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Differential Receptor Occupancy Requirements for Muscarinic Cholinergic Stimulation of Inositol Lipid Hydrolysis in Brain and in Neuroblastomas

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SUMMARY

The potency with which carbamoylcholine enhances phosphoinositide (PPI) hydrolysis in different brain regions (neostriatum, cerebral cortex, and hippocampus) and in two neuroblastomas (the murine N1E-115 and human SK-N-SH) differs by 10- to 20-fold. To determine whether the presence of a muscarinic receptor (mAChR) reserve might account for these differences, we have examined the effect of propylbenzilylcholine mustard (PrBCM) on mAChR number and on agonist-stimulated PPI hydrolysis. In the cerebral cortex, in hippocampus, and in N1E-115 cells, PrBCM treatment resulted in a loss of the PPI response, as measured by the release of [^{3}H]inositol phosphates, that was equal to or greater than the reduction in receptor number, as determined by the loss of either [^{3}H]quinuclidinylbenzilate- or [^{3}H]*N*-methylscopolamine-binding sites. From dose response curves for carbamoylcholine, it was determined that alkylation of mAChRs resulted in a reduction in the maximum release of inositol phosphates but had no effect on agonist potency. The K_A values for carbamoylcholine obtained following receptor inactivation were similar to those for the EC_{50} (120–316 μM). In contrast, in both the neostriatum and SK-N-SH cells, PrBCM treatment resulted in a greater loss of mAChR number than of stimulated inositol phosphate release, and dose response curves for carbamoylcholine were shifted to higher agonist concentrations. The K_A values (34–65 μM) were 2- to 9-fold higher than the comparable EC_{50} values. Moreover, in both tissues the PPI response elicited by partial agonists was more susceptible to receptor alkylation than that elicited by carbamoylcholine. The two groups of tissues also differ in their sensitivity to pirenzepine, which is a markedly weaker antagonist of stimulated PPI hydrolysis in SK-N-SH cells and neostriatum (K_i 160–250 nM), than in the cerebral cortex, hippocampus, and N1E-115 cells (K_i 10–20 nM). These results suggest: 1) that a population of "spare" receptors exists for mAChR-mediated inositol lipid hydrolysis in some neuronal tissues, 2) that both M_1 and M_2 mAChRs may be coupled to PPI turnover, and 3) that M_2 mAChRs appear to be more efficiently coupled to phosphoinositide hydrolysis than their M_1 counterparts.

The activation of guanylate cyclase (1, 2), inhibition of adenylate cyclase (1, 3, 4), and enhanced turnover of inositol phospholipids (5, 6) represent three of the known biochemical consequences of muscarinic receptor (mAChR) activation in neuronal tissues. Of these, inositol lipid breakdown via phospholipase C has received the most attention, principally due to the observation that phosphoinositide hydrolysis results in the formation of at least two intracellular second messenger molecules, namely, inositol 1,4,5-trisphosphate and diacylglycerol (7, 8). However, the transduction events which link mAChR occupancy to inositol lipid hydrolysis, factors which determine the extent of lipid breakdown and the pharmacological characteristics of the response, remain unclear. In most brain regions, e.g., cerebral cortex and hippocampus, the addition of full agonists at a concentration that occupies all of the available mAChR sites is required to achieve a maximum increase in PPI turnover (9, 10). Moreover, agonists such as oxotremorine and bethanechol are only weakly effective, even at optimum concentrations. Conversely, in the guinea pig neostriatum, carbamoylcholine is 20-fold more potent for stimulating PPI hydrolysis than in the cerebral cortex, and partial agonists are markedly more effective (11). One explanation for these tissue differences is that a muscarinic receptor reserve exists for PPI hydrolysis in the neostriatum, but not in the cerebral cortex. In the present study, we have directly determined the relationship between muscarinic receptor number and stimulated PPI

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; IP_1, D-*myo*-inositol monophosphate; IP_2, D-*myo*-inositol bisphosphate; IP_3, D-*myo*-inositol trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PPI, phosphoinositide (PI, PIP, PIP_2); PrBCM, propylbenzilylcholine mustard; QNB, quinuclidinylbenzilate; NMS, *N*-methylscopolamine; OXO-2, oxotremorine-2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

hydrolysis following an initial inactivation of a fraction of the mAChR complement with the alkylating agent, PrBCM. In addition to three guinea pig brain regions (cerebral cortex, hippocampus, and neostriatum), two neuroblastomas—murine N1E-115 and human SK-N-SH—were also chosen for study, since marked differences in muscarinic agonist affinity and partial agonist efficacy for PPI hydrolysis were also apparent in these two neuronally derived cell lines. The data demonstrate that, whereas in some tissues, occupancy of all of the mAChRs is necessary for a maximum functional response, in others (neostriatum and SK-N-SH neuroblastoma), there exists a population of "spare" mAChRs for PPI breakdown.

Materials and Methods

myo-[2-^{3}H]Inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]NMS (80 Ci/mmol) and PrBCM were obtained from New England Nuclear (Boston, MA). L-[^{3}H]QNB (30 Ci/mmol) was purchased from Amersham Corp. (Chicago, IL). Carbamoylcholine, atropine, bethanechol, and arecoline were obtained from Sigma Chemical Co. (St. Louis, MO). Pirenzepine was obtained from Boehringer Ingelheim (Ridgefield, CT). McN-A-343 was obtained from McNeil Laboratories (Springhouse, PA). Dowex-1 (100–200 mesh; X8 in the formate form) was obtained from BioRad (Rockville Centre, NY). Tissue culture supplies were obtained from Corning Glass Works (Corning, NY). Powdered Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Grand Island Biological Co. (Grand Island, NY). N1E-115 cells were obtained from Dr. E. Richelson, Department of Pharmacology, Mayo Clinic, Rochester, MN. SK-N-SH cells were obtained from Dr. W. Sadee, School of Pharmacy, University of California, San Francisco. The latter cells may also be obtained from American Type Culture Collection (Rockville, MD).

Preparation of Brain Slices

Cross-chopped slices (350 μm × 350 μm) of guinea pig cerebral cortex, hippocampus, and neostriatum (caudate nucleus and putamen) were prepared on a McIlwain tissue chopper, washed in a total of 50–100 ml of warm buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose, and 30 mM HEPES-N^+ buffer, pH 7.4). Fifty-μl aliquots of tissue slices (approximately 0.4–1.0 mg of protein) were used for [^{3}H]inositol labeling experiments.

Cell Culture Conditions

Mouse neuroblastoma cells, clone N1E-115 (passage 8–18) were grown in tissue culture flasks (75 cm^2/250 ml) in 20 ml of Dulbecco's modified Eagle's medium supplemented with 10% (by volume) fetal bovine serum (medium I). Cells were grown for 10–22 days at 37° in an atmosphere consisting of 10% CO_2 and 90% humidified air. Subculture was achieved by aspirating medium I and isolating the cells following incubation in a modified Puck's D_1 solution (medium II; Ref. 12). After centrifugation of the cell suspension for 1 min at 300 × *g*, the supernatant was removed and the cells were resuspended in 10 ml of medium I which was then inoculated into flasks at a density of 1–2 × 10^6 cells/flask on day 0. The culture medium was changed on day 4 and every day thereafter by the addition of 10 ml of fresh medium I and removal of 10 ml of medium. Human neuroblastma SK-N-SH cells were originally derived from a metastatic tumor in the bone marrow of a 4-year-old girl. The cells (of unknown passage number) were cultured under the same conditions as those employed for the N1E-115 cells with the exception that the cells were grown for 5–30 days and, following subculture, the culture medium was changed on day 4 and on alternate days thereafter.

