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PHARMACOLOGICAL DIFFERENCES BETWEEN MUSCARINIC RECEPTORS COUPLED TO PHOSPHOINOSITIDE TURNOVER AND THOSE COUPLED TO ADENYLATE CYCLASE INHIBITION

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Abstract—Pharmacological differences between muscarinic cholinergic receptors coupled to phosphoinositide turnover and those coupled to adenylate cyclase were studied. Stimulation of muscarinic receptors from SK-N-SH human neuroblastoma cells resulted in phosphoinositide hydrolysis, but not in inhibition of cAMP formation. As has been shown previously, stimulation of muscarinic receptors from NG108-15 neuroblastoma × glioma cells, on the other hand, resulted in inhibition of cAMP formation without any observable phosphoinositide hydrolysis. These two cell lines provide a useful model system in which to study differential coupling of muscarinic cholinergic receptors. Inhibition of [^{3}H]*N*-methyl scopolamine ([^{3}H]NMS) binding and inhibition of carbachol-stimulated function by the antagonists pirenzepine, AF-DX 116, and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) were studied in this system. Pirenzepine inhibited [^{3}H]NMS binding in both cell lines with low affinity (K_i of 130 and 160 nM in NG108-15 and SK-N-SH cells respectively), indicating that both cell lines express M_2 receptors. None of the three antagonists studied exhibited any clear selectivity for the receptors in one cell line over those of the other. In contrast, several agonists including acetylcholine, bethanechol and carbachol exhibited pronounced selectivity. These agonists inhibited [^{3}H]NMS binding to membranes from SK-N-SH cells with IC_{50} values that were 17-, 3- and 38-fold higher, respectively, than those of NG108-15 cells. This selectivity was still observed when whole cells rather than membranes were studied. These findings indicate that pharmacological differences between receptors coupled to phosphoinositide turnover and those coupled to cAMP inhibition can be detected with certain agonists, but not with the antagonists pirenzepine, AF-DX 116 or 4-DAMP.

Muscarinic cholinergic receptors have been subtyped using a variety of pharmacological criteria, the most common of which has been the M_1/M_2 classification based on the affinity of the receptors for the antagonist pirenzepine [1, 2]. Attempts to correlate this pharmacological classification of muscarinic receptors with the intracellular responses elicited from receptor activation have thus far been unsuccessful. It has now been well established that both the M_1 and M_2 receptor subtypes can couple to both adenylate cyclase and phosphoinositide turnover through appropriate G proteins [3, 4]. In addition to pirenzepine, several other muscarinic antagonists including AF-DX 116 and 4-DAMP have been used recently to further subtype muscarinic receptors [5, 6]. In the current study, we show that none of these three antagonists is clearly able to distinguish between an adenylate cyclase-coupled muscarinic receptor and one that is coupled to phosphoinositide turnover. However, we also show that certain muscarinic agonists, including carbachol, acetylcholine and bethanechol, were very effective in distinguishing these two receptors. These findings indicate that pharmacological differences between differentially coupled muscarinic receptors can be detected.

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Activation of muscarinic cholinergic receptors leads to a variety of biochemical responses, including inhibition of adenylate cyclase [7–9], stimulation of phosphoinositide hydrolysis [3, 8, 9] and stimulation of guanylate cyclase [8, 9]. In brain and in most cultured cells, activation of muscarinic receptors leads to several of these responses simultaneously [10, 11], thus making it difficult to determine whether each of these responses is mediated by a specific muscarinic receptor subtype. One approach used in addressing this issue has been to transfect muscarinic receptor genes into mammalian cells and to study the coupling of the expressed protein. It has been shown recently that the human m1 (Hm1) and human m4 (Hm4) muscarinic receptor subtypes couple to phosphoinositide turnover and not to adenylate cyclase inhibition, whereas the human m2 and m3 (Hm2 and Hm3) subtypes couple only to adenylate cyclase inhibition and not to phosphoinositide turnover [12]. This study clearly demonstrates specificity in the coupling of muscarinic receptor subtypes to effector systems. It is still not clear, however, whether the inability of Hm1 and Hm4 receptors to couple to adenylate cyclase turnover is due to the absence in the transfected cells of a specific subtype of G_i [13], a subtype that may not be required for Hm2 or Hm3 coupling.

