The EMBO Journal vol.7 no.10 pp.3031 – 3035, 1988

Cloned M1 muscarinic receptors mediate both adenylate cyclase inhibition and phosphoinositide turnover

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Communicated by A.Levitzky

The rat M1 muscarinic receptor gene was cloned and expressed in a rat cell line lacking endogenous muscarinic receptors. Assignment of the cloned receptors to the M1 class was pharmacologically confirmed by their high affinity for the M1-selective muscarinic antagonist pirenzepine and low affinity for the M2-selective antagonist AF-DX-116. Guanylyl imidodiphosphate [Gpp(NH)p] converted agonist binding sites on the receptor, from high-affinity to the low-affinity state, thus indicating that the cloned receptors couple to endogenous G-proteins. The cloned receptors mediated both adenylate cyclase inhibition and phosphoinositide hydrolysis, but by different mechanisms. Pertussis toxin blocked the inhibition of adenylate cyclase (indicating coupling of the receptor to inhibitory G-protein), but did not affect phosphoinositide turnover. Furthermore, the stimulation of phosphoinositide hydrolysis was less efficient than the inhibition of adenylate cyclase. These findings demonstrate that cloned M1 receptors are capable of mediating multiple responses in the cell by coupling to different effectors, possibly to different G-proteins.

Key words: biochemical responses/ligand binding/M1 muscarinic receptors

Introduction

Activation of muscarinic acetylcholine receptors (mAChR) in various cell lines and tissues elicits multiple biochemical responses, such as an increase in intracellular cGMP levels, induction of phosphoinositide (PI) turnover, inhibition of adenylate cyclase (AC), and opening or closing of potassium, calcium, sodium and chloride ion channels (for review see Sokolovsky, 1988).

Pharmacological investigations and studies with purified muscarinic receptors indicate that the mAChRs are coupled to several distinct G-proteins (Sokolovsky, 1988; Haga *et al.*, 1986). These coupled G-proteins mediate certain muscarinic responses such as inhibition of AC (Harden *et al.*, 1980), opening of potassium and sodium channels (Yatani *et al.*, 1987; Logothetis *et al.*, 1987; Cohen-Armon *et al.*, 1988) and PI hydrolysis in astrocytoma cells (Harden *et al.*, 1986).

Recent pharmacological work (for review see Sokolovsky, 1988) and genetic studies (Kubo *et al.*, 1986a,b; Bonner *et al.*, 1987; Peralta *et al.*, 1987) points to the existence of at least four distinct but highly homologous subtypes of mAChRs. These findings raise the question of whether all the subtypes share the same properties or whether each one possesses its own distinctive characteristics, such as coupling to a specific effector system or G-protein. Previous studies do not provide an answer to these questions because of the presence of mutliple receptor subtypes in the experimental material. Characterization of the molecular properties of the M1 muscarinic receptor subtype presents special difficulties because of the lack of tissues or cell lines which express the M1 receptor subtype exclusively. For example, the cerebral cortex, used in the past for studies on the M1 subtype, was found to contain M3 and M4 subtypes as well (Bonner *et al.*, 1987; Peralta *et al.*, 1987). Furthermore, the selective muscarinic antagonist pirenzepine (PZ), which distinguishes between the M1 and M2 subtypes, does not adequately distinguish between M1, M3 and M4. In order to determine the pharmacological and biochemical effects of the M1 subtype, material containing purely M1 is required.

Recently, the porcine M2 receptor subtype was cloned and stably expressed in cells that lack endogenous mAChRs. The pharmacological properties of the single recombinant M2 receptor subtype were found to be identical with those of M2 muscarinic receptor subtype from conventional sources. Furthermore, the recombinant receptor was found to be coupled to both AC inhibition and PI turnover (Ashkenazi *et al.*, 1987). In the present work we cloned and stably expressed the M1 muscarinic receptor subtype gene in a rat cell line, RAT-1, in order to study its molecular responses. We describe here the pharmacological and biochemical properties of the cloned M1 receptors, and show that they are coupled to G-protein and mediate both AC inhibition and PI hydrolysis.