Assay for Release of Labeled Inositol Phosphates

Brain slices. Both continuous labeling and prelabeling paradigms were employed (11). In the continuous labeling approach, tissue slices were incubated in buffer A which contained 5–10 μCi/ml of [^{3}H]inositol, 10 mM lithium chloride, and muscarinic agonist (or buffer A for controls), in a total volume of 0.5 ml. Reactions were initiated by the addition of tissue slices and terminated after 120 min by the addition of 1.5 ml of chloroform/methanol (1:2, by volume). Under these conditions the release of [^{3}H]inositol phosphates was approximately linear with time, after an initial lag period (11). Alternatively, brain slices were allowed to prelabel for 90 min at 37° in 5–10 ml of buffer A containing 50 μCi/ml of [^{3}H]inositol. Following prelabeling, the radioactive medium was aspirated and brain slices were washed twice with 20 ml of warm buffer A. Fifty μl of the labeled slices were then added to 400 μl of buffer A containing 10 mM lithium chloride and 50 μl of agonist (or buffer A). Incubations were terminated after 30 min at 37° by addition of 1.5 ml of chloroform/methanol (1:2 by volume). In the majority of the experiments, the continuous labeling paradigm was employed due to its simplicity and greater sensitivity. Results obtained with the two paradigms were comparable. A total water-soluble inositol phosphate fraction was separated from [^{3}H]inositol by ion exchange chromatography and quantitated as previously described (11). A 200-μl aliquot of the lower organic phase was routinely removed to determine the incorporation of [^{3}H]inositol into phospholipid. More than 90% of label incorporated into lipid was located in PI, with the remainder in phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. Results are expressed as radioactivity in inositol phosphates/10^4 dpm inositol lipids. This method of calculation corrects for small variations in the amount of tissue slices pipetted into each tube. Since the addition of agonists has little effect on lipid labeling (11), the radioactivity in the lipid fraction can be used to calculate a specific activity for inositol phosphate release.

Neuroblastomas. N1E-115 and SK-N-SH cells were harvested for assay by aspiration of tissue culture medium, addition of 10 ml of medium II to each culture flask, and incubation for 15 min at 37°. Following gentle agitation of the flask, the cells became detached and were collected by centrifugation at 300 × *g* for 1 min. The cells were then resuspended in buffer A containing [^{3}H]inositol (approximately 8 μCi/ml), at a protein concentration of approximately 0.4–2.0 mg/ml, and allowed to prelabel for 30 min at 37°. Aliquots (450 μl) of the labeled cells were then added to tubes containing 50 μl of agonist or buffer A, and reactions were allowed to continue for a further 30 min. Reactions were terminated by the addition of 1.5 ml of chloroform/methanol (1:2 by volume), and water-soluble inositol phosphates were isolated as described below. The release of labeled [^{3}H]inositol phosphates was linear with time over this period.

Product Identification

In the present study, a total inositol phosphate fraction was routinely isolated. In some experiments, the individual inositol phosphates were separated as previously described (11). For brain slices, the major product was IP_1, which represented 60–80% of total radioactivity, while 7–10% of radioactivity was located in IP_2 and IP_3. Approximately 10–20% of radioactivity was located in glycerophosphorylinositol. Agonist addition resulted in a significant increase in IP_1 radioactivity, with a smaller increase in IP_2 and IP_3. A similar product pattern was observed for both the N1E-115 and SK-N-SH neuroblastoma cells under the assay conditions described, with >65% of label present in IP_1, in agreement with a previous study (13). Thus, the increased release of [^{3}H]inositol phosphates observed in the presence of agonist represents predominantly an increased formation of [^{3}H]IP_1.

Receptor Alkylation

PrBCM was prepared as a 10 mM stock in absolute ethanol and allowed to cyclize at a concentration of 400 μM (14) in sodium phosphate buffer (pH 7.4) to form the active aziridium ion. Shortly before use, the cyclized PrBCM was diluted in buffer A to give a final concentration range of 1 nM–1 μM. Brain slices were then incubated with PrBCM for 15 min at 37° in a total volume of 4 ml, after which time the slices were washed six times with 5 ml of warm buffer A over a 10-min period to

remove unbound mustard. N1E-115 and SK-N-SH cells were treated identically with the exception that the cells were centrifuged at 300 × *g* for 1 min and unbound mustard was removed by washing the cells three times with 30 ml of warm buffer A over a similar time period. The alkylated brain slices or cells were then resuspended in buffer A prior to either radioligand binding or PPI turnover determinations. In some experiments, brain slices or neuroblastoma cells were allowed to prelabel prior to PrBCM treatment. Results so obtained did not differ from those in which receptor alkylation preceded labeling. In pilot studies, the degree of mAChR inactivation (as determined by receptor number) was found to remain constant during the time course of PPI or radioligand binding assays (30–120 min).

Radioligand Binding Assays

Untreated and alkylated brain slices were first homogenized in 10 mM sodium phosphate buffer (pH 7.4) and 50–100 μg of protein incubated in 2 ml of buffer A at 25° in the presence of a saturating concentration of 1.5–2.0 nM L-[^{3}H]QNB or 2–7 nM [^{3}H]NMS. Reactions were terminated after 120 min by vacuum filtration through GF/B filters, the filters were washed twice with 5 ml of 0.9% NaCl, and radioactivity was quantitated, as previously described (9, 10). Under these assay conditions, >99% of added ligand remained free in solution, and nonspecific binding (that unaffected by inclusion of 50 μM atropine) represented less than 5% of specific binding. Radioligand binding assays on N1E-115 and SK-N-SH neuroblastomas were performed on intact cells (200–500 μg of protein) under the same incubation conditions as described for brain slice homogenates. Nonspecific binding was <25% on all occasions with the exception of [^{3}H]QNB binding to N1E-115 cells, where this value approached 70% of total binding. For this reason, most of the radioligand binding assays performed with N1E-115 cells utilized [^{3}H]NMS as ligand. Protein was measured by the method of Geiger and Bessman (15).

Data Analysis

EC_{50} values for dose response curves were determined using probit analysis. In some experiments the data for stimulated inositol phosphate release as a function of agonist concentration was evaluated for evidence of a one- or two-site interaction as previously described (9). For determination of agonist equilibrium dissociation constant (K_A) values, equiactive concentrations of carbamoylcholine in control (A) and PrBCM-treated (A') cells were obtained graphically from the linear part of the response curves, and their reciprocals were plotted. The inverse of the slope (q) gives the fraction of active receptors, and K_A was calculated from the equation:

$$K_A = \frac{\text{slope} - 1}{\text{intercept}}$$

as described by Furchgott and Bursztyn (16). To optimize conditions for determination of K_A, conditions were chosen such that 30–80% of the available mAChR sites were inactivated. Percentage of receptor occupancy required for a half-maximal increase in PPI turnover was calculated from the equation:

