Biochemical Pharmacology, Vol. 36, No. 18, pp. 3075–3077, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

SHORT COMMUNICATIONS

An *in vivo* pharmacological study on muscarinic receptor subtypes regulating cholinergic neurotransmission in rat striatum

(*Received* 18 *November* 1986; *accepted* 3 *March* 1987)

Evidence of multiple muscarinic cholinergic receptors (MChR) has accumulated in recent years on the basis of binding studies with agonists and antagonists. Initial studies revealed complex interactions of the muscarinic receptors with agonists as attested, in rat cerebral cortex, by the ability of carbachol to interact with three subpopulations of binding sites which differed in affinity for potent agonists by up to 3000 times but had equal affinity for classical antagonists such as atropine [1]. More recently, receptor binding data obtained with pirenzepine (PZ) showed up muscarinic receptor heterogeneity for an antagonist [2]. PZ appears to distinguish between two classes of sites among those labelled homogeneously by classical antagonists; the site termed M-1 exhibits high affinity for PZ while the M-2 site shows low affinity for the compound [3, 4]. The heterogeneity of muscarinic receptors recognized by PZ was supported by pharmacological data in peripheral systems which revealed selective effects of the drug on functional responses [3, 5] but, for the brain, data correlating PZ binding sites with definite functions are scant.

Using *in vitro* neurotransmitter release from superfused synaptosomes as a functional response, Raiteri *et al.* [6] showed that PZ, acting at presynaptic muscarinic receptors located on different types of neurons, modulated dopamine release in striatum and acetylcholine (ACh) release in cerebral cortex and hippocampus, the compound being more potent in the former than in the latter regions. It was further brought out by this *in vitro* study that cholinergic nerve endings of the striatum lack a muscarinic autoreceptor mechanism regulating cholinergic neurotransmission even if *in vivo* pharmacological studies [7, 8] and *in vitro* slice techniques [9] did provide evidence of a feedback inhibition of striatal cholinergic neurons triggered by muscarinic receptor stimulation.

In the light of these observations, an *in vivo* pharmacological approach was used here to define the subtype(s) of muscarinic receptor modulating ACh release in striatum. We determined the changes in striatal ACh content elicited by typical and atypical muscarinic agents injected intracerebroventricularly (i.c.v). These changes, known to be associated with an inverse effect on the synthesis [10, 11] and release of ACh *in vivo* [8] and *in vitro* [12], were taken as an index of the feedback regulation of cholinergic neurotransmission.

Materials and methods

Female CD-COBS rats (Charles River, Italy), body weight 210–220 g, were used. The animals were housed under constant temperature (22°), humidity (60%) and 12 hr dark–light cycles and allowed free access to food and water. The rats were killed by fast focused microwave irradiation to the head (1.3 kW at 2.45 GHz for about 4 sec). The striatum was removed unilaterally from the brain and weighed before proceeding to the measurement of ACh and choline by the radioenzymatic method of Saelens *et al.* [13] with modifications [14].

Striatal concentrations of PZ and scopolamine (SCOP) were measured by radioreceptor assay. The animals were killed by microwave irradiation to the head 30 min after i.c.v. administration of the drugs. The striata were quickly dissected on ice and homogenized in 100 vol. of 50 mM Na^+-HEPES buffer, pH 7.5, at 20°. The homogenates were centrifuged at 20,000 rpm for 10 min and the supernatants were saved. PZ was determined by measuring the displacement of 0.5 mM (^{3}H)PZ specific binding, and SCOP by the displacement of 0.1 nM (^{3}H) 1-quinuclidinyl benzilate (QNB) specific binding from fresh striatal membranes (0.05 mg protein per assay tube) produced by appropriately diluted aliquots of the supernatants, comparing the displacement values with standard displacement curves of the drugs. In some experiments, homogenates of striata were incubated at 37° for 20 min to facilitate the enzymatic hydrolysis of endogenous ACh. Stereospecific muscarinic receptor binding was measured as previously described by Hammer *et al.* [2] for (^{3}H)PZ and by Watson *et al.* [4] for (^{3}H)QNB.

