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Cloned rat M3 muscarinic receptors mediate phosphoinositide hydrolysis but not adenylate cyclase inhibition

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Rat M3 mAChR subtype was stably expressed in RAT 1 cells. Investigation of the pharmacological and biochemical properties of the cloned M3 receptors revealed that they mediate phosphoinositide hydrolysis but not adenylate cyclase inhibition. The similarities and differences between the properties of cloned rat M1 and M3 receptors are discussed.

Muscarinic receptor; Receptor ligand binding; Receptor subtype comparison; Phosphoinositide hydrolysis

1. INTRODUCTION

Muscarinic acetylcholine receptors (mAChR) are a family of at least four distinct but highly homologous subtypes, coded for by four different genes and termed M1, M2, M3 and M4 ([1,2] and references therein). The binding of agonist to mAChR elicits multiple biochemical responses via activation of G proteins, such as inhibition of adenylate cyclase (AC), induction of phosphoinositide (PI) hydrolysis, and opening of potassium, calcium and sodium channels (reviews [3,4]).

It seems reasonable to assume that individual muscarinic receptor subtypes might display characteristic ligand-binding profiles as well as specific coupling to the various second-messenger signaling systems mediated by mAChR. However, correlation between a specific subtype and a particular second-messenger system(s) is difficult to demonstrate, partly because of the lack of a homogeneous source for each of the four subtypes. In order to determine the pharmacological and biochemical effects of a particular subtype and to compare them with those of others, each subtype must first be expressed separately in the same cellular context. Recently we isolated the rat M1 and M3 receptor genes and stably expressed the M1 subtype in a rat cell line lacking endogenous mAChR. The cloned M1 receptors were found to mediate both AC inhibition and PI turnover [5]. Since the M1 and M3 subtypes show greater homology to one another than to either M2 or M4 [1], it was of particular interest to determine and compare their ligand-binding profiles and biochemical responses. In the present study we stably expressed rat M3 mAChR subtype in the same rat cell line as that used earlier for the expression of M1 receptors. We describe here the pharmacological and biochemical properties of the cloned M3 receptor.

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2. MATERIALS AND METHODS

2.1. *Materials*

N-[^{3}H]Methylscopolamine ([^{3}H]NMS) (73 Ci/mmol) and [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) (43.6 Ci/mmol) were purchased from NEN. Pirenzepine hydrochloride (PZ) and AF-DX 116 were donated by Karl Thomae (FRG), and 4-diphenylacetyl-*N*-methylpiperidine methiodide (4-DAMP) was a gift from Dr R. Barlow. Atropine sulfate, carbamylcholine and oxotremorine were from Sigma.

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2.2. *Expression of rat M3 receptors*

M3 genomic *Acc*I-*Nde*I fragment was end-filled and subcloned into the end-filled *Eco*RI site of the mammalian expression vector pMV7 [6] (gift from Dr B. Weinstein). RAT-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells were plated in 10-cm Petri dishes (5×10^5 cells per dish) and transfected with 2 μg of pMV7-M3 vector by calcium phosphate precipitation [7]. Colonies were selected and grown in medium containing the antibiotic G418 (350 μg/ml).

2.3. *Ligand-binding assay*

Cells were scraped from the plates with phosphate-buffered saline (PBS), pH 7.4, and homogenates were prepared as described [5]. As also described [8], the binding of labeled muscarinic antagonists to homogenates of cell cultures was assayed by the filtration method [5,8], the binding of unlabeled muscarinic agonists was measured by competition with 1.8 nM [^{3}H]4NMPB [5,8], and binding isotherms and competition curves were analyzed by a nonlinear curve-fitting procedure using a model for either one or two binding sites [5,8].

2.4. *Assay for phosphoinositide hydrolysis*

Cells were labeled with 5 μCi/ml [^{3}H]inositol (17 Ci/mmol; Amersham) for 18 h, washed 3 times with PBS and incubated for 10 min with DMEM containing 8 mM $CaCl_2$, 20 mM Hepes buffer and 10 mM LiCl [5,9]. Different concentrations of carbachol were added for an additional 30 min. The reaction was terminated by resuspension of the cells in H_2O and the addition of chloroform/methanol (1:2). The water-soluble products of PI hydrolysis were separated by extraction of the aqueous phase from the chloroform phase followed by ion-exchange chromatography.

