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Evidence that muscarinic cholinergic receptors selectively interact with either the cyclic AMP or the inositol phosphate second-messenger response systems

John R. HEPLER, Arlene R. HUGHES and T. Kendall HARDEN
Program in Neurobiology and Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27514, U.S.A.

The relative capacities of muscarinic cholinergic receptor (MR) and bradykinin (BK)-receptor activation to increase phosphoinositide hydrolysis and to increase cytosolic Ca^{2+} were compared in NG108-15 neuroblastoma × glioma and 1321N1 human astrocytoma cells. In 1321N1 cells, the muscarinic cholinergic agonist carbachol and BK each stimulated a concentration-dependent accumulation of inositol phosphates ($K_{0.5} \sim 10\ \mu$M and ~ 10 nM respectively) and a rapid increase in cytosolic Ca^{2+} as determined by quin2 fluorescence. In NG108-15 cells, BK alone stimulated a pertussis-toxin-insensitive accumulation of inositol phosphates ($K_{0.5} \sim 10$ nM) under conditions in which pertussis toxin completely inhibited MR-mediated inhibition of adenylate cyclase. BK also stimulated a rapid increase in cytosolic Ca^{2+} in NG108-15 cells. In contrast, no MR-mediated increase in phosphoinositide hydrolysis or change in cytosolic Ca^{2+} concentration was observed in NG108-15 cells. These results support the idea that MR selectively interact with either the cyclic AMP or the inositol phosphate second-messenger systems.

INTRODUCTION

Stimulation of muscarinic cholinergic receptors (MR) results in the activation of various transmembrane signalling systems. These include (1) the direct inhibition of adenylate cyclase and a decrease in cyclic AMP (reviewed by Harden *et al.*, 1986), (2) the direct activation of phospholipase C and an increase in the cellular inositol phosphate (Ins*P*) and diacylglycerol concentrations (reviewed by Harden *et al.*, 1986), (3) an Ins*P*-induced increase in cytosolic Ca^{2+}, followed in turn by an increase in the cellular concentrations of arachidonate (DeGeorge *et al.*, 1987) and cyclic GMP (McKinney & Richelson, 1984), and (4) the regulation of a variety of membrane ion conductances (reviewed by North, 1986). Although the existence of MR subtypes has been proposed on the basis of pharmacological data (Goyal & Rattan, 1978; Hammer & Giachetti, 1982), a clear association of putative receptor subtypes with particular biochemical responses has not been established.

1321N1 human astrocytoma cells express a pharmacologically homogeneous population of MR that respond to cholinergic stimuli with an increase in Ins*P* formation and mobilization of intracellular Ca^{2+} (Masters *et al.*, 1984). MR-mediated inhibition of adenylate cyclase does not occur in these cells (Meeker & Harden, 1982), and, although fully functional G_i is present, inactivation of this protein by pertussis toxin has no effect either on MR-mediated responses of intact 1321N1 cells (Hughes *et al.*, 1984; Masters *et al.*, 1985) or on GTP-sensitive high-affinity binding of cholinergic agonists to washed 1321N1-cell membranes (Evans *et al.*, 1985). Thus MR on these cells apparently do not interact with G_i, the protein that couples inhibitory receptors to adenylate cyclase.