An alternative approach in determining whether each biochemical response is mediated by a different

muscarinic receptor subtype is through the use of cell lines that have muscarinic receptors coupled to a single effector system. Such receptors have been described recently in two cell lines [14]. NG108-15 neuroblastoma × glioma cells have muscarinic receptors that are coupled only to adenylate cyclase inhibition, and 1321N1 astrocytoma cells have muscarinic receptors that are coupled only to phosphoinositide turnover [14]. In the following report, we describe another cell line, SK-N-SH human neuroblastoma cells, that also has muscarinic receptors coupled to a single effector system. These human neuroblastoma cells have been shown previously to have muscarinic receptors coupled to phosphoinositide turnover [15, 16]. We show that the receptors in these cells did not couple to G_i or G_o, and, like the astrocytoma cells, had muscarinic receptors coupled only to phosphoinositide turnover. The pharmacological differences between the muscarinic receptors from NG108-15 cells and those of SK-N-SH cells were compared and found to differ.

MATERIALS AND METHODS

Materials. Carbamylcholine (carbachol) chloride, oxotremorine, methacholine, bethanechol, pilocarpine, atropine sulfate, IBMX*, and PGE_1 were from the Sigma Chemical Co. McN-A-343, arecoline and 4-DAMP were from Research Biochemicals Inc. (Wayland, MA) and pirenzepine and AF-DX 116 were gifts from Boehringer Ingelheim, Ltd.

Cell culture. NG108-15 cells were obtained from Dr. M. Nirenberg and grown at 37° in Dulbecco's modified Eagle's medium (Advanced Biotechnology, Silver Spring, MD) supplemented with 5% fetal bovine serum, 0.1 μM hypoxanthine and 0.016 μM thymidine. Cells of passages 21–32 were used in these studies. SK-N-SH human neuroblastoma cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells of passages 40–52 were used in these studies. All cells were subcultured weekly using a divalent cation-free phosphate-buffered saline solution containing 0.02% EDTA, and were maintained for at least 4 days as confluent cultures, before being subcultured further. The cells were refed 2 days after being subcultured and then daily until used.

Binding assays. Membranes from both cell lines were prepared as follows. The cells were washed three times with phosphate-buffered saline and then lysed in a solution of 2 mM Tris–HCl, pH 7.2, 1 mM EDTA for 30 min at 2°. The lysed cells were harvested and centrifuged at 15,000 rpm for 15 min. The membranes were resuspended in the Tris–EDTA solution and stored frozen (−70°). Before use, these cell membranes were thawed, centrifuged and resuspended in the Tris–EDTA solution by homogenizing in a teflon–glass homogenizer. Aliquots of cell membrane were incubated in Krebs solution (118 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 35 mM Tris–HCl, pH 7.4) with [^{3}H]NMS (New England Nuclear, Boston, MA; 87 mCi/mmol) at room temperature for 60 min. The binding reaction was terminated by rapid filtration over GF/B filters using a cell harvester (Brandel, Gaithersburg, MD). The filters were washed with ice-cold 0.9% NaCl, and counted in a scintillation counter after having equilibrated with the scintillation fluid. Non-specific binding was determined in the presence of 1 μM atropine and was subtracted from all values. The data were analyzed using the LIGAND and ALLFIT computer programs [17].

Binding to whole cells was performed as follows. The cells were washed three times with serum-free medium and then were detached from the flask using a divalent cation-free PBS solution containing 0.02% EDTA. An equal volume of serum-free medium was added and the cells were collected by centrifugation (200 *g*, 5 min). The cells were gently resuspended in serum-free medium containing 25 mM Hepes, pH 7.2, and used immediately. Aliquots of the cell suspension were incubated with the indicated drugs and [^{3}H]NMS for 60 min at 37° in serum-free medium containing Hepes. The incubation was terminated by rapid filtration over GF/B filters, which were washed and counted as described above.

cAMP accumulation assay. The growth medium from cell cultures that were 70–90% confluent was replaced with serum-free medium containing 1 mM IBMX, 25 mM Hepes pH 7.2, and the indicated drugs. The cells were equilibrated in this solution for 5–10 min at 37°; then PGE_1 was added, where indicated, to a final concentration of 5 μM. The cells were incubated for another 10 min at 37°, the medium was removed, and the cAMP was extracted with 0.1 M HCl. The HCl was lyophilized and the remaining cAMP was assayed by radioimmunoassay [18].

Protein determinations. Protein was determined using the assay described by Lowry *et al.* [19] with bovine serum albumin as standard.

Phosphoinositide turnover assay. Cells were prelabeled with 10 μCi/ml of [2-^{3}H(N)]*myo*-inositol from American Radiolabeled Chemicals, Inc. (St. Louis, MO) for about 24 hr. The growth medium containing the [^{3}H]*myo*-inositol was aspirated and the cells were washed twice with a solution of 10 mM LiCl in serum-free medium. The indicated drugs were then added to the cells along with 10 mM LiCl in serum-free medium containing 25 mM Hepes, and the cells were incubated at 37° for 30 min. The incubation was terminated by harvesting the cells in 1 ml of cold methanol. After adding chloroform and water, the mixture was sonicated and the two phases were separated by centrifugation at 1000 *g* for 15 min. An aliquot of the upper phase (1.2 ml) was applied to a 0.6 ml Dowex column (AG1 X8, Bio-Rad), the columns were washed with 15 ml of water, and the various inositol phosphates were eluted

* Abbreviations: IBMX, isobutylmethylxanthine; PGE_1, prostaglandin E_1; Hepes, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane; NMS, *N*-methyl scopolamine; PBS, Dulbecco's phosphate-buffered saline; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; SDS, sodium dodecyl sulfate; and DMEM, Dulbecco's modified Eagle's medium.