2.5. *Inhibition of cAMP accumulation*

Cells were incubated with DMEM medium containing 20 mM Hepes and 200 μM isobutylmethylxanthine for 20 min, followed by incubation with 1 μM isoproterenol for 1 min with different concentrations of carbachol. The reaction was then terminated by the addition of 50 mM hot (80°C) acetate buffer (pH 4) to the cells. The level of cAMP was determined as described [5].

3. RESULTS

3.1. *Expression of rat M3 receptor subtype in RAT-1 cells*

Rat M3 gene, referred to here as M3 [1], was isolated as described [5]. Since the protein-coding region of M3 is contained within a single exon [1], genomic clone was used to isolate a 3.8-kb genomic *Acc*I-*Nde*I fragment which contains the entire coding sequence for M3 mAChR and the polyadenylation signal. This fragment was subcloned into the *Eco*RI site of the mammalian expression vector pMV7, thus generating the muscarinic receptor construct pMV7-M3. RAT-1 cells were transfected with the muscarinic expression vector pMV7-M3, following the selection of stable transformants by employing the antibiotic G418. Colonies resistant to G418 were isolated and checked for the expression of M3 mRNA (not shown) and for binding of the muscarinic antagonist [^{3}H]4NMPB to their membranes. One of the colonies, M3-2, which was found to express [^{3}H]4NMPB binding sites at a level (110 fmol/mg protein) comparable to that of PRR-1 (cloned M1 receptor in the same cell line [5]), was selected for further analysis.

3.2. *Binding characteristics*

Membranes prepared from M3-2 cells were examined for the binding of muscarinic antagonists and agonists. The antagonists [^{3}H]4NMPB, [^{3}H]QNB and [^{3}H]NMS yielded saturable binding curves (not shown) with the following binding parameters: for [^{3}H]4NMPB, B_{max} = 110 fmol/mg protein, K_D = 1.6 nM; for [^{3}H]QNB, B_{max} = 120 fmol/mg protein, K_D = 1.3 nM; for [^{3}H]NMS, B_{max} = 54 fmol/mg protein, K_D = 0.54 nM. Competition for [^{3}H]4NMPB labeled sites by the selective muscarinic antagonist 4-DAMP, PZ and AF-DX 116 is illustrated in fig.1. Analysis of these curves (table 1) yielded two binding states of the M3 receptor, one of high and one of low affinity, for both 4-DAMP and PZ. For AF-DX 116, however, the analysis showed a single low-affinity binding state of the receptor with a K_D of 4 μM (n_H = 1.1).

The binding properties of the muscarinic agonists carbamylcholine, oxotremorine and acetylcholine were examined by competition with ^{3}H-antagonist. All three agonists showed both high- and low-affinity binding for [^{3}H]4NMPB-labeled sites (table 1). When Gpp(NH)p (300 μM) was included in the incubation mixture the carbamylcholine binding curve was shifted to the right (not shown), and analysis revealed that 57% of the high-affinity binding sites had been converted to the low-affinity state.

3.3. *Mediation of phosphoinositide hydrolysis by cloned M3 receptors*

PI hydrolysis was determined by measuring the accumulation of [^{3}H]inositol trisphosphate ([^{3}H]IP_3), [^{3}H]inositol bisphosphate ([^{3}H]IP_2) and [^{3}H]inositol monophosphate ([^{3}H]IP_1) in the presence of 10 mM LiCl (which blocks IP_1 [10]).