Results

Isolation and expression of rat M1 receptor subtype in RAT-1 cells

Screening of the λgt10 rat brain cDNA library with a 50 base synthetic oligonucleotide probe complementary to the porcine M1 mAChR gene (Kubo *et al.*, 1986a) resulted in the isolation of two distinct but homologous cDNAs. Sequence analysis identified one of them as the rat homologue for porcine M1 mAChR subtype and the other as a novel subtype gene (data not shown). While this work was in progress, Bonner *et al.* (1987) identified the rat mAChR subtype genes M1, M3 and M4. Comparison between these published sequences and our own indicates that we have independently isolated the rat M1 and M3 mAChR subtype genes. Since the protein coding region of M1 is contained within a single exon (Bonner *et al.*, 1987), genomic clones for M1 were obtained by screening the rat genomic library. These genomic clones were used to isolate a genomic DNA fragment which contains the entire coding sequence for M1 mAChR and the polyadenylation signal. This fragment was subcloned into the mammalian expression vector pRR23, thus generating the muscarinic receptor

construct pRR23-H6 (Figure 1A). In order to obtain rat cells expressing M1 mAChR, RAT-1 cells were stably transfected with the construct and with the bacterial neomycin phosphotransferase gene as a selective marker. The G418 (neomycin) resistant colonies were first checked for the expression of muscarinic M1 mRNA. Figure 1(B) demonstrates that M1 mRNA is present in transfected but not in untransfected RAT-1 cells. One of the colonies, PRR-1, was selected for further analysis.

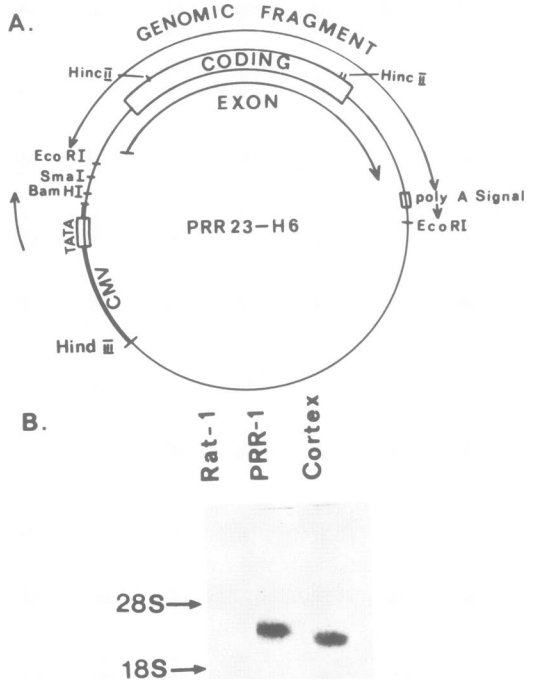

Fig. 1. Expression of M1 mAChR subtype gene in transfected RAT-1 cells. (**A**) Expression vector pRR23-H6. Genomic *Eco*RI DNA fragment was subcloned into the unique EcoRI site in pRR23 plasmid and placed under the transcription control of human cytomegalovirus promoter. The genomic DNA contains an exon which includes the entire coding region and a polyadenylation signal. (**B**) RNA blot analysis of RNA derived from (1) RAT-1; (2) PRR-1; (3) cortex. Total RNA (25 μg) was fractionated by formaldehyde/agarose gel electrophoresis, transferred to GeneScreen and assayed for hybridization to the ^{32}P-labelled *Hinc*II fragment shown in A.

Pharmacological characterization of the cloned M1 receptor

The ability of the cloned receptor to bind muscarinic ligands was examined using muscarinic antagonists and agonists. Figure 2(A) illustrates the binding of the muscarinic antagonist ^{3}H-*N*-methyl-4-piperidylbenzilate ([^{3}H]4NMPB) to membranes prepared from PRR-1 cells. Both ^{3}H-*N*-methylscopolamine ([^{3}H]NMS) (not shown) and [^{3}H]-4NMPB yielded saturable binding curves and the following parameters were obtained; for [^{3}H]4NMPB, B_{max} = 33 fmol/mg protein, K_D = 0.4 nM (n_H = 1.01); for [^{3}H]NMS, B_{max} = 29 fmol/mg protein, K_D = 0.29 nM (n_H = 0.99).

