Pharmacology of the putative M₄ muscarinic receptor mediating Ca-current inhibition in neuroblastoma × glioma hybrid (NG 108-15) cells

¹M.P. Caulfield & D.A. Brown

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

1 We have assessed the potency of a range of agonists and antagonists on the muscarinic receptor responsible for inhibiting the Ca-current (I_{Ca}) in NG 108-15 hybrid cells.

2 Acetylcholine (ACh), oxotremorine-M and carbachol were potent 'full' agonists (EC_{50} values were 0.11 μ M, 0.14 μ M and 2 μ M, respectively). Maximum inhibition of peak high-threshold I_{Ca} by these agonists was 39.5%. ($\pm$)-Muscarine, methylfurmethide and arecaidine propargyl ester (APE) were 'partial' agonists, with EC_{50} values of 0.54 μ M, 0.84 μ M and 0.1 μ M, respectively.

3 Atropine, pirenzepine and himbacine were potent antagonists of muscarinic inhibition of I_{Ca} , with apparent pK_B values of 9.8, 7.74 and 8.83, respectively. Methoctramine was relatively weak (pK_B = 7.63). Atropine and pirenzepine depressed maximum responses to agonists, probably because these antagonists have relatively slow dissociation rates.

4 The characteristic pharmacological profile found for the M₄ receptors in these functional experiments (himbacine high affinity, pirenzepine moderate to high affinity, methoctramine low affinity) corresponds well with data from earlier binding experiments (Lazareno *et al.*, 1990). Since mRNA hybridising to probes for the m4 receptor genotype can be detected in these cells, it is suggested that these pharmacological characteristics identify the equivalent expressed receptor subtype M₄.

Keywords: M₄ muscarinic receptor; Ca current; neuroblastoma cell; acetylcholine; himbacine; pirenzepine; methoctramine; muscarine; oxotremorine-M

Introduction

There are at least five muscarinic acetylcholine receptor genotypes, designated m1 to m5 (Kubo *et al.*, 1986a,b; Bonner *et al.*, 1987; Peralta *et al.*, 1987; Buckley *et al.*, 1989). Receptors expressed from genes m1, m2 and m3 may correspond to those defined pharmacologically as M₁, M₂ and M₃ (see Hulme *et al.*, 1990).

One cell type which appears to express mRNA from the m4 genotype is the NG 108-15 mouse neuroblastoma × rat glioma hybrid cell (Peralta *et al.*, 1987; Fukuda *et al.*, 1988). Recent ligand-binding experiments on muscarinic receptors expressed in these cells have shown a unique profile of antagonist binding constants, suggesting that it is now possible to define a corresponding pharmacological M₄ subtype (Lazareno *et al.*, 1990).

The functional role of this subtype is not yet clear, but one possibility is that it might mediate some of the presynaptic inhibitory effects of muscarinic agonists. The reason for suggesting this is that differentiated NG 108-15 cells express a variety of neural ion channels (see Brown & Higashida, 1988; Robbins & Sim, 1989) and that one effect of stimulating the endogenous muscarinic receptor in these cells is to inhibit a voltage-gated Ca-current (Higashida *et al.*, 1990). An effect of this type might (arguably) contribute to the inhibition of transmitter release (Miller, 1990).

Hence, in these present experiments, we have tested the effects of some different muscarinic agonists and antagonists on the Ca-current in differentiated NG 108-15 cells, in order to define further the pharmacological profile of this putative M₄ receptor as seen from a functional viewpoint.

Some of these data have been presented in preliminary form (Caulfield *et al.*, 1991).

Methods

Ca-currents

NG 108-15 cells (passage numbers 11-96) were 'differentiated' with either 10 μ M prostaglandin E₁ plus 50 μ M isobutylmethylxanthine for 4–10 days (Docherty, 1988) or 200 μ M dibutyryl cyclic AMP for 9–28 days (Higashida *et al.*, 1990) prior to recording. Dishes of cells were perfused at 5–10 ml min⁻¹ with a modified Krebs solution (pH 7.4; 22°C–24°C; pre-bubbled with 95% O₂/5% CO₂) containing (mM): NaCl 120, KCl 3, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 23, HEPES 5, D-glucose 11. Tetrodotoxin (0.5 μ M) was included in the medium to suppress Na⁺ currents. The whole-cell variant of the patch-clamp technique (Hamill *et al.*, 1981) was used to record voltage-activated Ca-currents. Patch electrodes (3–9 M Ω , usually 4–5 M Ω) were filled with a solution containing (mM): CsCl 110, tetraethylammonium chloride (TEA) 25, MgCl₂ 3, EGTA 3, HEPES 40 (adjusted to pH 7.4 with CsOH). After formation of a gigaohm seal (1 G Ω –4 G Ω) between electrode and cell membrane and breaking through into 'whole-cell' recording, the cell was voltage-clamped at –80 mV (occasionally –90 mV) with an Axoclamp-2A amplifier (Axon Instruments) in switch-clamp mode at a switching frequency of 4–10 kHz. Ca-currents were recorded as the composite currents evoked by stepping for 100 ms (occasionally 500 ms) to 0 mV or +10 mV (cf. Docherty, 1988).