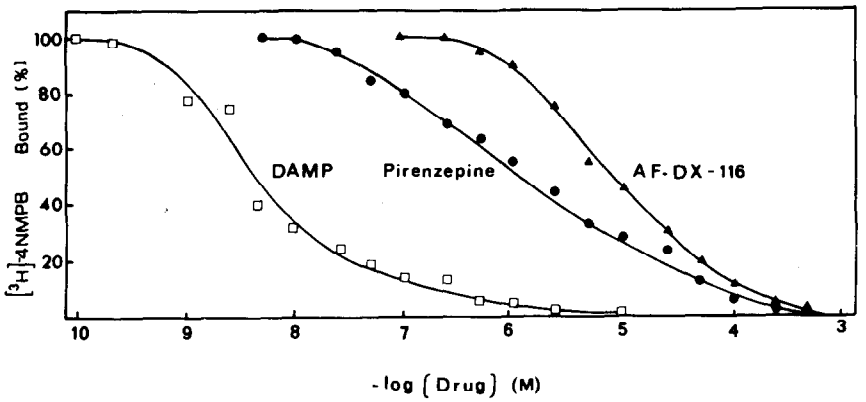

Fig.1. Concentration-dependent displacement of [^{3}H]4NMPB (1.8 nM) by the selective muscarinic antagonists 4-DAMP, pirenzepine and AF-DX 116. Binding assays were performed as described in section 2 in homogenates prepared from M3-2 cells.

Treatment of cells with the muscarinic agonist carbamylcholine (10^{-3} M), for various periods revealed time-dependent accumulation of IP_1, IP_2, IP_3; for example, incubation for 10 min increased their accumulation by 1.8-, 1.5- and 1.5-fold, respectively, while incubation for 30 min increased it by 3.6-, 3.3- and 1.5-fold, respectively. Accordingly, in subsequent experiments a 30 min period of incubation with carbamylcholine was adopted in order to allow maximal accumulation of [^{3}H]IP_1 as an index of PI hydrolysis. The concentration dependence of the carbamylcholine effect on PI hydrolysis is illustrated in fig.2. As shown, the ED_{50} of carbamylcholine-stimulated PI hydrolysis was 7×10^{-5} M. Maximum hydrolysis of 3.6-fold relative to the basal value was reached at 10^{-3} M

Table 1

Parameters obtained from displacement of [^{3}H]4NMPB (1.8 nM) by antagonists and agonists in homogenates of M3-2 cells

Ligands	K_H	K_L	R_H	n_H
4-DAMP	1.3 nM	116 nM	83%	0.7
Pirenzepine	76 nM	6.2 μM	49%	0.5
Acetylcholine	3.9 μM	57 μM	54%	0.5
Oxotremorine	8.3 μM	47 μM	46%	0.7
Carbamylcholine	74 μM	1.1mM	56%	0.8

Values are means of three experiments. R_H is the percentage of high-affinity binding sites. K_H and K_L are the dissociation constants for the binding of ligands to the high- and low-affinity sites, respectively. Values were determined by Scatchard analysis and nonlinear least-squares best-fit computer analysis

carbamylcholine. This carbamylcholine effect was completely blocked by 50 μM atropine, but was unaffected by pretreatment with pertussis toxin (100 ng/ml).

3.4. *Testing for inhibition of adenylate cyclase in M3-2 cells by cloned M3 receptors*

Examination of the effects of various concentrations (10^{-10}–10^{-3} M) of carbamylcholine on isoproterenol-induced cAMP levels revealed no significant reduction in cAMP levels over this concentration range. Thus, in control M3-2 cells

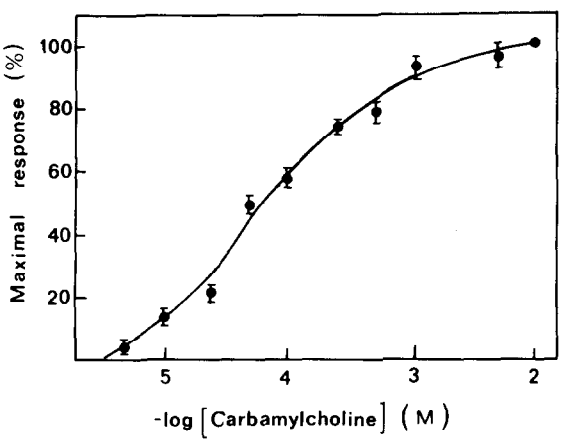

Fig.2. Concentration dependence of the carbamylcholine effect on PI hydrolysis in M3-2 cells. Cells were incubated with the indicated concentrations of carbamylcholine for 30 min at 37°C. [^{3}H]IP_1 was determined as described in section 2. The data points represent the percentages of maximal response in the presence of carbamylcholine.