The data for 1321N1 cells initially were surprising in that regulation of adenylate cyclase is a well-characterized property of MR in other tissues. One potential explanation for these results is that a MR subtype exists on 1321N1 cells that selectively interacts with the putative G-protein that regulates phospholipase C activity (Taylor & Merritt, 1986), but that does not interact with G_i, the G-protein that regulates adenylate cyclase. If this is true, then an analogous but opposite selectivity might be expected for the MR subtype that regulates adenylate cyclase. That is, this receptor would couple to G_i and adenylate cyclase, but not to the membrane machinery responsible for hormonal regulation of phosphoinositide mnetabolism. Consistent with this proposal, NG108-15 neuroblastoma × glioma cells express a pharmacologically homogeneous population of MR that interact with G_i to inhibit adenylate cyclase activity (Sabol & Nirenberg, 1979; Lichtshtein *et al.*, 1979; Kurose *et al.*, 1983; Smith & Harden, 1984). Although stimulation of bradykinin (BK) receptors on NG108-15 cells results in the activation of phospholipase C and formation of inositol phosphates (Yano *et al.*, 1985), it has not been shown whether activation of MR on these cells also results in the hydrolysis of phosphoinositides. We report that no MR-mediated regulation of Ins*P* formation or Ca^{2+} mobilization occurs in NG108-15 cells under conditions where a fully functional, pertussis-toxin-insensitive and BK-receptor-mediated phosphoinositide/Ca^{2+} response system is operative. Conversely, stimulation of both MR and BK receptors on 1321N1 cells results in the formation of Ins*P* and a mobilization of Ca^{2+}. These data are consistent with the idea that 1321N1 and NG108-15 cells each express a homogeneous population of MR that are functionally distinct, and that MR subtypes can be distinguished on the basis of their

Abbreviations used: MR, muscarinic cholinergic receptors; Ins*P*, total inositol phosphates, including inositol monophosphate, inositol bisphosphate, inositol trisphosphate and inositol tetrakisphosphate; BK, bradykinin; G_i, the guanine nucleotide binding protein that couples inhibitory receptors to adenylate cyclase; G-protein, any member of a related family of guanine nucleotide binding regulatory proteins.

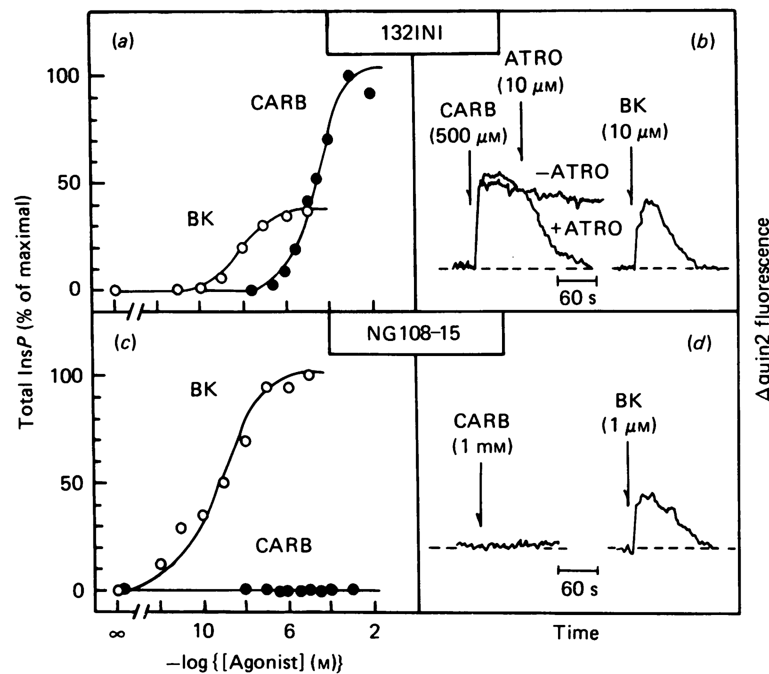

Fig. 1. Effects of carbachol or BK on InsP and cytosolic Ca^{2+} concentrations in 1321N1 and NG108-15 cells