It has previously been shown that the use of TEA to inhibit K⁺ currents activated concomitantly by the depolarizing voltage steps reduces the potency of muscarinic agonists and antagonists in this system (Caulfield, 1991). Elimination of contaminating outward K⁺ currents was therefore achieved by allowing sufficient time for the Cs in the pipette solution to diffuse into the cell. This could be monitored by the progressive increase in inward current seen during a 100 ms depolarizing command pulse and experiments were not started until this current had stabilized (cf. Caulfield, 1991).

¹ Author for correspondence.

Data analysis

The effects of muscarinic agonists were measured as the percentage reduction of peak inward Ca-current. Currents were measured with respect to the zero current line. Agonists were applied until no further reduction in peak current amplitude was seen. Stepwise cumulative additions of agonist concentrations without agonist-free periods between applications were used only where responses to each dose of agonist determined in this manner did not differ significantly from corresponding responses obtained by discrete application of each agonist concentration. Dose-effect relationships were analyzed by determining the least-squares fit of data to the function $y = y_{\max} x^n / (x^n + K^n)$, where y is percentage inhibition of currents, $y_{\max}$ is maximum inhibition, x is agonist concentration and n is a 'slope factor'. Where agonist dose-effect data were pooled, data obtained in each individual experiment were expressed as a percentage of the maximum response to ACh ($3 \mu\text{M}$ or $10 \mu\text{M}$) in that experiment and the least-squares fit to the pooled mean responses was weighted by the inverse of the corresponding variances. These and all subsequent curve fitting procedures were carried out by use of 'Sigma Plot 4' computer software (Jandel Scientific).

Antagonist potencies were measured in experiments in which antagonists were at apparent equilibrium, as assessed by stabilization of responses to a continually adjusted submaximal agonist dose. Effects of antagonists producing rightward parallel shifts of agonist dose-response curves were quantified by calculating the K_B value using the Gaddum-Schild equation: $K_B = B / (DR - 1)$, where K_B is the apparent antagonist dissociation constant, B is antagonist concentration and DR is the ratio of the EC_{50} value for the agonist dose-effect curve in the presence of antagonist to that for the control agonist curve.

Some antagonists reduced the maximum agonist response and shifted the dose-effect curve rightward. These data were analyzed by determining the least squares fit of the function:

$$A = \frac{(1 - p_B)}{p_B} \frac{K_A A'}{K_A / p_B + A'}$$

which is a rearrangement in hyperbolic form of the equation:

$$\frac{A}{A + K_A} = \frac{A'(1 - p_B)}{A' + K_A}$$

where A and A' are agonist concentrations producing matched responses in the absence and in the presence of antagonist, respectively, K_A is agonist dissociation constant and p_B is the proportion of receptors occupied by antagonist (see Rang, 1965; Furchgott, 1966).

The antagonist K_B was then estimated from:

$$K_B = \frac{B(1 - p_B)}{p_B}$$

which is a rearrangement of the Langmuir isotherm

$$p_B = \frac{B}{B + K_B}$$

where B is antagonist concentration.

Materials

The following compounds were obtained from Semat: methoctramine tetrachloride, ($\pm$)-muscarine chloride, oxotremorine-M methiodide. Atropine sulphate, ACh chloride, pirenzepine dichloride, carbachol chloride and isobutylmethylxanthine (IBMX) were purchased from Sigma Chemicals. BM-5 (N-methyl-N(1-methyl-4-pyrrolodino-2-butynyl)acetamide) was a generous gift of Dr A.D. Michel (Glaxo Group Research Ltd.). Dr R.B. Barlow very kindly supplied samples of APE (arecaidine propargyl ester) and McN A 343 (4-chlorophenyl-carbamoyloxy-2-butynyl tri-

methyl ammonium chloride). We thank Dr S.H. Lazareno (MRC Collaborative Centre, London) for a kind gift of himbacine. Prof. H. Higashida (Kanazawa University) generously sent supplies of high purity dibutyl cyclic AMP.