In contrast to PRR-1 cells, the untransfected cells did not specifically bind either [^{3}H]4NMPB or [^{3}H]NMS at the concentration range employed (0.1 – 10 nM). These findings, together with the hybridization data (Figure 1B), demonstrate that RAT-1 cells do not express endogenous muscarinic receptor.

Competition for sites labelled with [^{3}H]4NMPB by the selective muscarinic antagonists AF DX-116 and PZ is illustrated in Figure 2(B). Best-fit computer model and Scatchard analysis indicate a single low-affinity binding state for AF DX-116, with a K_D of 24 μM (n_H = 0.99). For PZ, however, the analysis yielded two binding states of the receptor (n_H = 0.4): 70% of the PZ-binding sites are of high affinity (K_H = 41 nM), and the rest are of low affinity (K_L = 4.5 μM).

Competition for sites labelled with [^{3}H]4NMPB by the muscarinic agonists carbachol, oxotremorine and acetylcholine are illustrated in Figure 3. The best-fit parameters of these curves are recorded in Table I. All three agonists showed both high and low binding affinity for the [^{3}H]4NMPB-labelled sites they displaced, with the high-affinity state accounting for 20–25% of the binding. When guanylyl imidodiphosphate [Gpp(NH)p] was included in the incubation mixture, the curve depicting carbachol binding was shifted to the right (Figure 3A), and analysis of the curves indicated that the high-affinity state had been entirely converted to the low-affinity state (K_L = 181 μM). This Gpp(NH)p effect suggests a coupling of the cloned muscarinic receptor to endogenous G-proteins in the transfected cell.

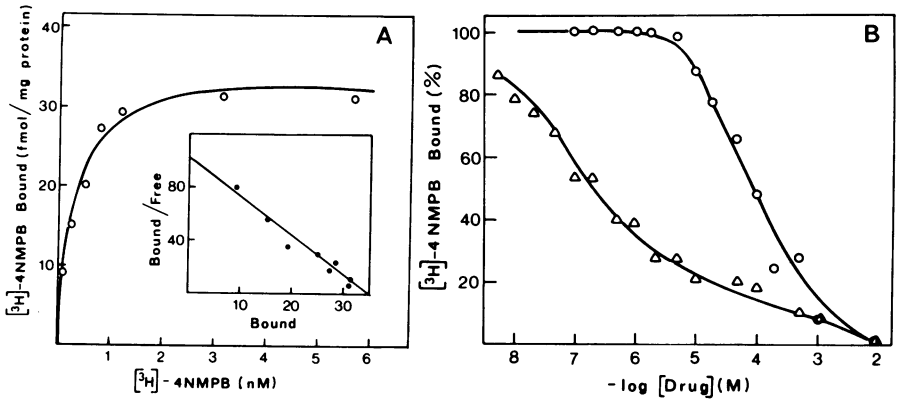

Fig. 2. Binding of muscarinic antagonist to homogenates prepared from PRR-1 cells. (**A**) Specific binding of [^{3}H]4NMPB to homogenates from PRR-1 cells (○). Scatchard plots of the [^{3}H]4NMPB-binding data are presented in the insert. (**B**) Concentration-dependent displacement of 1 nM [^{3}H]4NMPB by pirenzepine (△) and by AF DX-116 (○). Non-specific binding was determined with 10 μM atropine.

Mediation of adenylate cyclase inhibition by cloned M1 receptors

In order to determine whether the cloned M1 receptors can inhibit AC activity in PRR-1 cells, we examined the effects induced by carbachol on intracellular basal and isoproterenol-induced cyclic adenosine 3′,5′ monophosphate (cAMP) levels.

Isoproterenol increased the basal level of cAMP in PRR-1 cells by up to 9.3 ± 2.5-fold. As shown in Figure 4, carbachol inhibited the isoproterenol-induced cAMP levels in a dose-dependent manner with half-maximal inhibition (IC_{50}) at 1.1×10^{-8} M carbachol and maximal inhibition at 10^{-6} M carbachol. The carbachol response was blocked by 0.1−1 μM atropine, thus indicating that the inhibitory effect of carbachol on the cAMP level is mediated by muscarinic receptors. Pertussis toxin (PTX), which catalyses the adenosine diphosphate (ADP)-ribosylation of certain G-proteins, abolished the carbachol inhibitory effect when added 18 h prior to carbachol treatment. Carbachol also reduced the basal level of cAMP in the PRR-1 cell, with a maximum of 70% reduction at 10^{-7} M carbachol (data not shown). In similar experiments conducted on untransfected RAT-1 cells, carbachol had no effect on either basal or isoproterenol-induced cAMP levels.