$$\text{occupancy} = 100\left(\frac{EC_{50}}{K_A + EC_{50}}\right)$$

Results

Brain regions. Incubation of brain slices with increasing concentrations of PrBCM resulted in a progressive reduction in mAChR density, as determined by the loss of either [^{3}H]QNB- or [^{3}H]NMS-binding sites (Fig. 1). As observed for other tissues (17, 18), [^{3}H]NMS labeled substantially fewer sites (40–60%) than did [^{3}H]QNB. However, within each brain region the concentration of PrBCM required to elicit a 50% reduction in receptor density was similar for both ligands (10 nM), indicating that receptors in both hydrophilic and hydrophobic membrane environments were equally influenced by the mustard. Furthermore, mAChRs in the neostriatum, cerebral cortex, and hippocampus were equally susceptible to alkylation. A small population of mAChR sites was refractory to alkylation by concentrations of PrBCM as high as 1 μM (12–16% of [^{3}H]QNB- and 8–11% of [^{3}H]NMS-binding sites), as has been previously observed for rat brain (19) and chick heart cells (18). In some experiments we also determined whether PrBCM had a selective effect on the putative M_1 or M_2 mAChR subtypes. The ability of the M_1-selective antagonist pirenzepine to displace [^{3}H]QNB or [^{3}H]NMS binding was independent of the degree of receptor inactivation. Little selectivity of action of PrBCM on M_1 and M_2 subtypes was observed previously for rat cerebral cortex (20). Taken collectively, these results indicate that PrBCM has similar effects on mAChRs in all three brain regions.

Pretreatment of brain slices with PrBCM also resulted in a reduction of stimulated PPI turnover elicited by an optimum concentration of carbamoylcholine, although the extent of inhibition was dependent upon the brain region examined (Fig. 2). In the neostriatum, approximately 40% of mAChR sites could be inactivated before any reduction in the stimulated release of [^{3}H]inositol phosphates was detectable; at all concentrations of PrBCM, the reduction in receptor density (expressed as per cent reduction of [^{3}H]QNB-binding sites) exceeded the loss of the functional response, which was still clearly detectable at 1 μM PrBCM. However, in the cerebral cortex and hippocampus, receptor alkylation resulted in a reduction in [^{3}H]inositol phosphate release that was equal to or greater than the loss of receptor number. In these latter brain regions, stimulated PPI turnover elicited by the addition of carbamoylcholine was abolished by pretreatment with a 0.1 μM concentration of PrBCM, even though 10–20% of the mAChRs still remained. It would appear that these remaining mAChRs are not linked to PPI turnover. In all three brain regions, no change in the basal (no agonist) release of [^{3}H]inositol phosphates was detectable following PrBCM treatment, nor was there any inhibition of the stimulated release of inositol phosphates resulting from addition of a 10^{-4} M concentration of norepinephrine or ibotenate (data not shown). These results indicate that the effects of PrBCM are not due to a nonspecific effect of the alkylating agent.

To further evaluate the relationship between mAChR number and PPI response, dose response curves for carbamoylcholine were constructed for both untreated brain slices and slices in which a proportion (50–80%) of the mAChRs had been irreversibly inactivated by PrBCM. In the neostriatum, carbamoylcholine was a potent agonist ($EC_{50} = 8 \pm 4$ μM, $n = 3$). Receptor alkylation in the neostriatum resulted not only in a reduction in the maximum release of [^{3}H]inositol phosphates, but also in a shift in the dose response curve to higher agonist concentrations (Fig. 3). The equilibrium dissociation constant (K_A) calculated by the method of Furchgott and Bursztyn (16) for this experiment was 34 μM, a value 9-fold higher than the EC_{50} (3.5 μM). In a second experiment, the K_A value (45 μM) was 3-fold higher than the EC_{50} (15 μM). In the cerebral cortex and hippocampus, PrBCM treatment also resulted in a reduction in the maximum PPI response, but unlike neostriatum, there was no discernible shift in the dose response curves (Fig.

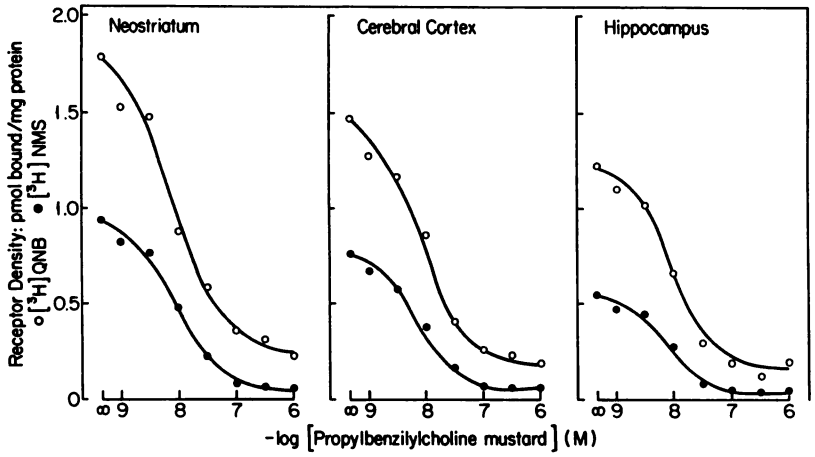

Fig. 1. Loss of mAChR number in PrBCM-treated brain slices. Cross-chopped slices (350 μM) of neostriatum, cerebral cortex, and hippocampus were incubated for 15 min at 37° in either buffer A alone or PrBCM at the concentrations indicated. Unbound mustard was then removed by washing the slices six times with 5 ml of warm buffer A over a 10-min period. Tissue slices were then homogenized in 10 mM sodium phosphate buffer (pH 7.4), and the number of remaining muscarinic receptors was determined by incubation in aliquots of the homogenates (100–150 μg of protein) with [^{3}H]QNB or [^{3}H]NMS. The concentrations of QNB and NMS were 1.31 and 2.13 nM, respectively, which are minimally 5 times the K_d values for these ligands. In all three tissues some receptor sites (approximately 10% of total) were refractory to alkylation (specific binding of [^{3}H]QNB and [^{3}H]NMS was > 1500 dpm at 1 μM PrBCM). Results shown are the means of quadruplicate replicates (standard error < 5%) from one of three experiments that gave similar results.