Results

Agonists

Application of muscarinic agonists produced reversible (usually within 5 min) and reproducible inhibition of the Ca-current (Figure 1). Prolonged application of agonist (> 2 min) resulted in some recovery from inhibition, which may have been due to desensitization of the agonist effect (data not shown). The agonists inhibited a transient high-voltage-activated component of the current, with little effect on the component of inward current remaining at the end of a 500 ms depolarizing step (Figure 1). This resembles the pattern of Ca-current inhibition by noradrenaline in these cells (Docherty & McFadzean, 1989).

The inhibitory effect of muscarinic agonists varied greatly between cells and batches of cells, even though all of the cells appeared morphologically 'differentiated'. In 61 cells exhibiting high-voltage activated currents (cf. Docherty, 1988), the mean maximum inhibition ($\pm$ s.e.mean) was $40\% \pm 2$, ranging from 15% to 76%. Muscarinic inhibition was small ($< 5\%$) or absent in recordings made over a 15 month period from 285 cells which only had a low voltage-activated Ca-current (cf. Docherty, 1988). This variability in responsiveness of batches of cells seemed not to be due to any of the following factors: passage number, culture conditions (e.g., serum concentration, culture substrate, CO_2 concentration in incubator atmosphere), differentiating agent (dibutyl cyclic AMP, prostaglandin E_1 (PGE_1)/IBMX), duration of exposure to differentiating agent (5 days to 4 months), pipette solution contents (with or without ATP 1 mM/GTP 0.1 mM, or an ATP-regenerating system) or recording temperature (22°C – 37°C). It stands in contrast to the high proportion of noradrenaline-responsive cells (193/205) reported by Docherty & McFadzean (1989), but is more in accord with the low mean inhibition (about 14%) seen in native NG 108-15 cells by Higashida *et al.* (1990).

Figure 1 Inhibition of calcium current (I_{Ca}) in NG 108-15 cells by acetylcholine (ACh). The downward deflections in the upper trace represent peak inward Ca-currents evoked by stepping for 100 ms to a command potential of 0 mV from a holding potential of -80 mV. Steps were made every 10 s. The lower expanded traces show the current evoked by the same command step applied for 500 ms, before and during the peak effect of ACh. For clarity, capacity transients at the beginning and end of the current have been masked.

Figure 2 (a) A representative experiment showing inhibition of Ca-current by increasing concentrations of oxotremorine-M. Downward deflections show peak Ca-current amplitude (see Figure 1). (b) Dose-effect relationships for agonist inhibition of Ca-current. Points represent mean normalized responses (see Methods for calculations and curve-fitting procedures) and vertical bars show s.e.mean values for acetylcholine (ACh) (●), oxotremorine-M (▼) and muscarine (◆). *K* and *n* values (see Methods) for the curves fitted to the pooled mean agonist responses were 0.1 μM and 1.07 for oxotremorine-M, 0.11 μM and 0.9 for ACh and 0.76 μM and 0.85 for muscarine.

Figure 2 shows dose-effect curves obtained with the muscarinic agonists ACh, oxotremorine-M and muscarine. The potency of ACh was increased about 3 fold in the presence of neostigmine, suggesting the presence of functionally-active cholinesterase on the cells (McGee *et al.*, 1978). Results obtained with these and other agonists are summarized in Table 1. Muscarine was a 'partial agonist' with a lower maximal effect than oxotremorine-M, ACh or carbachol. Estimates of the maximum effect of muscarine were obtained from the curve fitted to the pooled normalised data (see Methods). Methylfurmethide and APE were potent agonists, but also produced lower maximum responses than ACh or oxotremorine-M. The putative M₁ receptor-selective agonist McN A 343 did not reduce Ca-currents at concentrations up to 100 μM, but this latter concentration of McN A343 abolished the response to a subsequent dose of ACh (1 μM; *n* = 2). Similar effects were seen with the oxotremorine analogue, BM-5, which has been reported to be a selective agonist for the adenylate-cyclase coupled receptors in heart and in NG 108-15 cells (Baumgold & Drobnick, 1989). BM-5 had no inhibitory effects at concentrations up to 10 μM, but this concentration of BM-5 shifted the ACh dose-response curve to the right (49 and 52 fold, *n* = 2) in a parallel way, suggesting an apparent antagonist *K_B* of about 0.2 μM.