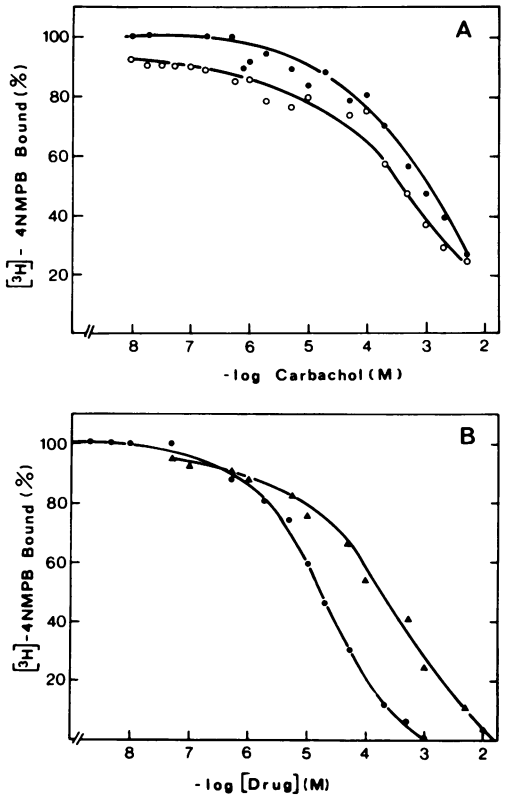

Fig. 3. Concentration-dependent displacement of 1 nM [^{3}H]4NMPB by muscarinic agonists in homogenates prepared from PRR-1 cells. (**A**) Displacement of [^{3}H]4NMPB by carbachol in the absence (○) and presence (●) of Gpp(NH)p. (**B**) Displacement of [^{3}H]4NMPB by oxotremorine (●) and by acetylcholine (▲). Homogenates were prepared from PRR-1 cells. Binding assays were performed as described in Materials and methods.

Table I. Parameters obtained from displacement of 1 mM [^{3}H]4NMPB by agonists in homogenates of PRR-1 cells

Agonist	K_H (nM)	K_L (μM)	R_H
Carbachol	92	129	25
Acetylcholine	52	117	20
Oxotremorine	15	8.4	23

The parameters derived from analysis of the competition displacement data are shown in Figure 3. Values are means of three experiments. R_H is the percentage of high-affinity agonist-binding sites. K_H and K_L are the dissociation constants for the binding of agonists to the high- and low-affinity sites, respectively. Values were determined by Scatchard analysis and non-linear least squares best-fit computer analysis.

Mediation of phosphoinositide hydrolysis by cloned M1 receptors

PI hydrolysis was determined by 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, Berridge *et al.*, 1982). Incubation of cells with 10^{-3} M carbachol for

