctramine tetrahydrochloride (gift of Prof. C. Melchiorre, University of Bologna, Italy), 4-DAMP (kindly provided by Prof. R.B. Barlow, Bristol, UK) and (±) [3H]-noradrenaline hydrochloride, [3H]-choline chloride (New England Nuclear, Boston, Mass, USA) and acetyl-AF-64 (AF64A) (Research Biochemicals, Wayland, Mass, USA).

Stock solutions (10 mmol/l) of drugs were made in distilled water, except AF-DX 116 which was dissolved in 0.05 mol/l HCl and in saline (0.9% NaCl)-ascorbic acid (0.01% w/v) solution. Stock solutions were kept at -20°C; those for AF-DX 116 and pirenzepine were protected from light and were used under sodium vapor light. Appropriate dilutions of these solutions were made in distilled water on the day of experiments.

Results are given as means±SEM. Statistical analysis was done by means of Student's t-test for unpaired observations; a probability of less than 5% was considered significant. To analyze the action of antagonists for presynaptic muscarinic receptors in bovine cerebral arteries, the pIC50 values (−log antagonist molar concentration causing 50% inhibition of the carbachol-evoked reduction of stimulated noradrenaline or acetylcholine release) were calculated by least square regression analysis (Remie et al. 1990). These values were expressed with the respective 95% confidence intervals, which were determined according to the method of Fleming et al. (1972).