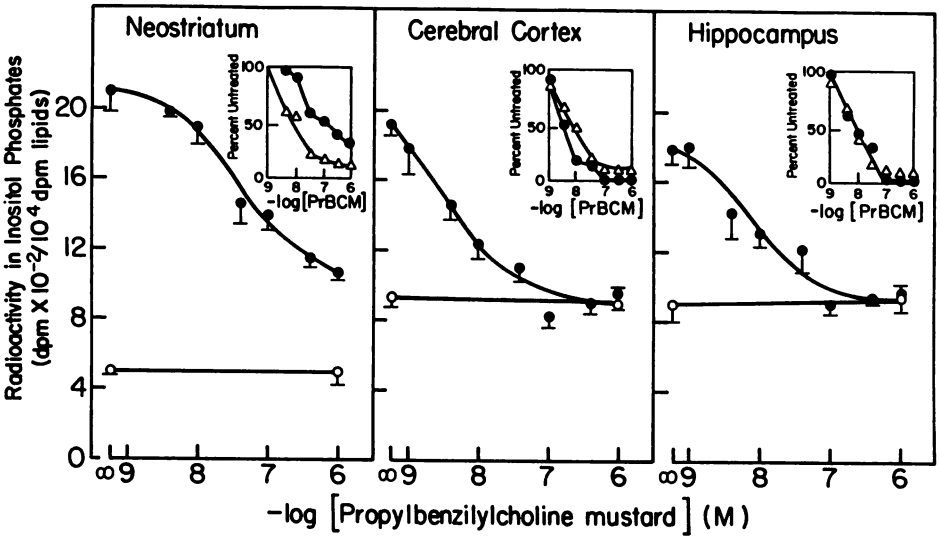

Fig. 2. Loss of mAChR-stimulated PPI turnover in PrBCM-treated brain slices. Cross-chopped slices of neostriatum, cerebral cortex, and hippocampus were incubated for 15 min in either buffer A or in the presence of PrBCM at the concentrations indicated. After wash-out of the unbound mustard, tissue slices (1–2 mg protein) were incubated with 6.2 μCi of [^{3}H]inositol in the absence (○) or presence (●) of 10 mM carbamoylcholine for 120 min. Reactions were then terminated and release of labeled inositol phosphates was measured. Muscarinic receptor density was also monitored by means of [^{3}H]QNB binding. Results shown are means ± standard errors for quadruplicate replicates from one of three experiments that gave similar results. The concentrations of PrBCM required to reduce the PPI response by 50% in neostriatum, cerebral cortex, and hippocampus were 100 nM, 7 nM, and 9 nM, respectively. *Insets:* The percentage of original PPI response (●) and mAChR density (△) were calculated and plotted as a function of mustard concentration. The densities of [^{3}H]QNB-binding sites in untreated slices of neostriatum, cerebral cortex, and hippocampus were 1.45, 1.17, and 1.06 pmol/mg protein, respectively.

4). The K_A values for carbamoylcholine in the cerebral cortex and hippocampus were 316 ± 115 and 200 ± 48 μM, respectively (mean ± SE, three to five separate experiments), and closely matched those for the EC_{50}.

In a subsequent series of experiments, we obtained further evidence to support the existence of a receptor reserve for PPI turnover in the neostriatum by measuring the susceptibility to PrBCM treatment of PPI stimulation elicited by full and partial muscarinic agonists (Table 1). A greater reduction in the response to a partial agonist following PrBCM pretreatment might indicate the presence of receptor reserve, since a partial agonist needs to occupy a greater proportion of the available receptor sites than a full agonist to achieve an optimum stimulation (21). Pretreatment of neostriatal slices with 40 nM PrBCM resulted in a 75% loss of [^{3}H]QNB- or [^{3}H]NMS-binding sites, but only in a 38 ± 2% reduction in carbamoylcholine-stimulated release of [^{3}H]inositol phosphates. Under these conditions, however, the responses elicited by the addition of a partial agonist, bethanechol (relative efficacy = 70% of that of carbamoylcholine), was reduced by 78 ± 2%, a value in keeping with the reduction in mAChR density. A similar reduction in response was observed for OXO-2. This analog of oxotremorine is a weak partial agonist in the cerebral cortex (10) but is a full agonist for PPI turnover in the neostriatum. Although the greater efficacy of OXO-2 in the neostriatum is presumably a reflection of the presence of a receptor reserve for PPI turnover in this tissue, it is evident that this agonist needs to occupy more available receptor sites than does carbamoylcholine. Previous studies have indicated that both full and partial agonists interact with the same population of mAChRs in the neostriatum (11). Pretreatment of slices of cerebral cortex and hippocampus with 10 nM PrBCM resulted in a 36–40% loss of mAChRs, whereas carbamoylcholine-stimulated [^{3}H]inositol phosphate release was reduced by >60% (Table 1). It was not feasible to measure the loss of partial agonist efficacy in the cerebral cortex or hippocampus following receptor alkylation, due to the very limited ability of these agents to promote inositol lipid breakdown.

Neuroblastomas. Muscarinic receptor activation in two neuroblastoma cell lines (murine N1E-115 and human SK-N-SH) also resulted in a large increase (5- to 20-fold) in the release of [^{3}H]inositol phosphates from prelabeled cells. Stim-

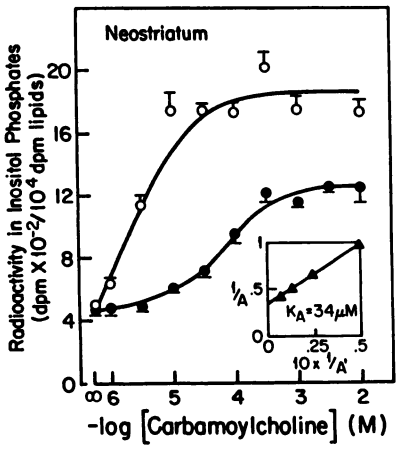

Fig. 3. Receptor alkylation of neostriatal slices shifts the carbamoylcholine dose response curve for PPI turnover to a higher concentration. Combined slices from four guinea pigs were divided into two aliquots. One was treated with 75 nM PrBCM for 15 min (●), while the other was incubated in the presence of buffer A (○). After removal of unbound mustard, untreated and alkylated slices were incubated with 7.2 μCi of [^{3}H]inositol for 120 min in the presence of carbamoylcholine at the concentrations indicated. Values shown are means ± standard errors for quadruplicate replicates. *Inset:* Equiactive concentrations (μM) of carbamoylcholine in control and PrBCM-treated slices were derived graphically and their reciprocals plotted. The K_A is 34 μM as derived from Furchgott analysis (16). Fraction of active receptors (q) = 0.75. The EC_{50} value is 3.5 μM. The concentrations of [^{3}H]QNB- and [^{3}H]NMS-binding sites were reduced by 79 and 80%, respectively, in PrBCM-treated slices. Similar K_d values for [^{3}H]QNB in control and alkylated tissues (40–45 pM) indicate that the unbound mustard is effectively removed by the washing procedure.

ulation of PPI turnover was more robust in the SK-N-SH cells, which may reflect the greater receptor density in this cell line (443 ± 71 fmol/mg of protein for [^{3}H]QNB and 298 ± 50 fmol/mg of protein for [^{3}H]NMS; mean ± SE for seven separate experiments). In the N1E-115 cell, there were 18 ± 2 fmol/mg of protein for [^{3}H]NMS-binding sites (mean ± SE for five separate experiments). As observed for the brain regions, marked differences in agonist potency and partial agonist efficacy were also observed between these two neuroblastomas. In the SK-N-SH cells, a 5- to 9-fold lower concentration of either carbamoylcholine or oxotremorine-M was required to elicit a half-maximal increase (EC_{50}) in the release of inositol phosphates than in the N1E-115 cells (Fig. 5). In the human neuroblastoma, several partial muscarinic agonists including bethanechol, arecoline, oxotremorine, and oxotremorine-1 were also more efficacious (Table 2). The putative M_1-selective agonist McN-A-343 was completely ineffective at the mAChRs on both neuroblastomas as previously observed for brain (11). To determine whether the differences in agonist potency and efficacy reflected the existence of a receptor reserve for PPI turnover in the SK-N-SH cell, a series of experiments was conducted. First, N1E-115 and SK-N-SH cells were pretreated with various concentrations of PrBCM (1–100 nM) and, following removal of the mustard, the reduction in mAChR density and carbamoylcholine-stimulated release of [^{3}H]inositol phosphates was monitored. In the N1E-115 cells, receptor alkylation resulted in a similar reduction in the number of [^{3}H]NMS-binding sites and of [^{3}H]inositol phosphate release (Fig. 6). In contrast, PrBCM treatment of SK-N-SH cells resulted in a greater loss of mAChR number than of stimulated PPI turnover. Lower concentrations of the mustard were required to inactivate the mAChRs on N1E-115 cells than on the SK-N-SH neuroblastoma. Second, dose response curves for carbamoylcholine were constructed for both cell types, in untreated and PrBCM-pretreated cells (Fig. 7). In the N1E-115 cell, alkylation of mAChRs resulted in a reduction in the maximum response but had no effect on agonist potency. The K_A value for carbamoylcholine (which is similar to that for the EC_{50}) was 120 ± 5 μM (mean ± SE, four separate experiments). In the SK-N-SH cell, the reduced response following alkylation was accompanied by a shift in the dose response curve to a higher agonist concentration. The K_A for carbamoylcholine in SK-N-SH cells was 65 ± 13 μM (mean ± SE, four separate experiments). The comparable EC_{50} values were 2- to 4-fold lower. Further indication of a receptor reserve for PPI turnover in SK-N-SH cells was obtained from experiments in which PrBCM treatment was found to have a greater inhibitory effect on [^{3}H]inositol phosphate release elicited by partial agonist addition than that resulting from carbamoylcholine addition. Pretreatment of SK-N-SH cells with 15 nM PrBCM resulted in a loss of 72 and 75% of [^{3}H]QNB- and [^{3}H]NMS-binding sites, respectively. Under the same conditions, there was a 55% loss of carbamoylcholine-stimulated release of [^{3}H]inositol phosphates, whereas the stimulatory effects of two partial agonists, bethanechol and arecoline, were reduced by 77 and 83% (Table 3). In the N1E-115 cells, the reduction in mAChR