(^{3}H)QNB (33 Ci/mmole) and (^{3}H)PZ (83 Ci/mmole) were purchased from New England Nuclear, Boston, MA; oxotremorine sesquifumerate (OTMN) and scopolamine hydrobromide trihydrate were purchased from Aldrich-Europe, Beerse, Belgium; pirenzepine dihydrochloride and McN-A-343 were kindly supplied by the Istituto De Angeli, Milan, Italy. The drugs were dissolved in saline and administered into the third ventricle in a volume of 15 μl through a Portex PP10 polyethylene guide cannula implanted 24 hr earlier, unless otherwise stated. Control rats received equivalent volumes of vehicle.

Results and discussion

The dose–response effect of muscarinic agonists (OTMN, McN-A-343) and antagonists (SCOP, PZ) on striatal ACh content, 30 min after i.c.v. administration, are illustrated in Fig. 1. The effects are expressed as the percentage of the ACh content (65.5 ± 3.2 nmoles/g) found in the striata of control rats.

OTMN, which shows high preference for the M-2 receptors [15], significantly raised ACh content (27%) at the dose of 7.5 nmoles and exhibited a maximal effect (about 40%) at the concentrations of 250 and 500 nmoles. The M-1 agonist McN-A-343, administered over the same dose range (7.5–500 nmoles) had no effect on ACh content. These findings are in agreement with binding data indicating that the ratio of the IC_{50} values for McN-A-343 to OTMN was 3.5 in striatum, an area containing almost equal proportions of the M-1 and M-2 subtypes, but 70 in the brainstem, a region reported to possess mostly M-2 muscarinic receptors [15]. Interestingly, in behavioral experiments, it was shown that McN-A-343 was inactive when microinjected into the brain directly at the site where carbachol and OTMN elicited tremor, analgesia and hypothermia [16].

The muscarinic antagonists SCOP and PZ, too, were compared for their effects on striatal ACh content. SCOP induced a dose related decrease in striatal ACh content (Fig. 1, upper panel). The drug had no effect at 1.5 and 3 nmoles, reduced ACh content by about 15% at 6 nmoles and exerted maximal ACh lowering action (50%) at 60 nmoles. The doses of SCOP were appropriately corrected for clearance from the brain which was found by radioreceptor assay to be about 20% during the 30 min