Table 1. Carbachol-stimulated phosphoinositide (PI) hydrolysis in two cell lines

	PI hydrolysis (fold stimulation)		
	[^{3}H]IP	[^{3}H]IP$_2$	[^{3}H]IP$_3$
NG108-15	1.13 ± 0.23	1.10 ± 0.10	1.07 ± 0.10
SK-N-SH	9.91 ± 0.30	5.40 ± 1.20	6.50 ± 0.91

Cells were prelabeled with [^{3}H]*myo*-inositol for 24 hr and then either exposed to 0.5 mM carbachol and 10 mM LiCl or just to LiCl for 30 min. The inositol phosphates were separated on ion exchange columns as described in Materials and Methods. The data shown represent the fold stimulation due to carbachol treatment and are means ± SD of three separate experiments. In a representative control experiment, the cpm in the [^{3}H]IP, [^{3}H]IP$_2$, and [^{3}H]IP$_3$ fractions were 1426, 240 and 236 in NG108-15 cells, and 188, 20 and 25 in SK-N-SH cells.

according to the procedure described by Berridge [20].

ADP-ribosylation. Pertussis toxin-catalyzed ADP-ribosylation of cell membranes was accomplished as follows. Membranes from each cell type were prepared as described above, and an aliquot (0.3 mg) was incubated for 90 min at 30° with 0.2 mCi of [^{32}P]NAD (ICN Radiochemicals, Irvine, CA; 238 Ci/mmol) in the presence or absence of 2 μg of pertussis toxin (List Biochemicals, Inc., Campbell, CA). SDS was then added and 60 μg of protein was separated on SDS-polyacrylamide gels. After drying the gel, the radioactivity was detected by autoradiography.

Data presentation. Except for the cAMP data, all values are means ± SD from at least three separate determinations, each performed in triplicate or quadruplicate. Since the intracellular cAMP levels in both cell lines varied from experiment to experiment, these values could not be averaged. Therefore, unless otherwise specified, the cAMP data represent means ± SD of triplicate determinations from a typical experiment that was repeated at least three times with qualitatively similar results.

RESULTS

*Coupling of muscarinic receptors to different effector systems in SK-N-SH and NG*108-15 *cells.* As previously demonstrated [15, 16], carbachol stimulation of SK-N-SH cells resulted in a strong phosphoinositide turnover response. When these cells were prelabeled with [^{3}H]*myo*-inositol and then stimulated with 0.5 mM carbachol for 30 min, [^{3}H]inositol mono-, bis-, and tris-phosphate levels were all elevated compared to unstimulated controls (Table 1). In contrast, exposure of prelabeled NG108-15 cells to carbachol failed to stimulate phosphoinositide hydrolysis (Table 1), even when short incubation times were used.

Carbachol stimulation of NG108-15 cells resulted in the previously observed [21] inhibition of both basal and PGE$_1$-stimulated cAMP levels (Table 2). In contrast, carbachol stimulation of SK-N-SH cells resulted in an increase of both basal and PGE$_1$-stimulated cAMP levels (Table 2). This increase was not sensitive to pertussis toxin pretreatment of the cells (100 ng/ml for 4 hr), indicating that it was not mediated via G_i or G_o. The pertussis toxin pretreatment did ADP-ribosylate G proteins, since these conditions abolish the morphine-induced inhibition of cAMP levels via opiate receptors in these cells [22]. Thus, NG108-15 cells have muscarinic receptors coupled to adenylate cyclase inhibition but not to phosphoinositide hydrolysis, whereas SK-N-SH cells have muscarinic receptor coupled to phosphoinositide hydrolysis, but not to adenylate cyclase inhibition.