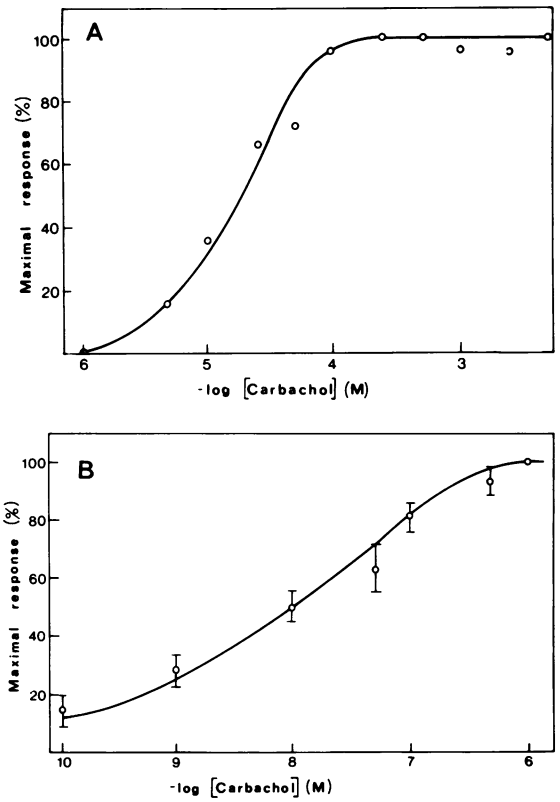

Fig. 4. Concentration dependence of the effect of carbachol on cAMP levels and PI hydrolysis in PRR-1 cells. (**A**) Effect of carbachol on IP_1 accumulation. PRR-1 cells were incubated with the indicated concentrations of carbachol for 30 min at 25°C. [^{3}H]IP_1 was determined as described in Materials and methods. The data shown are percentages of maximal response in the presence of carbachol. (**B**) Inhibition of isoproterenol induced stimulation of cAMP. PRR-1 cells were equilibrated with IBMX (100 μM) and isoproterenol (1 μM) and treated with the indicated concentration of carbachol for 1 min at 25°C. Levels of cAMP were determined as described in Materials and methods. The data shown are percentage inhibition of the cAMP level in the presence of carbachol. Values are means ± SEM of five experiments.

5 min increased the formation of $[^3H]IP_3$, $[^3H]IP_2$ and $[^3H]IP_1$ by 2-, 2.8- and 4.8-fold, respectively, while incubation for 30 min increased their formation by 1.7-, 4.4- and 7.5-fold, respectively. Thus, in subsequent experiments cells were incubated with carbachol for 30 min in order to allow maximal accumulation of $[^3H]IP_1$ as an index of PI hydrolysis. The concentration dependence of the carbachol effect on PI hydrolysis is illustrated in Figure 4. As shown, the ED_{50} for carbachol-stimulated PI hydrolysis was 5×10^{-5} M. Maximum hydrolysis of 7.5-fold relative to the basal value was reached at 10^{-3} M carbachol.

The carbachol effect was completely blocked by 50 μM atropine, but was unaffected by pretreatment with PTX (data not shown). In similar experiments conducted on untransfected RAT-1 cells, carbachol had no effect on PI hydrolysis.

Discussion

The rat cell line, RAT-1, was stably transfected with a vector directing the expression of rat M1 mAChR subtype gene. The transfected cells contain 6×10^3 receptors per cell. This receptor number is close to those observed for cell lines expressing endogenous muscarinic receptors such as: NG 108-15, N1E-115, PC-12 (Strange *et al.*, 1978; Jumblatt and Tischler, 1982; Akiyama *et al.*, 1984). These receptors are coupled to both AC inhibition and PI turnover. Identification of the isolated gene as that coding for the rat M1 mAChR subtype was based on its marked homology to the porcine M1 mAChR gene and its identity with the rat M1 sequence published while this work was in progress (Bonner *et al.*, 1987). The M1 mRNA transcript in the transfected cells is slightly larger than that in the cortex. The difference in size is due to our use of a genomic fragment for the expression of the receptor gene. Since this fragment contains, in addition to the entire coding region and the polyadenylation signal, a 550 bp intron sequence upstream of the exon (Figure 1A), the transcript predicted from the pRR23-H6 construct should be larger than the transcript obtained from the endogenous gene. However, the 550 bp added upstream of the coding region should not alter the receptor protein, since sequence analysis of this region reveals that the only correct open reading frame for M1 protein is the one which starts with the native AUG.

The binding affinity states of the selective muscarinic antagonists PZ and AF DX-116 for our cloned receptor are in agreement with that reported for the M1 muscarinic receptor subtype in rat cortex (Gurwitz *et al.*, 1985; Giraldo *et al.*, 1987) and consistent with the assignment of the cloned receptor to the M1 class. Moreover, pharmacological characterization of the cloned receptor by employing other muscarinic ligands revealed binding affinities similar to those previously reported for M1 receptors in the rat cortex (Gurwitz *et al.*, 1985) and for cloned rat M1 receptors in mouse A9 L cells (Brann *et al.*, 1987). Interestingly, our data indicate two binding states for the M1-slective antagonist PZ, one of high (70% of the sites) and the other of low affinity. This finding of a cloned muscarinic receptor with two binding states for antagonist is not altogether unexpected in view of an earlier report that cloned porcine M2 receptors bind AF DX-116 both with low and with high affinities (Peralta *et al.*, 1987).