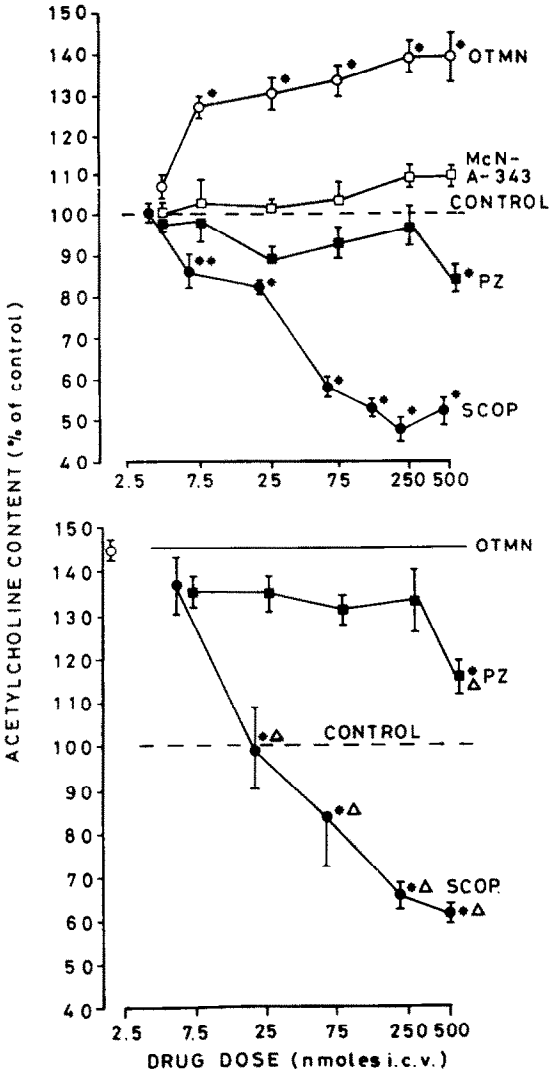

Fig. 1. Dose–response effects of the muscarinic receptor agonists oxotremorine and McN-A-343 and of the muscarinic receptor antagonists scopolamine and pirenzepine in altering acetylcholine content (upper panel) and the dose–response effects of scopolamine and pirenzepine in blocking the acetylcholine increasing action of oxotremorine (lower panel). The ordinate shows the ACh content as a percent of the control value which was 65.4 ± 3.2 nmoles/g. The abscissa gives the drug doses on a logarithmic scale. The drugs were administered i.c.v. to freely moving rats through a cannula implanted into the third ventricle 24 hr earlier. The animals were killed after 30 min by focused microwave irradiation to the head and acetylcholine content in the striatum was assayed by a radioenzymatic method. In the lower panel experiment, oxotremorine was administered i.p., 1 μmole/kg, the lowest parenteral dose yielding the maximal increase of acetylcholine, 146% above control (open circle and solid line), 10 min before either scopolamine or pirenzepine. The symbols and vertical bars represent the means and SEM, respectively (N = 12–20). **$P < 0.05$ and *$P < 0.01$ vs control by Dunnett's test. Δ, $F = P < 0.01$ by ANOVA (2 × 2) factorial analysis followed by Tukey's test for unconfounded means.

experimental period. PZ had no effect on ACh at any of the doses from 5 up to 250 nmoles i.c.v., 30 min. At 500 nmoles, the drug induced a significant decrease (17%) in ACh content. A higher dose of 1000 nmoles of PZ produced a similar ACh-decreasing effect (data not shown) but hypersensitivity to touch, kangaroo jumping and convulsions were observed.

Radioreceptor assay showed that PZ was not cleared from striatum during the 30 min course of the experiment and it achieved within 15 min striatal concentrations expected from theoretical calculation. This finding indicates that there was no concentration gradient from the intraventricular compartment to the extracellular mileu of the striatum.

None of the agonists or antagonists altered the striatal level of choline which was 25.6 ± 1.8 nmoles/g in control rats.

Taken together the data suggest that SCOP reduces ACh through an action at sites with low affinity for PZ and OTMN raises ACh through an action at McN-A-343 insensitive sites. These results fit with the finding that PZ required a several thousand fold greater concentration than atropine to display similar activity in inhibiting ACh-induced depolarization of locus coeruleus neurons in pontal slices [17], an area uniformly rich in low affinity PZ sites [2].

The dose–response effects of SCOP and PZ in preventing the increase in striatal ACh induced by OTMN are illustrated in Fig. 1 (lower panel). The drugs were given i.c.v. 10 min before OTMN, 1 μmole/kg i.p. SCOP prevented the effect of OTMN at the dose of 20 nmoles whereas PZ did so only at the dose of 500 nmoles ($P < 0.01$).

In conclusion, the M-1/M-2 antagonist SCOP, which binds to a homogeneous population of sites, reduced striatal ACh by itself and antagonized the cholinergic action of OTMN whereas the M-1 antagonist PZ, at doses assumed to interact with high-affinity subpopulations of MChR in striatum, failed to change ACh content or to antagonize the ACh-accumulating activity of OTMN.

However, PZ at the dose considered not selective for the M-1 receptor population, counteracted the cholinergic action of OTMN. Interestingly, the dose of PZ required to produce the *in vivo* cholinergic effect fell roughly within the PZ concentration range reported to interact *in vitro* with the low-affinity M-2 sites [6]. It is likely that the hydrophilicity of PZ is one reason why a high dose of the compound is required for cholinergic activity. However, N-methylscopolamine, a quaternary M-1/M-2 antagonist sharing the hydrophilic property of PZ, did interact *in vivo* with the receptors modulating ACh release at the i.c.v. dose of 7.5 nmoles as reflected by its ability to lower the level of striatal ACh (manuscript in preparation). Thus, the high dose of PZ needed to act at muscarinic receptors subserving ACh release cannot be solely a reflection of the compound's hydrophilic nature.

These findings provide *in vivo* pharmacological evidence that the M-2 muscarinic receptor is probably involved in the feedback regulation of cholinergic neurotransmission in rat striatum. It is now being investigated whether this receptor is of the cardiac M-2 or glandular M-2 subtype since both have recently been distinguished in different brain regions with uneven distribution [18].

Acknowledgements—We thank Mr P. Cicioni for skilled technical assistance. This work was partly supported by the CNR, Rome, Italy, Pharmacology and Therapeutics Group, Contract No. 8500323.04.