Table 2. Effect of carbachol on cAMP accumulation in two cell lines

	cAMP accumulation (pmol/mg protein/10 min)	
	NG108-15	SK-N-SH
Basal		
Control	37.2 ± 6	30.9 ± 1
Carbachol	23.6 ± 3	46.7 ± 3
Carbachol + atropine	30.0 ± 3	17.2 ± 2
PGE$_1$-stimulated		
Control	7134 ± 318	1363 ± 163
Carbachol	3323 ± 219	3168 ± 24
Carbachol + atropine	6314 ± 419	1661 ± 259

Cells were preincubated for 10 min at 37° with 1 mM IBMX and the indicated drugs (0.5 mM carbachol; 1 μM atropine). PGE$_1$ (5 μM) was added, and the cells were incubated for another 10 min. cAMP was extracted and assayed as described in Materials and Methods. cAMP data represent means ± SD of triplicate determinations from a typical experiment that was repeated at least three times with qualitatively similar results.

One possible explanation for the absence of observable muscarinic receptor-mediated inhibition of intracellular cAMP levels in SK-N-SH cells may be that these cells lack a functional G_i or G_o. To demonstrate that the α subunit of these G proteins is present in SK-N-SH cells, we ADP-ribosylated membranes from these cells in the presence of pertussis toxin. As shown in Fig. 1, pertussis-toxin-catalyzed ADP ribosylation of membranes from both SK-N-SH and NG108-15 cells led to the phosphorylation of a protein in the 41,000 dalton range, demonstrating that the α subunit of G_i or G_o is present in both of these cell types.

To determine whether carbachol stimulation of SK-N-SH cells activates a calcium-dependent phosphodiesterase similar to that previously observed in 1321N1 astrocytoma cells [23], we compared the effect of carbachol stimulation of these cells in the presence and the absence of the phosphodiesterase inhibitor IBMX. Table 3 shows that carbachol stimulation of SK-N-SH cells resulted in increased cAMP levels whether or not IBMX was present, indicating that, in contrast to the astrocytoma cells, activation of muscarinic receptors in these cells did not activate a phosphodiesterase. Similarly, carbachol stimulation of NG108-15 cells resulted in decreased cAMP levels, whether or not IBMX was present (Table 3). In both cell lines, cAMP levels were higher in the presence of IBMX than in its absence.

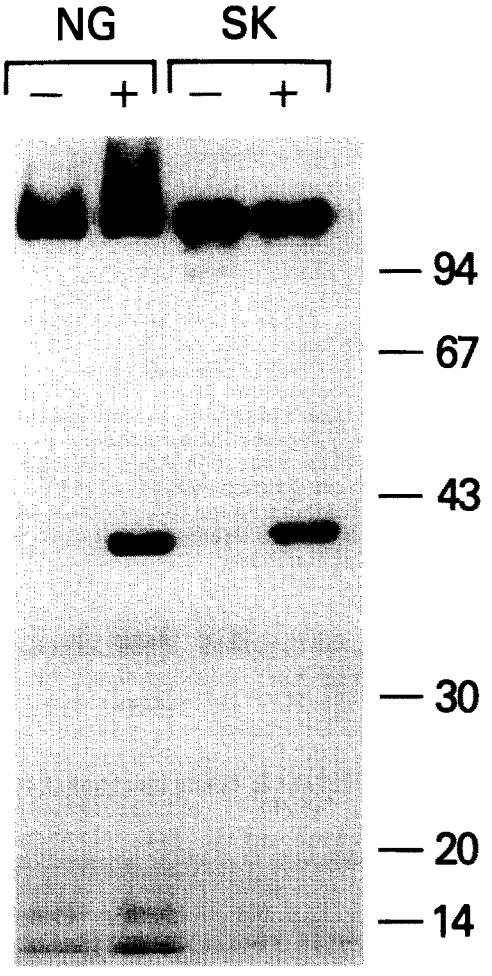


Fig. 1. Pertussis toxin-catalyzed ADP-ribosylation of cell membranes from NG108-15 and from SK-N-SH cells. Membranes from each cell type were incubated with [^{32}P]NAD in the absence (−) or presence (+) of 2 μg of activated pertussis toxin, dissolved in SDS, and separated by SDS–polyacrylamide gel electrophoresis. The figure shows the autoradiogram of the gel.