(without carbamylcholine) the level of isoproterenol-induced cAMP was 26.6 ± 5.9 pmol/10^6 cells, whereas in cells treated for 1 min with 10^{-7} M or 10^{-6} M carbamylcholine the cAMP levels were 20.4 ± 4.2 and 26.9 ± 6.0 pmol/10^6 cells, respectively. It should be noted that in some experiments carbamylcholine at 10^{-3} M increased isoproterenol-induced cAMP levels by 25–50% above the control.

4. DISCUSSION

Our data demonstrate that cloned M3 receptors from M3-2 cells (RAT-1 cells stably transfected with M3 muscarinic expression vector) bind muscarinic antagonist and agonist, couple to G-protein and mediate PI turnover but not AC inhibition. M3-2 cells were found to express 1.4×10^4 M3 receptors per cell. This receptor number is close to that observed for cell lines expressing endogenous muscarinic receptors ([5] and references therein) as well as for PRR-1 (RAT-1 cells in which cloned rat M1 receptors are stably expressed at 2×10^4 receptors per cell). One may thus reliably compare the binding profiles and biochemical responses of cloned M3 receptors with those of endogenous receptors and of cloned rat M1 receptors.

The demonstration of multiple muscarinic receptor subtypes suggests the possible existence of a mechanism by which a single ACh neurotransmitter can elicit distinct cellular responses, i.e. by the coupling of individual receptor subtypes to different G proteins and/or different signaling systems. A comparison of our data concerning the biochemical responses of cloned M1 and M3, when expressed within the same cellular context, indicates that this may indeed be the case. Thus, as we reported recently [5], rat M1 receptors mediate both PI hydrolysis and AC inhibition, while rat M3 receptors mediate PI hydrolysis only. In addition, human cloned M2 is reportedly coupled to both AC inhibition and PI turnover [11].

In cloned rat M1 receptors the inhibition of cAMP accumulation was atropine blocked and dose dependent (10^{-9}–10^{-6} M carbamylcholine), with a maximum effect at about 10^{-6} M carbamylcholine. At higher agonist concentration however, we observed in both rat M1 and M3 receptor subtypes an increased accumulation of cAMP (see also below), which was blockable by 1 μM atropine.

Although M1 and M3 receptors differ in their response to AC inhibition, both of them mediate PI hydrolysis with similar ED_{50} values. Their difference in maximal response (3.3-fold for M3 in M3-2 cells versus 7.5-fold for M1 in PRR1 cells [5]) can be explained by the 30% larger receptor number observed for M1 in PRR1 cells than for M3 in M3-2 cells. Previous studies on cloned muscarinic receptors [2,11] have already demonstrated that the maximal effect of PI hydrolysis may depend on the receptor number. A comparison of the binding profiles of M1 and M3 receptors reveals similarities in their binding affinities for the selective muscarinic antagonists AF-DX 116, PZ and 4-DAMP; this finding is consistent with the assignment of cloned M3 receptors to the M1 class [1]. However, agonist binding affinities are about 10-fold higher for M1 than for M3 cloned receptors.

While this paper was in preparation, Peralta et al. [2] reported that cloned human M3 and M1 receptors in human embryonic kidney cells mediate PI hydrolysis but not AC inhibition. Their results are in agreement with ours with regard to the rat M3 receptor but differ for the M1 receptor, where inhibition of cAMP accumulation was observed in addition to PI hydrolysis. This apparent discrepancy between our findings and those reported for human M1 may result from one or more of the following. (i) Differences in properties of the cell lines used for the expression of the M1 receptors. (ii) Difference in receptor number: e.g., 14.1×10^4 [^{3}H]QNB sites per cell in HM1 cells versus 2×10^4 [^{3}H]QNB sites per cell in PRR1 cells. (iii) In HM1 carbamylcholine concentrations higher than 10^{-6} M lead to a muscarinically mediated increase in cAMP accumulation, rather than a reduction in cAMP levels, as also observed for rat M1 receptors. However, in contrast to the maximal increase of 9.8-fold in cAMP accumulation reported for HM1, we observed a maximal increase of only 2-fold in the PRR-1 cells; thus, perhaps the large increase in cAMP level on the one hand masks the effect of cAMP reduction on the other. The nature of this cAMP-muscarinic mediated increase in both types of mAChR subtypes is currently under investigation in our laboratory.

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