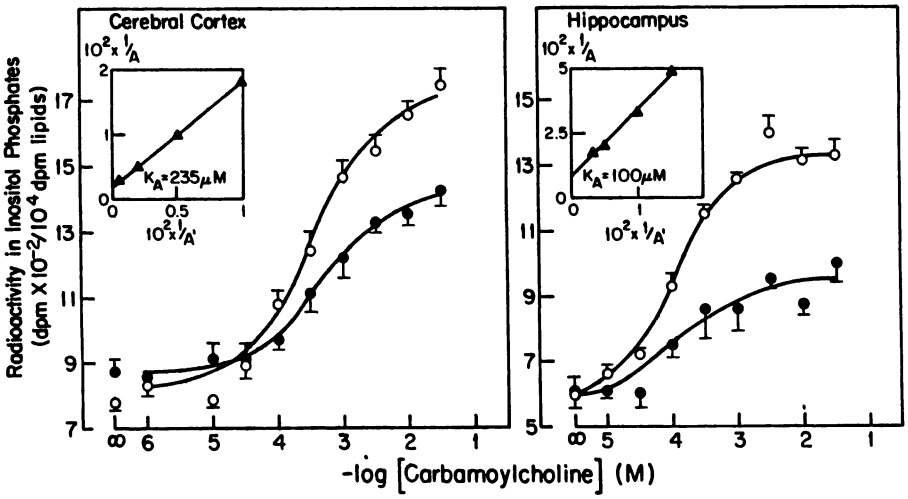

Fig. 4. Effect of PrBCM treatment on carbamoylcholine dose response curves for PPI turnover in cerebral cortex and hippocampus. Slices from cerebral cortex and hippocampus were treated with either buffer A (○) or 10 nM PrBCM (●) for 15 min. After removal of unbound mustard, untreated and alkylated slices were incubated with [^{3}H]inositol in the presence of carbamoylcholine at the concentrations indicated, and release of inositol phosphates was determined. Values shown are means ± standard errors for triplicate replicates. *Insets:* Equiactive concentrations of carbamoylcholine (μM) in control and PrBCM-treated slices were obtained graphically and reciprocals were plotted. Calculated K_A values are 235 and 100 μM for cerebral cortex and hippocampus, respectively. Corresponding EC_{50} values were 200 and 90 μM, respectively.

TABLE 1

Effect of muscarinic receptor alkylation on receptor density and on the release of inositol phosphates in three brain regions

Tissue slices prepared from neostriatum, cerebral cortex, and hippocampus were divided into two aliquots. One was treated with PrBCM for 15 min at 37° at the concentrations indicated, while the other was incubated in the presence of buffer A. Both untreated and alkylated slices were then washed with buffer A and incubated with [^{3}H]inositol in the presence of either 10 mM carbamoylcholine (CARB), 10 mM bethanechol (BETH), or 1 mM OXO-2 for 120 min. Reactions were then terminated and radioactivity in the inositol phosphate fraction was determined. The per cent reduction in stimulated PPI turnover following receptor alkylation was calculated from the equation:

$$100 - (100 \times \frac{{}^{*}I_A - {}^{*}I_C}{I_A - I_C})$$

where I represents the release of inositol phosphates (expressed as dpm $\times 10^{-2}/10^4$ dpm lipids) in the presence (A) or absence (C) of agonist for alkylated (*) or untreated tissues. In absolute terms, the addition of carbamoylcholine, bethanechol, and OXO-2 increased the release of inositol phosphates in the neostriatum to 344 ± 32, 247 ± 21, and 348 ± 46% of control, respectively. In the cerebral cortex and hippocampus, addition of carbamoylcholine resulted in an increase in the release of inositol phosphates to 252 ± 22 and 219 ± 18% of controls, respectively. The densities of [^{3}H]QNB-binding sites in the neostriatum, cerebral cortex, and hippocampus in untreated tissues were 1.42 ± 0.17, 1.30 ± 0.07, and 1.22 ± 0.09 pmol/mg of protein. The corresponding values for [^{3}H]NMS-binding sites were 0.95 ± 0.22, 0.64 ± 0.07 and 0.61 ± 0.10 pmol/mg of protein. Values shown are means ± standard errors for the number of separate experiments indicated in parentheses.

Brain region	[PrBCM]	Release of [^{3}H]inositol phosphates (per cent reduction)			Receptor density (per cent reduction)	
		CARB	BETH	OXO-2	[^{3}H]QNB	[^{3}H]NMS
Neostriatum (n = 4)	40 nM	38 ± 2[a]	78 ± 2	76 ± 1	75 ± 5	72 ± 4
Cerebral cortex (n = 5)	10 nM	66 ± 5[b]	ND[c]	ND	36 ± 5	33 ± 4
Hippocampus (n = 6)	10 nM	64 ± 5[b]	ND	ND	40 ± 7	38 ± 5

[a] Less than reduction in bethanechol and OXO-2 responses, $p < 0.001$.
[b] Per cent reduction in carbamoylcholine-mediated PPI response is greater than reduction in mAChR density, $p < 0.01$.
[c] ND, not determined.