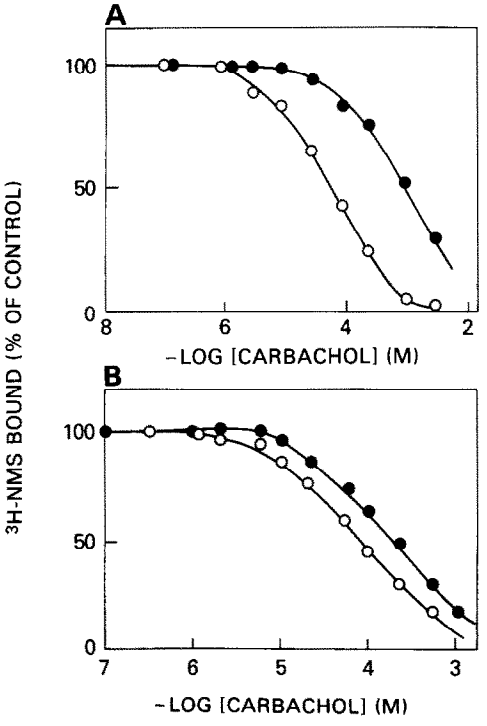


Fig. 2. (A) Carbachol inhibition of [^{3}H]NMS binding to membranes from SK-N-SH (●) and from NG108-15 cells (○). (B) Carbachol inhibition of [^{3}H]NMS binding to intact SK-N-SH (●) and NG108-15 (○) cells. Cells or membranes from cells were incubated for 90 min at 37° in DMEM-Hepes containing the indicated concentrations of carbachol and 0.5 nM [^{3}H]NMS. Data represent mean values of triplicate determinations from single experiments that were repeated four (top) or three (bottom) times with similar results. See text for IC_{50} and K_i values. Control values: (panel A) SK-N-SH cells, 1540 cpm/aliquot; NG108-15 cells, 760 cpm/aliquot; (panel B) SK-N-SH cells, 1000 cpm/250,000 cells; NG108-15 cells, 1100 cpm/375,000 cells.

Table 3. Effect of IBMX on PGE_1-stimulated cAMP accumulation in SK-N-SH and NG108-15 cells

		cAMP accumulation (pmol/mg protein/10 min) SK-N-SH	NG108-15
−IBMX	Control	828 ± 34	2704 ± 207
	Carbachol	1350 ± 106	927 ± 83
+IBMX	Control	3633 ± 124	7213 ± 638
	Carbachol	6126 ± 476	4406 ± 406

Cells were preincubated in DMEM-Hepes either with or without 1 mM IBMX for 5 min at 37°. Carbachol was then added where indicated and the PGE_1-stimulated cAMP levels were determined as described in Materials and Methods. cAMP data represent means ± SD of triplicate determinations from a typical experiment that was repeated at least three times with qualitatively similar results.

Agonist inhibition of [^{3}H]*NMS binding to differently coupled muscarinic receptors.* Figure 2A shows that carbachol was considerably more potent in inhibiting [^{3}H]NMS binding to membranes from NG108-15 cells compared to membranes from SK-N-SH cells. The IC_{50} values were 931 ± 98 μM (N = 4) and 61 ± 54 μM (N = 4) in membranes from SK-N-SH cells and from NG108-15 cells respectively. Conversion of the IC_{50} values to K_i values using the Cheng–Prusoff equation [24] yielded K_i values of 326 ± 34 μM (N = 4) and 15 ± 13 μM (N = 4) respectively. This represented a 21.7-fold difference in K_i values. When whole cells rather than membranes were used, significant differences in the binding of carbachol were still found (Fig. 2B), although the magnitude of this difference was smaller than that found in membranes. In three separate experiments, the average IC_{50} values were 230 ± 60 μM for SK-N-SH cells and 70 ± 40 μM for NG108-15 cells. Conversion of these values, using the Cheng–Prusoff equation, yielded K_i values of 51 ± 15 and 7 ± 4 μM in SK-N-SH and NG108-15 cells respectively.

To determine whether other muscarinic agonists

Table 4. Agonist inhibition of [^{3}H]NMS binding to membranes from two cell lines

Agonist	IC_{50} (μM)		n_H	
	NG108-15	SK-N-SH	NG108-15	SK-N-SH
Acetylcholine*	10 ± 2	174 ± 33	0.6 ± 0.1	0.6 ± 0.1
Bethanechol*	337 ± 81	1162 ± 240	0.7 ± 0.1	0.9 ± 0.2
Carbachol†	39 ± 15	1500 ± 280	0.5 ± 0.1	0.7 ± 0.1
Oxotremorine‡	29 ± 17	8 ± 1	0.5 ± 0.1	0.9 ± 0.1
Arecoline‡	62 ± 13	71 ± 11	0.7 ± 0.1	0.9 ± 0.1
Pilocarpine‡	18 ± 6	26 ± 10	0.9 ± 0.1	1.0 ± 0.1
Methacholine‡	160 ± 86	174 ± 31	0.8 ± 0.3	1.0 ± 0.2
McNeil-A-343‡	69 ± 13	55 ± 4	0.8 ± 0.1	1.1 ± 0.1

Cell membranes were preincubated with agonist for 30 min. [^{3}H]NMS was then added to a final concentration of 0.5 nM, and the membranes were incubated for another 60 min before being filtered and counted. Data are means ± SD of at least three independent determinations.

*‡ IC_{50} values of the two cell lines that are significantly different at *$P < 0.01$, or †$P < 0.05$ (Student's t-test); a double dagger (‡) denotes no significant difference between IC_{50} values.