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Biochemical Pharmacology, Vol. 36, No. 18, pp. 3077–3081, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Antipyrine metabolism in cultured rat hepatocytes

(*Received* 1 *September* 1986; *accepted* 17 *March* 1987)

Antipyrine is metabolized to three main oxidative products by the cytochrome P-450 system. These products are 3-hydroxymethyl antipyrine (3OHA), 4-hydroxyantipyrine (4OHA) and norantipyrine (NORA). Antipyrine has, therefore, been proposed as a probe of drug oxidation in animals and man [1]. Selective effects on the formation of these metabolites have been shown with inducers such as phenobarbital and β-naphthoflavone in rats [1–3] and in man [4] and by the use of inhibitors [5]. Several groups have investigated the effects of induction upon antipyrine metabolite pattern in rat urine [1, 3, 5, 6]. All of these authors found selective changes in metabolite profile as a consequence of induction suggesting that several isozymes of cytochrome P-450 metabolize antipyrine, although significant variability in urinary metabolite patterns was found between groups. Rhodes and Houston, [3] showed that induction with phenobarbital increased the *in vivo* formation rate constant of 4OHA by 200%. As a consequence 4OHA was the major urinary metabolite of antipyrine in rats pretreated with phenobarbital.

The use of isolated and cultured hepatocytes to generate metabolite profiles of novel drugs has recently been proposed [7]. However, the partial loss of differentiated function of hepatocytes in culture is well-documented [8]. In particular, losses in cytochrome P-450 levels are commonly observed within the first day of culture [9] although these losses may be modified by the addition of ligands to the culture medium [10]. Little information is available on the metabolism of drugs and xenobiotics by hepatocytes maintained in culture. Thus, the aim of this study is to investigate the utility of antipyrine metabolite formation as a probe of changes in cytochrome P-450 content of hepatocytes maintained in culture.

Materials and methods

Chemicals. The chemicals and reagents used in this study were of the highest quality generally available. 3-hydroxymethyl-antipyrine, 4-hydroxyantipyrine and norantipyrine were synthesized by the Department of Synthetic and Isotope Chemistry, SK&F.

Animals. Rats (Wistar, male) were obtained in the range 180–200 g from the SK&F colony and were housed on grade 6 greenwood granules in polypropylene cages. Free access to tap water and PRD pellets (Labsure, Poole, Dorset, U.K.) was provided.

Induction of rat liver microsomal activity. Rats were administered phenobarbital for 7 days in the drinking water ($1\ mg \cdot ml^{-1}$) [11]. β-Naphthoflavone (βNF) was administered on two consecutive days ($80\ mg \cdot kg^{-1}$ in corn oil, i.p.) and hepatocytes were prepared 24 hr after the last dose.

Preparation of hepatocytes. Hepatocytes were prepared by the technique of Strom *et al.* [12], and were then counted in a haemocytometer in the presence of 0.04% trypan blue. Yields of 30–40 $\times$ 10^6 cells were commonly achieved from each lobe with a viability in excess of 90%.

Hepatocytes were quickly diluted into culture medium consisting of Williams Medium E containing L-glutamine (4 mM), penicillin ($100\ IU\ ml^{-1}$), streptomycin ($100\ \mu g \cdot ml^{-1}$), neomycin ($100\ \mu g \cdot ml^{-1}$), insulin ($0.02\ IU\ ml^{-1}$) and newborn calf serum (10% v/v). The cell suspension was then seeded on to 35 mm wells (Sterilin) which had been coated with soluble collagen. The culture dishes were then introduced into a 37° incubator (T. R. Heraeus) containing 5% CO_2 in a water saturated atmosphere. Cell attachment could be detected after about one hour in culture. When cells were maintained in culture the medium was renewed every 24 hr.

Measurement of cytochrome P-450. Cell culture medium was removed and the cell monolayer washed with saline. A solubilising buffer was then added to each culture plate (1 ml to a 35 mm well). The buffer was exactly as described by Warner *et al.* [13] except that Renex 690 (Atlas Chemicals, Surrey, U.K.) was substituted for Emulgen 911. After 5 min at room temperature the cell monolayer was scraped into the buffer and the contents of two wells combined.

The buffer was then centrifuged (1000 *g*, 5 min, 21°, Sorvall RT-6000) to remove debris. The buffer, containing the solubilised cytochrome P-450, was then transferred to a clean tube. Cytochrome P-450 was measured by the method of Omura and Sato, [14] utilising a Hewlett–Packard 8450A diode array spectrophotometer.

Measurement of enzyme activities. 7-Ethoxycoumarin O-deethylation (ECOD) was measured by the formation of