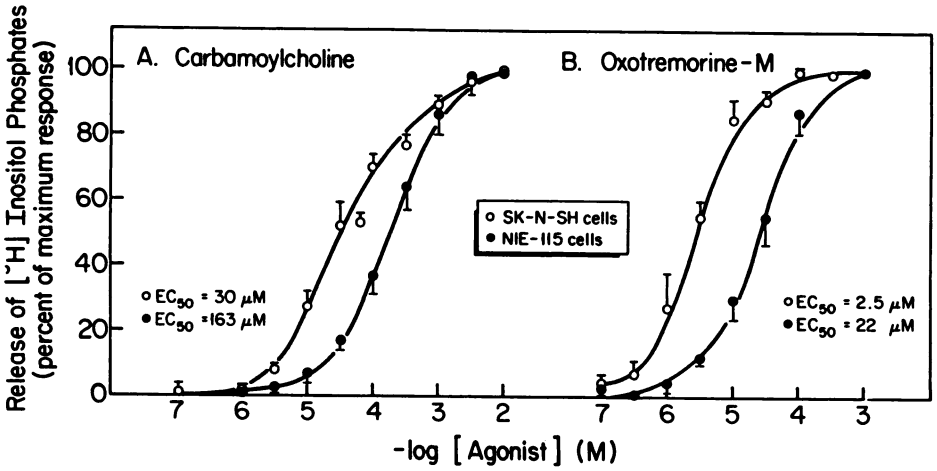

Fig. 5. Carbamoylcholine and oxotremorine-M are more potent stimulants of PPI hydrolysis in SK-N-SH cells than in N1E-115 cells. Cells were first allowed to prelabel with [^{3}H] inositol for 30 min and then incubated in the presence of the agonists at the indicated concentrations for a further 30 min. After termination of the reactions, labeled inositol phosphates were extracted and quantitated. Results are expressed as percentage of the maximum response (obtained at 10 mM carbamoylcholine or 1 mM oxotremorine-M) as a function of agonist concentration. Values shown are means ± standard errors for 4–10 separate experiments.

density was accompanied by a comparable loss of carbachol-, arecoline-, and bethanechol-stimulated inositol phosphate release.

In a previous study (11) we concluded, based upon pirenzepine inhibition, that mAChR stimulation of PPI hydrolysis is mediated by the M_2-mAChR subtype in the neostriatum, while an M_1-mAChR subtype mediates PPI turnover in the cerebral cortex and hippocampus. It was thus of interest to determine whether inositol lipid hydrolysis in mouse and human neuroblastomas was mediated by the same or different mAChR subtypes. In the N1E-115 cell, pirenzepine inhibited PPI hydrolysis with an apparent K_i value calculated from the Cheng and Prusoff equation (22) of 15.4 nM, whereas in the SK-N-SH cell the corresponding value was 187 nM (Fig. 8). The apparent K_i values for atropine were similar in the two cell types (0.34 and 1.03 nM, respectively). To examine more rigorously the pirenzepine inhibition of PPI turnover in the two neuroblastomas, dose response curves for carbamoylcholine-stimulated inositol phosphate release were constructed for control incubations and in the presence of three different concentrations of pirenzepine. Increasing concentrations of pirenzepine resulted in a parallel shift in the dose response curves characteristic of a competitive antagonist. Schild regression analysis of the data yielded a K_i value of 234 nM in the SK-N-SH cell (Fig. 9), whereas a K_i value of 10 nM was obtained for N1E-115 cells (Fig. 10). These values are similar to those obtained from the data in Fig. 8. Thus, in SK-N-SH cells (and in neostriatum), PPI turnover is a function of activation of the M_2 subtype, whereas in N1E-115 cells (and in cerebral cortex and hippocampus), it is the M_1 receptor that is coupled to PPI hydrolysis.

Discussion

The principal conclusion to emerge from the present study is that full mAChR occupancy is not invariably required for a maximum increase in the hydrolysis of inositol lipids. Whereas PPI turnover is linearly related to receptor occupancy in the cerebral cortex, in hippocampus and in N1E-115 cells, in the neostriatum and in SK-N-SH cells, three lines of experimental evidence point to the existence of a mAChR reserve for inositol lipid hydrolysis. First, alkylation of 30–40% of the available mAChRs failed to reduce the stimulated release of [^{3}H]inositol phosphates, and at each concentration of PrBCM, there was a greater reduction of receptor number than of functional re-

TABLE 2

Muscarinic agonist stimulation of labeled inositol phosphate release from SK-N-SH and N1E-115 neuroblastomas

Cells were first allowed to prelabel for 30 min with [^{3}H]inositol followed by exposure to the individual muscarinic agonists for a further 30 min. Reactions were terminated by the addition of chloroform/methanol (1:2 by volume), and inositol phosphates were separated from [^{3}H]inositol by ion exchange chromatography. For each agonist, the results are expressed in terms of stimulated release of inositol phosphates relative to that elicted by the addition of 1 mM oxotremorine-M. A 1 mM concentration was employed for all agonists except for bethanechol (10 mM) and OXO-2 (3 mM). Values shown are the means ± standard errors for four to seven separate experiments.

Agonist	Release of [^{3}H]inositol phosphates (per cent of maximum response)	
	SK-N-SH	N1E-115
a) Full		
Oxotremorine-M	(100)	(100)
Oxotremorine-4	96 ± 3	100 ± 0
Oxotremorine-2	92 ± 2	88 ± 6
b) Partial		
Bethanechol	77 ± 3[a]	27 ± 5
Arecoline	40 ± 1[a]	15 ± 3
Oxotremorine-1	28 ± 4[a]	7 ± 3
Oxotremorine	22 ± 1[a]	8 ± 2
Pilocarpine	18 ± 1	9 ± 1
c) Inactive		
McNA-343	5 ± 2	0 ± 0

[a] Different from stimulated PPI turnover in N1E-115 cells, $p < 0.01$.

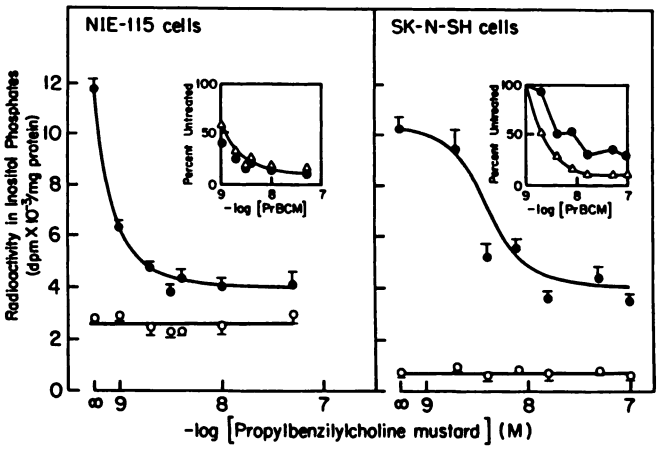

Fig. 6. Loss of mAChR number and of stimulated PPI turnover in PrBCM-treated neuroblastomas. N1E-115 and SK-N-SH cells were incubated for 15 min in either buffer A or in the presence of PrBCM at the concentrations indicated. After wash-out of unbound mustard, cells (0.75–0.90 mg of protein) were prelabeled with 4 μCi of [^{3}H]inositol and then incubated for a further 30 min with either buffer A (○) or 10 mM carbamoylcholine (●). Reactions were terminated and the labeled inositol phosphate fraction was isolated. Values shown are means ± standard errors for quadruplicate replicates. *Insets:* Percentages of original PPI response (●) and mAChR density (△) were calculated and plotted as a function of mustard concentration. The densities of [^{3}H]NMS-binding sites in untreated N1E-115 and SK-N-SH cells were 20 and 275 fmol/mg of protein, respectively.