Table 5. Relative effectiveness of muscarinic agonists in two cell lines

Agonist	cAMP formation in NG108-15 cells (% maximum response)	PI turnover in SK-N-SH cells (% of ACH response)
Acetylcholine	100	100
Arecoline	95	39
Methacholine	94	112
Oxotremorine	93	34
Carbachol	90	106
Bethanechol	73	73
McN-A-343	57	4
Pilocarpine	42	14

cAMP levels were determined by incubating NG108-15 cells in DMEM-Hepes containing 1 mM IBMX for 5 min at 37°. The indicated agonists were then added, and the PGE_1-stimulated cAMP levels were determined as described in Materials and Methods. The phosphoinositide hydrolysis was determined by incubating SK-N-SH cells in 10 mM LiCl and the indicated agonist for 30 min at 37°. Total [^{3}H]inositol phosphates were extracted and determined as described in Materials and Methods. The concentrations of agonists used were: 0.5 mM acetylcholine, 1 mM arecoline, 6 mM methacholine, 0.5 mM oxotremorine, 0.5 mM carbachol, 6 mM bethanechol, 1 mM McN-A-343, and 0.6 mM pilocarpine.

can differentiate receptors that are coupled to phosphoinositide turnover from those that are coupled to cAMP inhibition, we determined the IC_{50} for the displacement of [^{3}H]NMS binding by seven other agonists. As shown in Table 4, acetylcholine, bethanechol and carbachol were the only agonists tested that showed any statistically significant differences in IC_{50} values in the two cell lines. The displacement of [^{3}H]NMS binding by oxotremorine, arecoline, pilocarpine, methacholine and McN-A-343 was not statistically different in the two cell lines (Table 4).

To determine whether the differences in IC_{50} values for acetylcholine, bethanechol and carbachol were reflected in the potencies of the agonists in eliciting appropriate responses, we compared the potencies of the eight agonists in the two cell lines. As shown in Table 5, acetylcholine, methacholine and carbachol were full agonists in both cell lines; arecoline and oxotremorine were full agonists in NG108-15 cells but were only partially effective in eliciting phosphatidylinositol turnover in SK-N-SH cells, and bethanechol, McN-A-343 and pilocarpine were partial agonists in both systems, although McN-A-343 and pilocarpine were somewhat more effective in NG108-15 cells than in SK-N-SH cells. Thus, the relative potencies of acetylcholine, bethanechol and carbachol were similar in the two cell lines.

Comparison of antagonist inhibition of [^{3}H]*NMS binding and of carbachol-stimulated responses in two cell lines.* The non-classical muscarinic antagonist pirenzepine inhibited [^{3}H]NMS binding to membranes from both NG108-15 and SK-N-SH cells (Fig. 3A). Computer analysis of these binding data was best fit by a one-site model which yielded K_i values of 130 and 160 nM in NG108-15 and SK-N-SH cells respectively. In both cases, the Hill coefficient for this binding was close to unity. Since the K_d values