1321N1 and NG108-15 cells were labelled overnight with *myo*-[^{3}H]inositol (*a* and *c*), or loaded with the fluorescent Ca^{2+} indicator quin2 (*b* and *d*) as described in the Methods section. (*a*) *myo*-[^{3}H]inositol-labelled 1321N1 or (*c*) NG108-15 cells were exposed to vehicle, the indicated concentrations of carbachol (CARB; ●), or the indicated concentrations of BK (○) for 10 min in the presence of 10 mM-LiCl. Data are presented as the percentage of the maximal increase in [^{3}H]Ins*P*-associated radioactivity stimulated by carbachol in 1321N1 cells (1506 ± 176 c.p.m.; mean ± S.E.M.) or BK in NG108-15 cells (1762 ± 85). Basal radioactivity (207 ± 9 and 271 ± 11 c.p.m. for 1321N1 and NG108-15 cells respectively) was subtracted from the data presented. Data are means of triplicate determinations and are representative of three experiments. (*b*) Quin2-loaded 1321N1 cells were exposed to vehicle, 500 μM-carbachol, carbachol followed by 10 μM-atropine (ATRO), or 10 μM-BK. Resting free Ca^{2+} concentration (mean ± S.E.M.) was 111 ± 16 nM ($n = 8$), and carbachol and BK stimulated a 3.2- and a 2.8-fold rise in cytosolic free Ca^{2+} concentration respectively. (*d*) Quin2-loaded NG108-15 cells were exposed to vehicle, 1 mM-carbachol or 1 μM-BK. Resting free Ca^{2+} concentration was 123 ± 24 nM ($n = 7$), and BK stimulated a 2-fold rise in cytosolic free Ca^{2+} concentration.

selectivity of interaction with the cyclic AMP or Ins*P* second-messenger systems.

METHODS

Cell culture

1321N1 human astrocytoma and NG108-15 neuroblastoma × glioma cells were grown as previously described (Hughes *et al.*, 1984).

Measurement of inositol phosphate accumulation

Labelling of the cells with *myo*-[^{3}H]inositol and measurement of Ins*P* by anion-exchange chromatography were as previously described (Nakahata *et al.*, 1986). Briefly, 1321N1 and NG108-15 cells were labelled overnight with 1 μCi of *myo*-[^{3}H]inositol/ml and exposed to carbachol, BK or vehicle for 10 min in the presence of 10 mM-LiCl. The reaction was stopped by the addition of methanol, inositol phosphates were extracted by the addition of chloroform and water, and water-soluble [^{3}H]inositol phosphates were collected by anion-exchange chromatography.

Measurement of intracellular Ca^{2+}

Cells were suspended in Hepes-buffered Eagle's minimal essential medium and then loaded with the fluorescent Ca^{2+} indicator quin2 by incubation with 50 μM-quin2/AM for 30 min at 37 °C in the dark as previously described (Tsien *et al.*, 1982). Cells were washed free of extracellular quin2/AM by centrifugation (twice for 5 min at 100 *g*), resuspended in Hepes-buffered saline (pH 7.5), and maintained at room temperature. Cells (2×10^6–3×10^6 cells/ml) were monitored for changes in quin2 fluorescence with a Perkin–Elmer Hitachi MPF-2.A fluorescence spectrophotometer. Under these conditions, over 85% of the cells remained viable, as determined by Trypan Blue exclusion under microscopic examination. Determination of intracellular free Ca^{2+} concentration on the basis of quin2 fluorescence was as previously described (Tsien *et al.*, 1982).

Pertussis-toxin treatment of cells

Cells were treated overnight with various concentrations of pertussis toxin as described previously (Hughes *et al.*, 1984).

Materials

Carbachol and atropine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), BK and quin2/AM from Calbiochem (San Diego, CA, U.S.A.), [^{3}H]adenine (40 Ci/mmol) was from ICN (Irvine, CA, U.S.A.), and *myo*-[^{3}H]inositol (15 Ci/mmol) from ARC

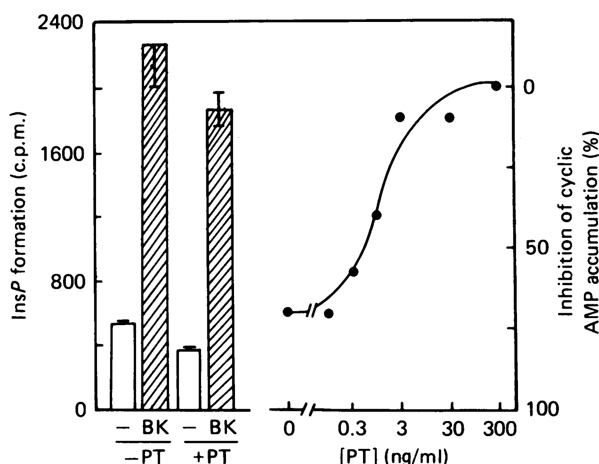

Fig. 2. Differential effects of pertussis toxin on BK-stimulated InsP accumulation and MR-mediated inhibition of cyclic AMP accumulation in NG108-15 cells