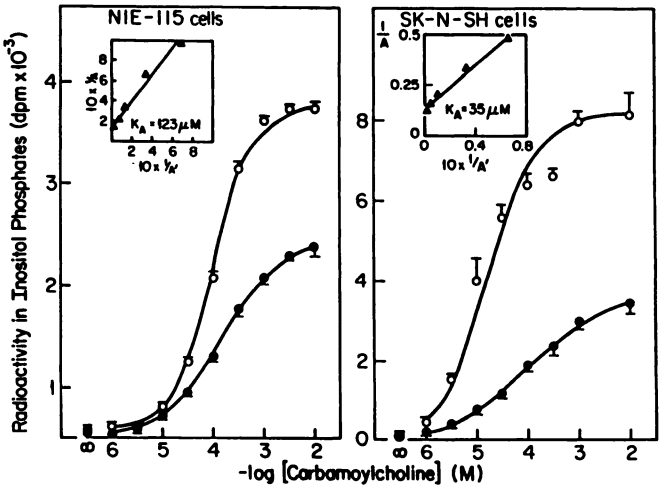

Fig. 7. Effect of PrBCM treatment on carbamoylcholine dose response curves for PPI turnover in N1E-115 and SK-N-SH cells. Cells were treated with either 2 nM (N1E-115) or 15 nM (SK-N-SH) PrBCM (●) or buffer A (○) for 15 min. After removal of unbound mustard, untreated and alkylated cells were prelabeled with [^{3}H]inositol and then incubated for a further 30 min in the presence of carbamoylcholine at the concentrations indicated. Results shown are means ± standard errors for triplicate replicates. *Insets:* Equiactive concentrations of carbamoylcholine (μM) in control and PrBCM-treated slices were obtained graphically and reciprocals plotted. Calculated K_A values were 123 μM (N1E-115 cells) and 35 μM (SK-N-SH cells). The corresponding EC_{50} values were 105 and 10 μM, respectively.

sponse. Second, following inactivation of 50–80% of mAChRs in the neostriatum and in SK-N-SH cells, there was both a reduction in the maximum response to carbamoylcholine and a shift in the dose response curves to higher agonist concentrations. The equilibrium dissociation constants (K_A) obtained following receptor inactivation were 2- to 9-fold higher than the comparable EC_{50} values, a result in keeping with the presence of a receptor reserve for PPI turnover. Third, the stimulation of PPI turnover elicited by partial agonists was more susceptible to receptor alkylation than that evoked by carbamoylcholine. Partial agonists are less able to transduce receptor occupancy into a response and must occupy more receptor sites than full agonists to achieve an optimum effect (21). It thus follows that, for responses which exhibit a receptor reserve, partial agonists will be more adversely affected than full agonists by receptor loss. Although the most parsimonious interpretation of the data is that a receptor reserve exists for PPI turnover in the neostriatum and in SK-N-SH cells, the possibility remains that the discrepancy between receptor number and response reflects an inability of the chosen radioligands to label all of the functionally coupled mAChRs. However, this explanation would not account for the shifts in agonist dose response curves, nor the differential susceptibilities of full and partial agonists to receptor alkylation. It has been concluded previously that there is little receptor reserve for stimulated PPI turnover in brain (6, 23, 24). However, spare receptors may exist for α_1-adrenergic stimulation of inositol lipid turnover in the vas deferens and caudal artery (25) and in both denervated iris smooth muscle (26) and hippocampus (23).

Occupancy of 10–33% of available mAChR sites in the neostriatum and in SK-N-SH cells by carbamoylcholine is required for a half-maximal stimulation of PPI turnover. Considerably fewer receptors need to be occupied for other consequences of mAChR activation such as the inhibition of adenylate cyclase in chick heart cells (<1%; Ref. 27), activation of cyclic AMP phosphodiesterase in 1321N1 astrocytoma cells (<5%; Ref. 28), or contraction of the ileum (0.5%; Ref. 29). Although the receptor reserve for PPI turnover, when present, is relatively small, it may be germane to the interpretation of desensitization studies involving inositol lipid hydrolysis. For example, brief exposure of 132N1 astrocytoma cells to carbamoylcholine results in a 20% reduction in mAChR number but not of stimulated inositol phosphate release (30). Thus, in astrocytoma

TABLE 3

Effect of muscarinic receptor alkylation on receptor density and on the release of inositol phosphates in two neuroblastomas

SK-N-SH and N1E-115 cells were divided into two aliquots. One was treated with PrBCM for 15 min at 37° at the concentrations indicated, while the other was incubated in the presence of buffer A. Untreated and alkylated cells were then prelabeled with [^{3}H]inositol for 30 min and then incubated in the presence of 10 mM carbamoylcholine (CARB), 10 mM bethanechol (BETH), or 1 mM arecoline (AREC) for a further 30 min. Reactions were then terminated and radioactivity in inositol phosphates was determined. The loss of agonist stimulation following PrBCM treatment was calculated as described in the legend to Table 1. Values shown are the means ± standard errors for the number of separate experiments indicated in parentheses, except for experiments with N1E-115 cells which are derived from a single experiment at each concentration of PrBCM.

Neuroblastoma	[PrBCM]	Release of [^{3}H]inositol phosphates (per cent reduction)			Receptor density (per cent reduction)	
		CARB	BETH	AREC	[^{3}H]QNB	[^{3}H]NMS
SK-N-SH	15 nM (n = 5)	55 ± 5[a,b]	77 ± 3	83 ± 3	72 ± 5	77 ± 6
N1E-115	2 nM	26	26	29	ND[c]	29
	4 nM	80	76	78	ND[c]	72

[a] Less than reduction in bethanechol and arecoline responses, $p < 0.01$.
[b] Per cent reduction in carbamoylcholine-mediated PPI response is less than reduction in mAChR density, $p < 0.05$.
[c] ND, not determined.

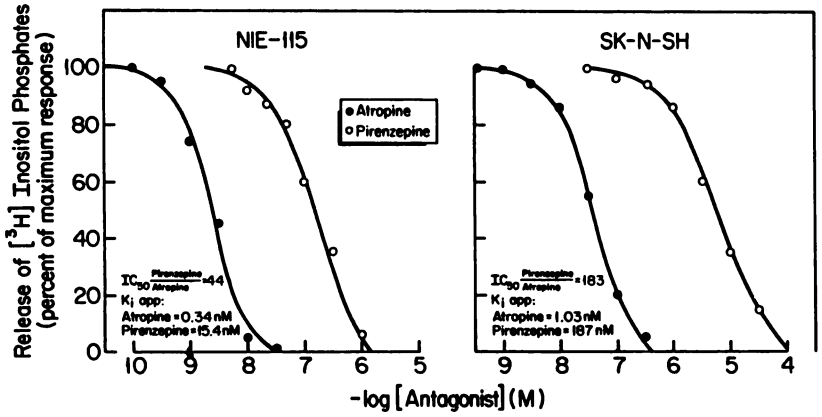

Fig. 8. Pirenzepine and atropine inhibition of PPI turnover in neuroblastomas. N1E-115 and SK-N-SH cells were prelabeled with [^{3}H]inositol for 30 min and then incubated for a further 30 min in the presence of 1 mM carbamoylcholine and atropine (●) or pirenzepine (○) at the concentrations indicated. Results are expressed as percentage of maximum response (obtained with agonist alone) as a function of antagonist concentration. Apparent K_i values were calculated from the Cheng and Prusoff equation (22). The K_A values for carbamoylcholine in N1E-115 and SK-N-SH cells were 120 μM and 65 μM, respectively. Values shown are means for either three separate experiments (pirenzepine) or two experiments (atropine).