Antagonists

In 8 out of 17 experiments, antagonist application increased the peak Ca current (Figure 3). This enhancement by antago-

Table 1 Potencies of muscarinic agonists as inhibitors of the Ca-current in NG 108-15 cells

Agonist	-log EC ₅₀ (s.e.mean)	EC ₅₀ (μM)	n	Maximum response (s.e.mean)
Acetylcholine	6.49 (0.1)	0.32	4	100
Acetylcholine + neostigmine 1 μM	6.95 (0.1)	0.11	11	100
Oxotremorine-M	6.85 (0.18)	0.14	4	100 (3)
Muscarine	6.27 (0.3)	0.54	4	73 (7)
APE	6.71 (0.28)	0.19	4	81 (6)
Methylfurmethide	6.1	0.84	2	57
Carbachol	5.7	2	2	100

APE = arecaidine propargyl ester.

nists was not observed in the remaining 9 experiments. The mean increase (± s.e.mean) in these 8 experiments was 18% ± 4. There was no apparent relation between this effect and either the antagonist used or the antagonist concentration. One explanation might be that the antagonist relieved a state of partial inhibition resulting from spontaneous or induced release of ACh from these cholinergic cells (McGee *et al.*, 1978). Notwithstanding, the overall effect of the antagonists on agonist dose-response curves was consistent, regardless of whether or not this enhancement occurred.

The antagonists studied fell into two groups. Himbacine (Gilani & Cobbin, 1986) and methoctramine shifted agonist dose-effect curves to the right without any significant reduction in maximum agonist response (Figure 4). This enabled calculation of apparent *K_B* values for these compounds from the estimated dose-ratios (Table 2). At a low concentration (30 nM), pirenzepine shifted the agonist curves rightward without reducing maximum responses, but at higher concentrations, the maximum was decreased (Figure 5a). Atropine (1–10 nM) also reduced maximum agonist responses (data not shown). This effect is not due to irreversible binding of antagonist to receptor (e.g. Furchgott, 1966; Barlow *et al.*, 1991), as the inhibitory effects of pirenzepine (Figure 5b) and atropine (data not shown) reversed within 30 min on perfusing with antagonist-free medium.

It is more likely that the reduction in maximum agonist response results from a low receptor reserve and slow dissociation of antagonist from the receptor, so that the antagonist behaves as if it were irreversible, as previously described by Rang (1965). The data were therefore analyzed using the function set out by Rang (1965) and Furchgott (1966), as modified by Barlow *et al.* (1991; see Methods section), allowing estimation of apparent antagonist *K_B* values (Figure 6 and Table 2). Included for comparison in Table 2 are data from the radioligand binding experiments of Lazareno *et al.* (1990) on NG

Figure 3 Pirenzepine increases Ca-current amplitude. Downward deflections represent peak Ca-current, as in Figure 1.

Figure 4 Sample experiments showing acetylcholine (ACh) dose-effect curves for Ca-current inhibition in the absence (●) and (a) in the presence of 10 nM (▼) and 30 nM (◆) himbacine and (b) in the presence of 100 nM (▽), 300 nM (◇) and 1 μM (▲) methoctramine. Curves were fit by least squares procedures (see Methods) where there were four or more data points; otherwise curves were drawn by eye. The control curve for the himbacine experiment was fit with a K value of $0.07 \mu\text{M}$, n of 0.81 and y_{max} of 75.8%. Estimated himbacine dose-ratios were 4.5 (10 nM) and 17.7 (30 nM). The control curve for the methoctramine experiment was fit with a K value of $0.07 \mu\text{M}$, n of 0.94 and y_{max} of 42.1%. Estimated methoctramine dose-ratios were 4 (100 nM), 8 (300 nM) and 21 (1 μM).

108-15 cells and rabbit lung (which express the m_4 receptor gene) and on M_1 , M_2 and M_3 receptor-containing preparations. The pK_B value for pirenzepine obtained directly from the dose-ratio shifts in the two experiments with 30 nM pirenzepine was slightly higher than the value estimated by the Rang/Furchgott analysis of data from experiments with higher pirenzepine concentrations, although the difference was not significant ($P > 0.05$, t test).

The use of the Rang/Furchgott analysis, which regards the

Figure 5 Representative experiments showing the effect of 30 nM (●), 100 nM (▼) and 300 nM (■) pirenzepine on respective acetylcholine (ACh) dose-effect curves (○, ▽, □) for Ca-current inhibition. Data from 3 experiments were pooled by normalizing responses to the fitted ACh maximum response in each experiment and curves were generated by least-squares procedures (see Methods). Fitted K values for the three control curves were $0.93 \mu\text{M}$, $0.98 \mu\text{M}$ and $0.92 \mu\text{M}$; fitted n values were 0.96, 1.1 and 0.76. Fitted parameters for the pirenzepine curves were: $K = 0.43 \mu\text{M}$, $n = 1.1$, $y_{\text{max}} = 0.98$ (pirenzepine 30 nM); $K = 0.45 \mu\text{M}$, $n = 1.1$, $y_{\text{max}} = 0.69$ (pirenzepine 100 nM). The curve for the experiment with 300 nM pirenzepine was drawn by eye. (b) Oxtremorine-M (Oxo-M) inhibition of Ca-current (represented by downward deflections, as in Figure 1) is reduced by pirenzepine. Recovery of response is shown after 30 min perfusion with antagonist-free medium.