NG108-15 cells were labelled overnight with *myo*-[^{3}H]inositol in the presence (+PT) or absence (−PT) of 300 ng of pertussis toxin/ml. Cells were challenged with vehicle (−) or 1 μM-bradykinin (BK) for 10 min in the presence of 10 mM-LiCl. Companion NG108-15 cells were incubated overnight with vehicle or the indicated concentrations of pertussis toxin as described by Hughes *et al.* (1984). The cells were incubated for 60 min with 1 μCi of [^{3}H]adenine/ml, and challenged with 1 μM-prostaglandin E_1 alone or in the presence of 100 μM-carbachol for 10 min. The results are plotted as the percentage inhibition of cyclic AMP accumulation observed in the presence of carbachol (●). The data are from quadruplicate determinations and are representative of three experiments.

(St. Louis, MO, U.S.A.). Pertussis toxin was prepared as previously described (Hughes *et al.*, 1984).

RESULTS

Carbachol is an efficacious stimulator of Ins*P* formation in 1321N1 human astrocytoma cells (Masters *et al.*, 1984; Fig. 1*a*). In addition, BK markedly stimulated Ins*P* formation in these cells (Fig. 1*a*). In confirmation of the data of Yano *et al.* (1985), BK also markedly stimulated Ins*P* formation in NG108-15 cells (Fig. 1*c*). The $K_{0.5}$ values for BK were approximately the same (~ 3–10 nM) for NG108-15 and 1321N1 cells. In contrast with the results obtained in 1321N1 cells, carbachol at concentrations up to 10 mM had no effect on Ins*P* formation in NG108-15 cells (Fig. 1*c*).

Since it has been demonstrated that Ins*P* formation is linked to the mobilization of Ca^{2+} in a wide variety of tissues (Berridge, 1987), it was also important to determine if stimulation of MR produced an increase in cytosolic Ca^{2+} in NG108-15 cells. Therefore Ca^{2+} concentrations measured with the fluorescent Ca^{2+} indicator quin2 were compared in hormone-stimulated 1321N1 and NG108-15 cells. Activation of MR on 1321N1 cells increased intracellular Ca^{2+} (Fig. 1*b*). This effect was maintained for at least 5 min in the presence of agonist and was reversed by addition of atropine. Consistent with its observed effects on Ins*P* formation, BK also increased intracellular Ca^{2+} in 1321N1 cells (Fig. 1*b*). Although BK also increased intracellular Ca^{2+} in NG108-15 cells, carbachol was without effect (Fig. 1*b*).

NG108-15 cells express a relatively high density of MR that markedly inhibit adenylate cyclase activity on activation (Sabol & Nirenberg, 1979; Lichtshtein *et al.*, 1979; Smith & Harden, 1984). As previously demonstrated in these cells (Kurose *et al.*, 1983), carbachol caused a 70% inhibition of cyclic AMP accumulation in NG108-15 cells that was completely reversed by pertussis toxin at concentrations as low as 3 ng/ml (Fig. 2). In contrast, preincubation of NG108-15 cells with 300 ng of pertussis toxin/ml had no effect on BK-stimulated Ins*P* formation (Fig. 2).

DISCUSSION

The presence of G_i or a 'G_i-like' protein in NG108-15 neuroblastoma × glioma cells apparently fully accounts for MR–G-protein coupling in these cells, in that pertussis toxin or low concentrations of *N*-ethylmaleimide, which both have been shown to inactivate G_i, abolish GTP-sensitive high-affinity binding of agonists to MR (Kurose *et al.*, 1983; Smith & Harden, 1984; Evans *et al.*, 1985) and MR-mediated inhibition of adenylate cyclase activity (Kurose *et al.*, 1983; and Fig. 2). The current study confirms the report by Yano *et al.* (1985), which demonstrated that BK markedly stimulates phospholipase C activity in NG108-15 cells. Moreover, as illustrated above, inactivation of G_i with pertussis toxin had no effect on BK-stimulated Ins*P* formation. Thus, as with the phosphoinositide response to muscarinic agonists (Hepler & Harden, 1986) and BK (J. R. Hepler, unpublished work) observed in 1321N1 cells, BK-receptor-mediated activation of phospholipase C in NG108-15 cells apparently does not involve G_i or a similar pertussis-toxin substrate.