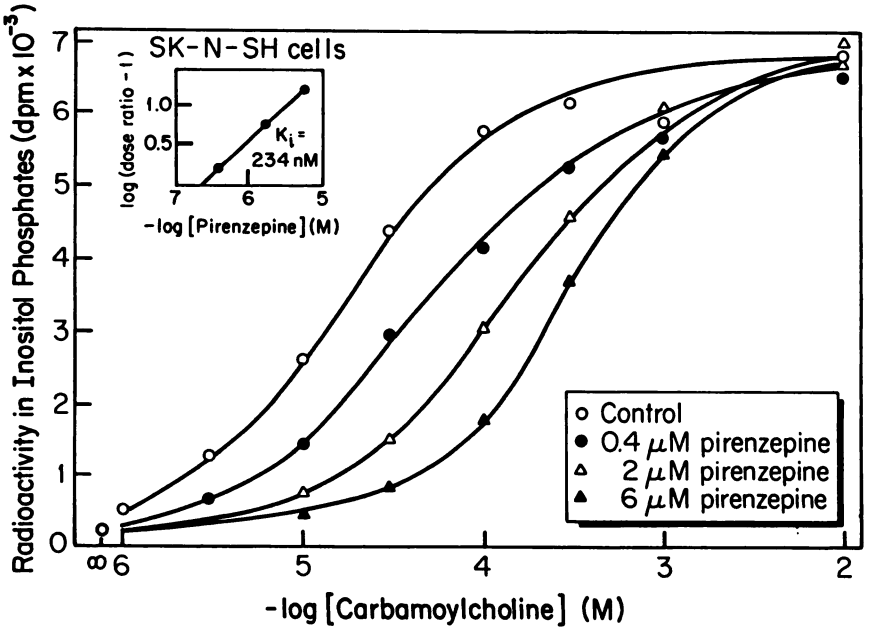

Fig. 9. Schild analysis of pirenzepine-inhibited carbamoylcholine-stimulated PPI turnover in SK-N-SH cells. Dose response curves for stimulated inositol phosphate release were determined in the absence of pirenzepine (○), or at pirenzepine concentrations of 0.4 (●), 2 (△), and 6 μM (▲). Values shown are the means of triplicate replicates from a single experiment. *Inset:* The log of the dose ratio−1 is plotted as a function of pirenzepine concentration. The calculated K_i value is 234 nM. In a second experiment using the same experimental conditions, a K_i value of 250 nM was calculated.

cells, as in the neostriatum or SK-N-SH cells, it appears that not all mAChRs need be occupied for a maximum breakdown of inositol lipids to occur. Conversely, in the cerebral cortex, where PPI hydrolysis is tightly coupled to muscarinic receptor occupancy, a loss of mAChRs that follows the chronic administration of a cholinesterase inhibitor is accompanied by a comparable desensitization of stimulated inositol lipid turnover (31).

Available evidence suggests that the primary biochemical event in increased PPI turnover in both neural and non-neural tissues is the breakdown of phosphatidylinositol 4,5-bisphosphate to release IP_3 and diacylglycerol (6). Whether a subsequent phosphodiesteratic breakdown of PI and phosphatidylinositol 4-phosphate also occurs remains the subject of debate (32). The principal water-soluble inositol metabolite identified in the present study was IP_1. This could conceivably have been

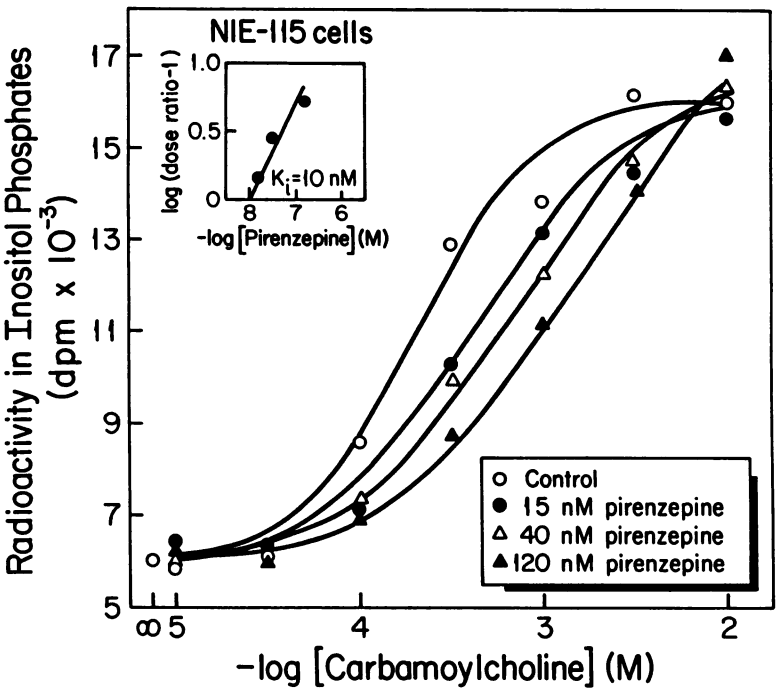

Fig. 10. Schild analysis of pirenzepine-inhibited carbamoylcholine-stimulated PPI turnover in N1E-115 cells. Dose response curves for stimulated inositol phosphate release were determined in the absence (○), or at pirenzepine concentrations of 15 (●), 40 (△), and 120 nM (▲). Values shown are the means of triplicate replicates from a single experiment. *Inset:* The log of the dose ratio−1 is plotted as a function of pirenzepine concentration. The calculated K_i value is 10 nM.

derived from the direct breakdown of PI or, alternatively, from sequential dephosphorylation of IP_3 and IP_2 (33). The results in the present study do not eliminate the possibility that the initial formation of IP_3 is linearly related to mAChR occupancy in all tissues, but that not all of the available receptors need be occupied to support a subsequent phosphodiesteratic breakdown of PI. This consideration, however, does not negate our primary conclusion that submaximal receptor occupancy can lead to maximum second messenger formation, since the breakdown of PI will liberate diacylglycerol, itself a second messenger molecule and activator of protein kinase C (8). Although the molecular basis for differences in the coupling efficiency of mAChRs is presently unknown, it remains possible that the various mAChRs interact with the intervening guanine nucleotide-binding protein with different stoichiometries.

A second conclusion to result from the present study is that both putative muscarinic receptor subtypes (M_1 and M_2) can couple to PPI hydrolysis in neuronal tissues. Based on the relative inability of pirenzepine to antagonize PPI turnover in the neostriatum (11) and in SK-N-SH cells (K_i = 160–250 nM), we conclude that it is the M_2 subtype that is coupled to inositol lipid hydrolysis in these tissues. M_2-mAChRs linked to PPI hydrolysis have also been identified in 132N1 astrocytoma and embryonic chick heart cells (34), tracheal smooth muscle (35), the parotid gland (3), and the avian salt gland (36). Conversely, it is the M_1 subtype that is coupled in most brain regions (3, 11, 37), mouse pituitary tumor cells (38), and murine N1E-115 neuroblastoma cells (K_i = 10–20 nM). The ability of mAChR subtypes to couple to PPI turnover is often difficult to predict from radioligand binding studies alone (3, 11, 34). In the present study we observed that the M_1 subtype is linked to PPI turnover in tissues in which there is an absence of significant receptor reserve, whereas it is the M_2 receptor that is coupled in tissues which exhibit spare receptors. One interpretation of this finding is that the coupling of M_2-mAChRs to PPI turnover may in general be more efficient than that of the M_1-mAChR.

In summary, the results obtained in the present study indicate that different degrees of receptor reserve can be demonstrated for mAChRs coupled to inositol lipid hydrolysis in neuronal tissues. The presence of a receptor reserve for PPI turnover may have functional relevance during conditions of muscarinic receptor desensitization.

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