antagonists as effectively irreversible, also allows estimation of agonist dissociation constants (see Methods). The mean pK_A ($\pm$ s.e.mean) estimated for ACh in this manner was 6.05 ± 0.24 , corresponding to a K_A of $0.89 \mu\text{M}$.

Discussion

The profile of antagonist affinities deduced from these experiments accords quite closely with those predicted from previous ligand-binding experiments (Michel *et al.*, 1989; Lazareno *et al.*, 1990). Thus, the absolute and relative

Table 2 Antagonist potencies against muscarinic inhibition of Ca-current in NG 108-15 cells

Antagonist	pK_B ($\pm$ s.e.mean)	K_B (nM)	n	pK_B from radioligand binding*				
				M_4 sites		M_1	M_2	M_3
				NG	Lung			
Atropine	9.8 (0.39)	0.15	3		9.56	9.55	9.01	9.51
Pirenzepine	7.74 (0.23)	18.2	7	7.2	7.55	8.02	6.48	7.09
(100 nM–300 nM)	7.53 (0.27)	29.5	5					
(30 nM)	8.25 (0.5)	5.6	2					
Methoctramine	7.63 (0.23)	23	4	8.09	7.83	7.6	8.34	6.88
Himbacine	8.83 (0.13)	1.5	3	8.53	8.47	7.16	8.34	7.09

* Data from antagonist displacement experiments of Lazareno *et al.* (1990). NG = NG 108-15 cells; Lung = rabbit lung, M_1 = [^3H]-pirenzepine binding to rat cerebral cortex; M_2 and M_3 = [^3H]-N-methylscopolamine binding to rat heart and rat submandibular gland, respectively.

Figure 6 (a) A representative experiment showing the reduction of maximum inhibition of Ca-current by acetylcholine (ACh) by 100 nM pirenzepine. Curves were fit by least-squares procedures (see Methods) and the best-fit parameters were: control $K = 0.07 \mu\text{M}$, $n = 1.9$, $y_{\text{max}} = 63\%$; pirenzepine, $K = 0.31 \mu\text{M}$, $n = 1$, $y_{\text{max}} = 44\%$. (b) The same data fit to the hyperbolic function

$$A = \frac{(1 - p_B) K_A A'}{p_B K_A / p_B + A'}$$

(see Barlow *et al.*, 1991)

where A is plotted on the ordinate and A' on the abscissa scale. A and A' are ACh concentrations in the absence and presence of antagonist, respectively; K_A is agonist dissociation constant and p_B is the proportion of receptors occupied by antagonist. The curve was drawn with K_A and p_B values of $0.2 \mu\text{M}$ and 0.6 , respectively.

potencies of himbacine, pirenzepine and methoctramine measured against muscarinic inhibition of I_{Ca} are very similar to the ligand displacement potencies on putative M₄ receptors in both NG 108-15 cells and rabbit lung (Lazareno *et al.*, 1990), and clearly differ from their potencies at M₁, M₂ or M₃ binding sites (Lazareno *et al.*, 1990) or at the corresponding receptor subtypes in functional assays (e.g. Brown *et al.*, 1980; Lazareno & Roberts, 1989). Although the potency of pirenzepine against I_{Ca} inhibition ($\text{p}K_B$ 7.74) was slightly higher than its binding potency in NG 108-15 cells ($\text{p}K_B$ 7.2; Lazareno *et al.*, 1990), the accuracy of functional $\text{p}K_B$ calculations is probably limited by the non-parallel shift of the dose-response curve (see below). Also, equivalent differences between binding and functional potencies for pirenzepine have been noted at M₁ receptors (Brown *et al.*, 1980) and it is possible that other factors such as uptake into cells may affect measurements. Therefore, M₄ receptors seem to preserve their unique antagonist profile when measured by a functional assay. Our experiments further suggest that they might best be distinguished operationally from M₁ receptors by their high sensitivity to himbacine rather than by their slightly lower sensitivity to pirenzepine.

In contrast to the unique profile for antagonist potencies, the potencies of the agonists we tested were roughly comparable with those observed in a functional assay for M₁ receptor

activation (e.g. Brown *et al.*, 1980). However, several points are noteworthy.