The cAMP levels in the transfected cells were reduced in the presence of the muscarinic agonist carbachol. The observed alteration in cAMP levels was not due to muscarinic activation of cAMP phosphodiesterase (Harden *et al.*, 1986), since the phosphodiesterase inhibitor IBMX was included in the reaction mixture. Gpp(NH)p converted the high-affinity carbachol binding sites to the low affinity state, thus indicating that the cloned M1 receptors couple to G-proteins. In addition, since PTX promotes the ADP-ribosylation of certain G-proteins, the PTX-induced abolition of the carbachol effect on cAMP levels points to the involvement of a G-protein in this effect. It thus seems likely that the reduction of cAMP levels by the activated M1 receptors is mediated via coupling to the inhibitory G-protein (G_i), which inhibits AC activity. On the other hand, PTX had no effect on carbachol-stimulated PI hydrolysis, indicating that the effect of the cloned M1 receptors on PI hydrolysis is not mediated via coupling to PTX-sensitive G-proteins. The inability of such G-proteins to mediate muscarinic PI effects is in line with findings from studies conducted on mAChR from tissues and cell lines (Masters *et al.*, 1985; Harden *et al.*, 1986; Lo and Hughes, 1987).

The coupling of cloned M1 receptors to multiple effector systems, e.g. AC inhibition and PI turnover (as discussed in this paper) and induced current (as recently shown by Brann *et al.*, 1987), appears to be a common feature of the muscarinic subtype family. For example, Ashkenazi *et al.* (1987) showed that cloned porcine M2 receptors are coupled both to PI hydrolysis and to AC inhibition in hamster CHO cell line. Furthermore, our results, like those of Ashkenazi *et al.* (1987), demonstrate that in addition to the coupling of one muscarinic receptor subtype to multiple effector systems, different muscarinic receptor subtypes are able to activate the same effector system. This finding is in contrast to the view suggesting that each receptor subtype is coupled to a different effector system. The ability of different muscarinic receptor subtypes to activate the same effector system raises the question of why different subtypes should exist. One possible answer is that although M1 and M2 are capable of evoking the same responses, they induce different responses in different cell types. In other words, the type and the potency of different mAChR subtype responses might be determined by the contents of the cell which expresses the receptors. In this way the M1 mAChR subtype for example, will induce PI hydrolysis in one cell type and AC inhibition in the other. Possible examples of such cellular contents are G-proteins or membrane components that interact with the mAChRs.

While it appears that different muscarinic receptor subtypes activate the same effector systems, it is conceivable that in addition to the shared responses each receptor subtype may also induce a specific response. This possibility is supported by the finding that *Xenopus* oocytes injected with mAChR M1 specific mRNA or with mAChR M2 specific mRNA, expressed muscarinic receptor subtypes that differ in their inward current responses (Fukuda *et al.*, 1987).

The system described here, namely, cloned muscarinic receptors which are successfully coupled to endogenous cellular effector systems, provides a useful tool for further studies on the pharmacological and biochemcial properties of these and other muscarinic receptor subtypes. A study of the correlation between receptor structure and function,

by means of a carefully designed series of mutageneses and subsequent transfection, is currently under way.

Materials and methods

Screening of rat brain cDNA and rat genomic libraries

The λgt10 rat brain cDNA library (supplied by D.J.Anderson, Department of Biology, Caltech, CA) was screened with 5′-end-labelled 50-base oligonucleotide derived from the porcine M1 muscarinic receptor sequence (Kubo *et al.*, 1986a) complementary to nucleotides 1198–1248. Hybridization was performed at 42°C for 20 h in a solution containing 35% formamide, 5 × SSCPE and 1 ×Denhardt's solution. Filters were washed to 2 × SSC at 60°C. Two different cDNA clones, λgt10-M1 and λgt10-M3, were isolated. The inserts were purified, digested and subcloned into M13mp18/19 for sequence analysis. The rat genomic *Eco*RI partial library (Sargent *et al.*, 1979) was screened with nick-translated probes derived from λgt10-M1 and λgt10-M3 inserts. Hybridization and washing were performed under standard conditions (Maniatis *et al.*, 1982).