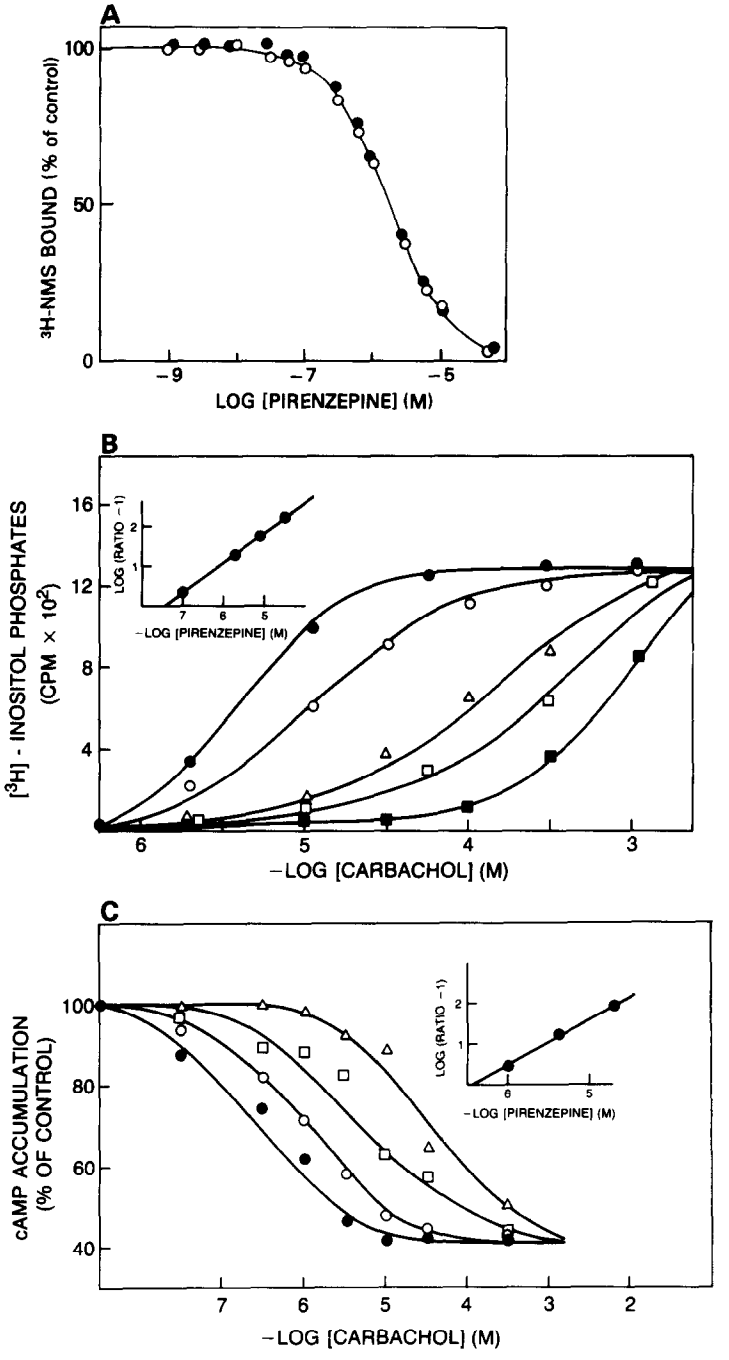


Fig. 3. Pirenzepine inhibition of [^{3}H]NMS binding and of carbachol-activated responses in SK-N-SH and NG108-15 cells. (A) Pirenzepine inhibition of [^{3}H]NMS binding to membranes from SK-N-SH (●) and NG108-15 (○) cells. Membranes were incubated with the indicated concentrations of pirenzepine and 0.5 nM [^{3}H]NMS for 60 min at 37°. Control values: 1500 cpm/aliquot for SK-N-SH cells and 900 cpm/aliquot for NG108-15 cells. (B) Schild analysis of pirenzepine inhibition of carbachol-activated phosphoinositide turnover in SK-N-SH cells. Cells were incubated in 10 mM LiCl in either the absence (●) or the presence of 0.1 μM (○), 2.0 μM (△), 8.0 μM (□) or 30 μM (■) pirenzepine for 15 min at 37°. Carbachol was then added at the indicated concentrations and the cells were incubated for another 30 min at 37°. Values shown are the averages of duplicate determinations from one experiment that was repeated three times (see text for K_i values). (C) Schild analysis of pirenzepine inhibition of the carbachol effect on cAMP accumulation in NG108-15 cells. Cells were incubated in 1 mM IBMX and either no (●), 1 μM (○), 4 μM (□) or 20 μM (△) pirenzepine for 15 min at 37°. Carbachol was then added at the indicated concentrations and the cells were incubated for another 5 min at 37°. PGE_1 was then added to a final concentration of 5 μM, and the cells were incubated for 10 min at 37°. Values shown are the averages of duplicate determinations from a single experiment that was repeated three times with similar results (see text for K_i values). Control value: 4675 pmol/mg protein/10 min.

for [^{3}H]NMS binding to membranes were slightly different in these two cell lines (37 ± 12 pM [N = 3] in SK-N-SH cells and 61 ± 17 pM [N = 3] in NG108-15 cells), the K_i values differed slightly, even though the competition curves were superimposable. Pirenzepine also inhibited the carbachol-stimulated phosphoinositide hydrolysis in SK-N-SH cells. Schild analysis [25] of this inhibition yielded a K_i value of 135 ± 63 nM (N = 3) (Fig. 3B), a value similar to that previously reported [16]. Furthermore, pirenzepine inhibited the carbachol effect on cAMP levels in NG108-15 cells with a K_i of 250 ± 32 nM (N = 3), as determined from Schild analysis (Fig. 3C).

AF-DX 116 has been shown recently to be selective for cardiac M_2 muscarinic receptors [5]. To determine whether AF-DX 116 exhibited any selectivity for the differentially coupled muscarinic receptors, we analyzed the ability of this drug to compete with [^{3}H]NMS binding in the two cell lines. Figure 4A shows the competition curves for AF-DX 116 inhibition of [^{3}H]NMS binding to intact cells. These data were best fit by a one-site model with K_i values of 456 ± 16 nM (N = 3) for NG108-15 cells and 435 ± 9 nM (N = 3) for SK-N-SH cells. Scatchard analysis of [^{3}H]NMS binding to intact cells revealed K_d values of 110 ± 28 pM (N = 3) in NG108-15 cells and 281 ± 19 pM (N = 3) in SK-N-SH cells. AF-DX 116, therefore, did not exhibit selectivity for the receptors on these two cell lines.