Firstly, there seems to be a rather low receptor reserve for Ca-current inhibition, even for 'full' agonists such as acetylcholine, carbachol and oxotremorine-M, such that high concentrations of pirenzepine and atropine reduced the maximum agonist effect. This is to be expected from the interaction of an agonist with a slowly dissociating antagonist when there are few 'spare receptors' (Rang, 1965). At first sight this seems an inadequate explanation for the depression, since methoctramine and himbacine had similar or lower apparent K_B values than pirenzepine, so might be expected to dissociate slowly, yet produced a parallel shift of the agonist dose-response curve. However, pirenzepine appears to have a much slower dissociation rate than that predicted from its equilibrium constant. Thus, the rate constant for dissociation of [³H]-pirenzepine from M₄ receptors in rabbit lung is only 1.5 times greater than that for dissociation of [³H]-N-methylscopolamine, whereas the ratio of the equilibrium constants is 480 (Lazareno *et al.*, 1990). Analysis of the data for pirenzepine block yielded an apparent dissociation equilibrium constant for acetylcholine of $0.89 \mu\text{M}$, so that a just-maximal concentration of $3 \mu\text{M}$ would have to occupy about 77% of the available receptors. Although this occupancy is somewhat greater than that predicted from the dose-ratio shift produced by concentrations of pirenzepine below those which depressed the maximum response, the concept of a low receptor reserve is substantiated by the fact that muscarine, methylfurmethide and APE appeared to behave as 'partial' agonists, with a lower maximum effect than that produced by acetylcholine or oxotremorine-M.

Secondly, and perhaps related to the apparent low receptor reserve, McN A 343 and BM-5 did not exhibit clear agonist activity but instead behaved as antagonists. BM-5 has previously been reported to exert partial agonist activity in respect of inhibition of prostaglandin-stimulated adenylate cyclase in NG 108-15 cells, with an EC_{50} value of $0.4 \mu\text{M}$ (Baumgold & Drobnick, 1989). This accords reasonably well with the apparent antagonist K_B ($0.2 \mu\text{M}$) obtained in the present experiments. This suggests that the same receptor may be responsible both for inhibition of adenylate cyclase and inhibition of the Ca-current, but that it couples less efficiently to the latter. It should be noted that inhibition of I_{Ca} is not due to inhibition of adenylate cyclase, since it is not affected by high intracellular concentrations of cyclic AMP (Higashida *et al.*, 1990) and further that the two responses are likely to be mediated by different GTP-binding proteins (see McFadzean *et al.*, 1989).

The present results have some bearing on the pharmacological identification of muscarinic receptors responsible for Ca-current inhibition (and possibly for inhibiting transmitter release) in other cells. Thus, whereas in most cells inhibition of I_{Ca} is resistant to high concentrations of pirenzepine (e.g. Mochida & Kobayashi, 1986; Gahwiler & Brown, 1987; Song *et al.*, 1989; Tse *et al.*, 1990), in rat sympathetic neurones inhibition is potently antagonized by pirenzepine, leading to the suggestion that it is mediated by an M₁ receptor (Wanke *et al.*, 1987). However, experiments on DNA-transfected NG 108-15 cells suggest that only m2 or m4 genotypic receptors are capable of inhibiting I_{Ca} and that neither m1 nor m3 genotypic receptors can couple efficiently to this current (Higashida *et al.*, 1990). The present data offer a way of resolving this difficulty, since they suggest that M₄ receptors could be responsible for the high pirenzepine sensitivity of I_{Ca} -block in sympathetic neurones. Likewise, if Ca-current inhibition does indeed contribute to presynaptic inhibition, then the high potency of pirenzepine against muscarinic presynaptic inhibition in the hippocampus (Dutar & Nicol, 1988; Sheridan & Sutor, 1990), olfactory cortex (Williams & Constanti, 1988) and septum (Hasuo *et al.*, 1988) might also reflect involvement of the putative M₄ receptor. Other instances of high presynaptic potency of pirenzepine include muscarinic inhibition of sympathetic transmission in rabbit ear artery (Choo *et al.*,

1986), acetylcholine release in chick heart (Jeck *et al.*, 1988) and sympathetic transmission at rabbit vas deferens (Eltze, 1988). In the latter case, the argument in favour of an M_4 receptor is strengthened by the equivalent high potency of himbacine.

Thus, the present observations provide a functional

counterpart to previous ligand-binding studies which may indicate a role for M_4 receptors in a variety of nerve cells.