Expression of rat M1 receptors

M1 genomic *Eco*RI fragment (Figure 1B) was subcloned into the *Eco*RI site of the mammalian expression vector pRR23 [the generous gift of B.Fleckestein (Boshart *et al.*, 1985)]. 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 at a density of 5×10^5 cells per dish and co-transfected with 2 μg pRR26-H6 vector and 100 ng pIBW (a plasmid containing the gene for neomycin resistance) by the calcium phosphate precipitation method (Wigler *et al.*, 1979). Colonies were selected and grown in medium containing G418 at 350 μg/ml.

Ligand-binding assay

Cells were scraped from the plates with phosphate buffered saline (PBS), pH 7.4, and homogenates were prepared as previously described (Kloog and Sokolovsky, 1978). Protein concentration was determined according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Binding of the labelled muscarinic antagonist [^{3}H]4NMPB to homogenates of cell cultures was assayed essentially as described (Kloog *et al.*, 1979). Briefly, aliquots of the homogenates were incubated at 25°C with different concentrations of [^{3}H]4NMPB. The reaction was terminated by rapid filtration through Whatman GF/C filters, followed by washing three times with cold modified Krebs buffer and counting the radioactivity by liquid scintillation spectrometry (Packard Tri-carb 300). Nonspecific binding was determined in the presence of 10 μM unlabelled atropine and subtracted from the total binding to yield the specific binding. The binding of unlabelled muscarinic agonists was measured as previously described (Kloog *et al.*, 1979) by competition with 1 nM [^{3}H]4NMPB.

Binding isotherms and competition curves were analysed by a non-linear curve-fitting procedure using a model for either one or two binding sites, as detailed in previous reports (Gurwitz and Sokolovsky, 1980; Kloog *et al.*, 1979), employing the curve-fitting program LIGAND (May 1987 version) developed by P.J.Munson (Laboratory of Theoretical and Physical Biology, National Institutes of Health, Bethesda, MD). In the case of [^{3}H]4NMPB binding, which yields linear Scatchard plots, a one-site model was employed for the analysis (Gurwitz and Sokolovsky, 1980; Kloog *et al.*, 1979). The binding of unlabelled agonists in competition with [^{3}H]4NMPB was analysed by a two-site model incorporating high- and low-affinity state, to which antagonists bind with equal affinity (Kloog *et al.*, 1979).

Inhibition of cAMP accumulation

Cells were incubated with DMEM medium containing 20 mM Hepes and 100 μM isobutylmethylxanthine (IBMX) for 20 min, followed by incubation of 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 using a modification (Naor *et al.*, 1975) of the competition protein-binding assay described by Gilman (1970).

Assay for phosphoinositide hydrolysis

The protocol used was as previously described (Gurwitz and Sokolovsky, 1987). Briefly, cells were labelled with 5 μCi/ml [^{3}H]inositol (17 Ci/mmol; Amersham) for 18 h, washed three times with PBS and incubated for 10 min with DMEM containing 8 mM $CaCl_2$, 20 mM Hepes buffer and 10 mM LiCl. Different concentrations of carbachol were added for an additional 30 min. The reaction was terminated by resuspending the cells in H_2O and adding 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.

Acknowledgements

Mrs Ronit Galron provided excellent technical assistance. We are grateful to Dr Yoel Kloog for enlightening discussion and suggestions. We thank Mrs Shirley Smith for excellent editorial assistance.

References

Akiyama,K., Watson,M., Roeske,W.R. and Yamamura,H.I. (1984) *Biochem. Biophys. Res. Commun.*, **119**, 289–297.

Ashkenazi,A., Winslow,J.W., Peralta,E.G., Peterson,G.L., Schimerlik,M.I., Capon,D.J. and Ramachandan,J. (1987) *Science*, **238**, 672–675.

Berridge,M.J., Downes,C.P. and Hanely,M.R. (1982) *Biochem. J.*, **206**, 587–609.

Bonner,T.I., Buckley,N.J., Young,A.C. and Brann,M.R. (1987) *Science*, **237**, 527–531.