Although Siman & Klein (1981) have reported that carbachol stimulates a small increase in ^{32}P labelling of phosphatidylinositol in a clone of NG108-15 cells, the present paper demonstrates that activation of MR on these cells does not result in phosphoinositide hydrolysis and Ins*P* formation. This lack of response to carbachol was further supported by the fact that Ca^{2+} concentrations, measured with quin2, did not change in response to MR agonists. Thus, although a functional population of MR and a fully functional BK-receptor-responsive phospholipase C are both present in NG108-15 cells, stimulation of MR does not activate phospholipase C and phosphoinositide hydrolysis. These results are the opposite of those observed in 1321N1 cells, in which MR interact with components of the phosphoinositide response cascade but do not interact with the adenylate cyclase system. Thus, at least in data obtained with two cloned cell lines, MR couple to either the Ins*P* or the cyclic AMP response system, but not to both. Owing to problems of cellular heterogeneity and lack of availability of MR antagonists that selectively block one or the other of the second-messenger responses, it has not been possible to make similar conclusions from studies with mammalian tissues. The rigid signalling specificity expressed by the two cell lines potentially could be explained by some vagary of cultured cell lines. However, the phosphoinositide and cyclic AMP response systems of both cell lines are, in all ways tested to date, fully functional and receptor-operated. Thus it seems appropriate to generalize from the data presented here and to

suggest that it is indicative of the existence of two forms of MR. One type of receptor protein possesses a polypeptide sequence that readily interacts with G_i, and thus inhibits adenylate cyclase. The other proposed receptor type expresses a polypeptide sequence that selectively interacts with the pertussis-toxin-insensitive G-protein involved in the activation of phospholipase C. Whether this selectivity of MR interaction with different G-proteins is absolute under all conditions and in all tissues cannot be concluded from the results reported here. There is evidence that the receptor proteins of NG-108-15 and 1321N1 cells are structurally distinct. That is the MR of the two cell lines have been labelled covalently with [^{3}H]propylbenzylcholine mustard (Liang *et al.*, 1987), and the apparent size of the specifically labelled receptors differed by approx. 20000 Da after SDS/polyacrylamide-gel electrophoresis under a variety of conditions.

Putative M_1- and M_2-muscarinic receptor subtypes have been proposed on the basis of pharmacological and radioligand-binding data obtained with the antagonist pirenzepine (Goyal & Rattan, 1978; Hammer & Giachetti, 1982). Although Gil & Wolfe (1985) have presented data with rat brain indicating that pirenzepine blocks the Ins*P* response to MR agonists at lower concentrations than those necessary to block the cyclic AMP response, several studies using other tissues have failed to confirm a selectivity of pirenzepine for blockade of these responses (Lazareno *et al.*, 1985; Brown *et al.*, 1985; Fisher, 1986). Thus it is yet to be established whether the MR subtypes proposed on the basis of selectivity of interaction with second-messenger systems can be segregated according to the previously proposed M_1–M_2 MR subtypes. The observations (Logothetis *et al.*, 1987; Yatani *et al.*, 1987) that MR activate K^+ channels directly in some tissues, apparently by interacting with a G-protein, underscores further the difficulties of characterizing MR subtypes.

It is clear that more selective ligands are needed to establish unequivocally the existence of pharmacologically distinct MR subtypes. However, our data with cultured NG108-15 and 1321N1 cells suggest that two MR can be delineated at the level of interaction with second-messenger systems. As such, these two cell lines should prove useful in further development of understanding of the pharmacological and biochemical properties of MR.

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