This work was supported by a grant from the Medical Research Council. We are grateful to Dr R.B. Barlow for invaluable help with computations and to Dr J. Robbins, Dr R.J. Docherty and Dr S.H. Lazareno for encouragement and helpful discussion. We thank Prof. H. Higashida for his advice on tissue culture.

References

- BARLOW, R.B., McMILLEN, L.S. & VEALE, M.A. (1991). The use of 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine (4-DAMP mustard) for estimating the apparent affinities of some agonists acting at muscarinic receptors in guinea-pig ileum. *Br. J. Pharmacol.*, **102**, 657–662.
- BAUMGOLD, J. & DROBNICK, A. (1989). An agonist that is selective for adenylyl cyclase-coupled muscarinic receptors. *Mol. Pharmacol.*, **36**, 465–470.
- BONNER, T.I., BUCKLEY, N.J., YOUNG, A.C. & BRANN, M.R. (1987). Identification of a family of muscarinic receptor genes. *Science*, **237**, 527–532.
- BROWN, D.A., FORWARD, A. & MARSH, S. (1980). Antagonist discrimination between ganglionic and ileal muscarinic receptors. *Br. J. Pharmacol.*, **71**, 362–364.
- BROWN, D.A. & HIGASHIDA, H. (1988). Voltage- and calcium-activated potassium currents in mouse neuroblastoma × glioma hybrid cells. *J. Physiol.*, **397**, 149–165.
- BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M. & BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.*, **35**, 469–476.
- CAULFIELD, M.P. (1991). Muscarinic receptor-mediated inhibition of voltage-activated Ca current in neuroblastoma × glioma hybrid (NG 108-15) cells—reduction of muscarinic agonist and antagonist potency by tetraethylammonium (TEA). *Neurosci. Lett.* (in press).
- CAULFIELD, M.P., BROWN, D.A. & BARLOW, R.B. (1991). A pharmacological study of the M_4 muscarinic receptor mediating inhibition of the voltage-gated Ca-current in neuroblastoma × glioma hybrid (NG 108-15) cells. *Br. J. Pharmacol.*, **102**, 149P.
- CHOO, L.K., MITCHELSON, F. & VONG, Y.M. (1986). Interaction of some muscarinic agonists and antagonists at the prejunctional muscarinic receptor in the rabbit ear artery preparation. *Br. J. Pharmacol.*, **87**, 733–740.
- DOCHERTY, R.J. (1988). Gadolinium selectively blocks a component of calcium-current in rodent neuroblastoma × glioma hybrid (NG108-15) cells. *J. Physiol.*, **398**, 33–47.
- DOCHERTY, R.J. & McFADZEAN, I. (1989). Noradrenaline-induced inhibition of voltage-sensitive calcium currents in NG108-15 hybrid cells. *Eur. J. Neurosci.*, **1**, 132–140.
- DUTAR, P. & NICOL, R.A. (1988). Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second-messenger systems: electrophysiological studies *in vitro*. *J. Neurosci.*, **8**, 4214–4224.
- ELTZE, M. (1988). Muscarinic M_1 - and M_2 -receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol.*, **151**, 205–221.
- FUKUDA, K., HIGASHIDA, H., KUBO, T.I., MAEDA, A., AKIBA, I., BUJO, H., MISHINA, M. & NUMA, S. (1988). Selective coupling with K^+ currents of muscarinic acetylcholine receptor subtypes in NG 108-15 cells. *Nature*, **335**, 355–358.
- FURCHGOTT, R.F. (1966). The use of β -haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. *Adv. Drug Res.*, **3**, 21–55.
- GAHWILER, B.H. & BROWN, D.A. (1987). Muscarine affects calcium-currents in rat hippocampal pyramidal cells *in vitro*. *Neurosci. Lett.*, **76**, 301–306.
- GILANI, S.A.H. & COBBIN, L.B. (1986). The cardio-selectivity of himbacine: a muscarine receptor antagonist. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **332**, 10–20.
- HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfugler's Arch.*, **391**, 85–100.
- HASUO, H., GALLAGHER, J.P. & SHINNICK-GALLAGHER, P. (1988). Disinhibition in the rat septum mediated by M_1 muscarinic receptors. *Brain Res.*, **438**, 323–327.
- HIGASHIDA, H., HASHII, M., FUKUDA, K., CAULFIELD, M.P., NUMA, S. & BROWN, D.A. (1990). Selective coupling of different muscarinic acetylcholine receptors to neuronal calcium currents in DNA-transfected cells. *Proc. R. Soc. B.*, **242**, 68–74.
- HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.
- JECK, D., LINDMAR, R., LOFFELHOLZ, K. & WANKE, M. (1988). Subtypes of muscarinic receptor on cholinergic nerves and atrial cells of chicken and guinea-pig hearts. *Br. J. Pharmacol.*, **93**, 357–366.
- KUBO, T., MAEDA, A., SUGIMOTO, K., AKIBA, I., MIKAMI, A., TAKAHASHI, H., HAGA, T., HAGA, K., ICHIYAMA, A., KANGAWA, K., MATSUO, H., HIROSE, T. & NUMA, S. (1986a). Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence. *FEBS Lett.*, **209**, 367–372.
- KUBO, T., FUKUDA, K., MIKAMI, A., MAEDA, A., TAKAHASHI, H., MISHINA, M., HAGA, T., HAGA, K., ICHIYAMA, A., KANGAWA, K., KOJIMA, M., MATSUO, H., HIROSE, T. & NUMA, S. (1986b). Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature*, **323**, 411–416.
- LAZARENO, S., BUCKLEY, N.J. & ROBERTS, F.F. (1990). Characterisation of muscarinic M_4 binding sites in rabbit lung, chicken heart and NG 108-15 cells. *Mol. Pharmacol.*, **38**, 805–815.
- LAZARENO, S. & ROBERTS, F.F. (1989). Functional and binding studies with muscarinic M_2 -subtype selective antagonists. *Br. J. Pharmacol.*, **98**, 309–317.
- McFADZEAN, I., MULLANEY, I., BROWN, D.A. & MILLIGAN, G. (1989). Antibodies to the GTP binding protein, G_o , antagonize noradrenaline-induced calcium current inhibition in NG108-15 hybrid cells. *Neuron*, **3**, 177–182.
- McGEE, R., SIMPSON, P., CHRISTIAN, C., MATA, M., NELSON, P. & NIRENBERG, M. (1978). Regulation of acetylcholine release from neuroblastoma × glioma hybrid cells. *Proc. Nat. Acad. Sci. U.S.A.*, **75**, 1314–1318.
- MICHEL, A.D., DELMENDO, R., STEFANICH, E. & WHITING, R.L. (1989). Binding characteristics of the muscarinic receptor subtype of the NG108-15 cell line. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **340**, 62–67.
- MILLER, R.J. (1990). Receptor-mediated regulation of calcium channels and neurotransmitter release. *FASEB J.*, **4**, 3291–3299.
- MOCHIDA, S. & KOBAYASHI, H. (1986). Activation of M_2 muscarinic receptors causes an alteration of action potentials by modulation of Ca entry in isolated sympathetic neurons of rabbits. *Neurosci. Lett.*, **72**, 199–204.
- PERALTA, E.G., ASHKENAZI, A., WINSLOW, J.W., SMITH, D.H., RAMACHANDRAN, J. & CAPON, D.J. (1987). Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO J.*, **6**, 3923–3929.
- RANG, H.P. (1965). The kinetics of action of acetylcholine antagonists in smooth muscle. *Proc. R. Soc. B.*, **161**, 488–510.
- ROBBINS, J. & SIM, J. (1990). A transient outward current in NG 108-15 neuroblastoma × glioma hybrid cells. *Pfugler's Arch.*, **416**, 130–137.
- SHERIDAN, R.D. & SUTOR, B. (1990). Presynaptic M_1 muscarinic cholinceptors mediate inhibition of excitatory synaptic transmission in the hippocampus *in vitro*. *Neurosci. Lett.*, **108**, 273–278.
- SONG, S.Y., SAITO, S., NOGUCHI, K. & KONISHI, S. (1989). Different GTP-binding proteins mediate regulation of calcium channels by acetylcholine and noradrenaline in rat sympathetic neurons. *Brain Res.*, **494**, 383–386.
- TSE, A., CLARK, R.B. & GILES, W.R. (1990). Muscarinic modulation of calcium current in neurones from the interatrial septum of bullfrog heart. *J. Physiol.*, **427**, 127–149.
- WANKE, E., FERRONI, A., MALGAROLI, A., AMBROSINI, A., POZZAN, T. & MELDOLESI, J. (1987). Activation of muscarinic receptor selectively inhibits a rapidly inactivated Ca^{2+} current in rat sympathetic neurons. *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 4313–4317.
- WILLIAMS, S.H. & CONSTANTIN, A. (1988). A quantitative study of the effects of some muscarinic antagonists on the guinea-pig olfactory cortex slice. *Br. J. Pharmacol.*, **93**, 855–862.

(Received March 20, 1991)

Revised May 8, 1991

Accepted May 9, 1991