Boshart,M., Weber,F., Jahn,G., Dorsch-Hasler,K., Flecknstein,B. and Schaffner,W. (1985) *Cell*, **41**, 521–530.

Brann,M.R., Buckley,N.J., Penelope Jones,S.V. and Bonner,T.I. (1987) *Mol. Pharmacol.*, **32**, 450–455.

Cohen-Armon,M., Garty,H. and Sokolovsky,M. (1987) *Biochemistry*, **27**, 368–374.

Fukuda,K., Kubo,T., Akiba,I., Maeda,A., Mishima,M. and Numa,S. (1987) *Nature*, **327**, 623–625.

Gilman,A.G. (1970) *Proc. Natl. Acad. Sci. USA*, **67**, 305–312.

Giraldo,E., Hammer,R. and Ladinsky,H. (1987) *Life Sci.*, **40**, 833–840.

Gurwitz,D. and Sokolovsky,M. (1980) *Biochem. Biophys. Res. Commun.*, **96**, 1293–1301.

Gurwitz,D. and Sokolovsky,M. (1987) *Biochemistry*, **26**, 633–638.

Gurwitz,D., Kloog,Y. and Sokolovsky,M. (1985) *Mol. Pharmacol.*, **28**, 295–297.

Haga,K., Haga,T. and Ichiyama,A. (1986) *J. Biol. Chem.*, **361**, 10133–10140.

Harden,T.K., Tanner,L.I., Martin,M.W., Nakahata,N., Hughes,A.R., Hepeler,J.R., Evans,T., Master,S.B. and Brown,J.H. (1986) *Trends Pharmacol. Sci.*, **7** (Suppl.), 14–18.

Jumblatt,J.E. and Tischler,A.S. (1982) *Nature*, **247**, 152–154.

Kloog,Y. and Sokolovsky,M. (1978) *Biochem. Biophys. Res. Commun.*, **81**, 710–717.

Kloog,Y., Egozi,Y. and Sokolovsky,M. (1979) *Mol. Pharmacol.*, **15**, 545–558.

Kubo,T., Fukuda,K., Mikami,A., Maeda,A., Takahashi,H., Mishima,M., Haga,T., Haga,K., Ichiyama,A., Kangawa,K., Kojima,M., Matsuo,H., Hirose,T. and Numa,S. (1986a) *Nature*, **323**, 411–416.

Kubo,T., Maeda,A., Sugimoto,K., Akiba,I., Mikami,A., Takahashi,H., Haga,T., Haga,K., Ichiyama,A., Kangawa,K., Matsuo,H., Hirose,T. and Numa,S. (1986b) *FEBS Lett.*, **209**, 367–372.

Lo,W.W.Y. and Hughes,J. (1987) *FEBS Lett.*, **220**, 115–151.

Logothetis,D.E., Kurachi,Y., Galper,J., Neer,E.J. and Clapham,D.E. (1987) *Nature*, **325**, 321–326.

Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265–275.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Masters,S.B., Martin,M.W., Harden,T.K. and Brown,J.H. (1985) *Biochem. J.*, **227**, 933–937.

Naor,Z., Koch,Y., Chasieng,P. and Zor,U. (1975) *FEBS Lett.*, **58**, 318–321.

Peralta,E.G., Ashkenazi,A., Winslow,J.W., Smith,D.H., Ramachandran,J. and Capon,D.J. (1987) *EMBO J.*, **6**, 3923–3929.

Sargent,T.D., Wu,J.-R., Sala-Trepat,J.M., Wallace,R.B., Reyes,A.A. and Banner,J. (1979) *Proc. Natl. Acad. Sci. USA*, **78**, 3521–3525.

Sokolovsky,M. (1988) *Adv. Drug Res.*, **18**, in press.

Strange,P.G., Birdsall,N.J.M. and Burgen,A.S.V. (1978) *J. Biochem.*, **172**, 495–501.

Wigler,M., Pellicer,A., Silverstein,S., Axel,R., Urlaub,G. and Chasin,L. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1373–1376.

Yatani,A., Codina,Y., Brown,A.M. and Birnbaumer,L. (1987) *Science*, **235**, 207–211.

Received on May 9, 1988; revised on June 23, 1988