AF-DX 116 also inhibited the appropriate carbachol-activated responses in both cell lines. Schild analysis of AF-DX 116 inhibition of carbachol-stimulated phosphoinositide turnover in SK-N-SH cells yielded a K_i of 1.7 ± 0.6 μM (N = 3) (Fig. 4B). Schild analysis of AF-DX 116 inhibition of the carbachol-mediated cAMP response in NG108-15 cells yielded a K_i value of 480 ± 30 nM (N = 3) (Fig. 4C).

4-DAMP had been shown recently to be selective for M_3 muscarinic receptors from exocrine tissue [6]. To determine whether this drug exhibits selectivity for differentially coupled receptors, we looked at the ability of this drug to inhibit [^{3}H]NMS binding and carbachol-stimulated responses in the two cell lines. The competition curves for 4-DAMP inhibition of [^{3}H]NMS binding to intact cells, shown in Fig. 5A, yielded K_i values of 1.52 ± 0.16 nM (N = 3) and 1.75 ± 0.45 nM (N = 3) in NG108-15 and SK-N-SH cells respectively. These binding data were fit best by a one-site model. 4-DAMP also inhibited carbachol-stimulated phosphoinositide turnover in SK-N-SH cells. Schild analysis of this inhibition yielded a K_i of 1.2 ± 0.4 nM (N = 3) (Fig. 5B). In addition, 4-DAMP inhibited the carbachol-induced inhibition of cAMP levels in NG108-15 cells with a K_i, from Schild analysis, of 2.5 ± 1.8 (N = 3) nM (Fig. 5C).

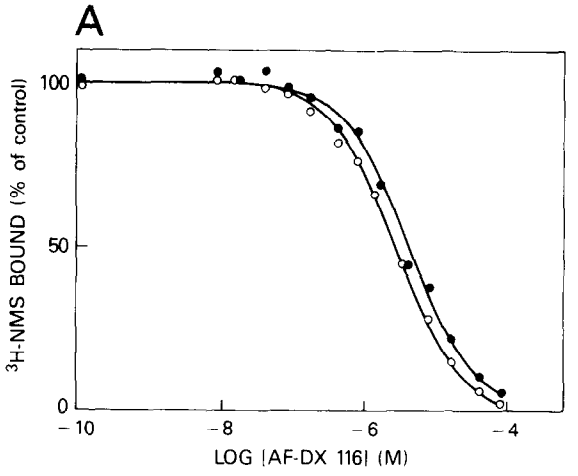
DISCUSSION

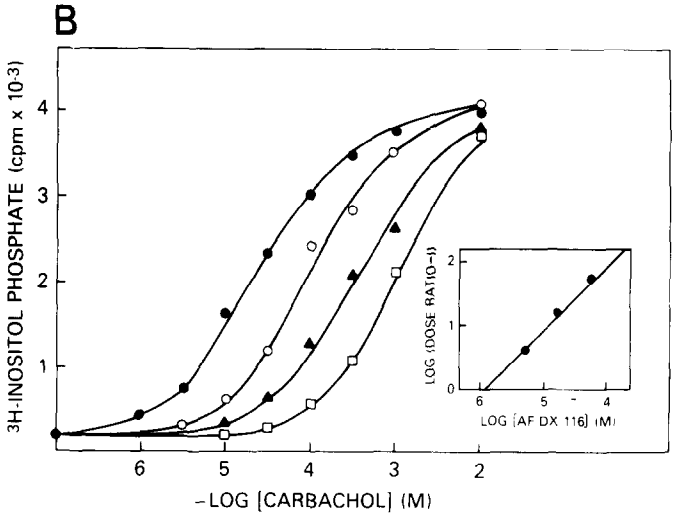
Two cell lines that each have muscarinic receptors coupled to a different effector system are described and compared in the current work. Activation of muscarinic receptors in NG108-15 glioma × neuroblastoma cells inhibited adenylate cyclase activity; no phosphoinositide hydrolysis in response to muscarinic agonists was observed. In contrast, SK-N-SH human neuroblastoma cells exhibited a phosphoinositide hydrolysis response when exposed to muscarinic agonists, but displayed no inhibition of cAMP accumulation. The non-classical antagonists pirenzepine, AF-DX 116, and 4-DAMP did not exhibit any clear selectivity for these differentially coupled receptors. In contrast, the agonists acetylcholine, bethanechol and carbachol displayed significant differences in their binding affinity for these two receptors. These findings show that certain agonists, but not antagonists, can detect pharmacological differences between these differentially coupled receptors.

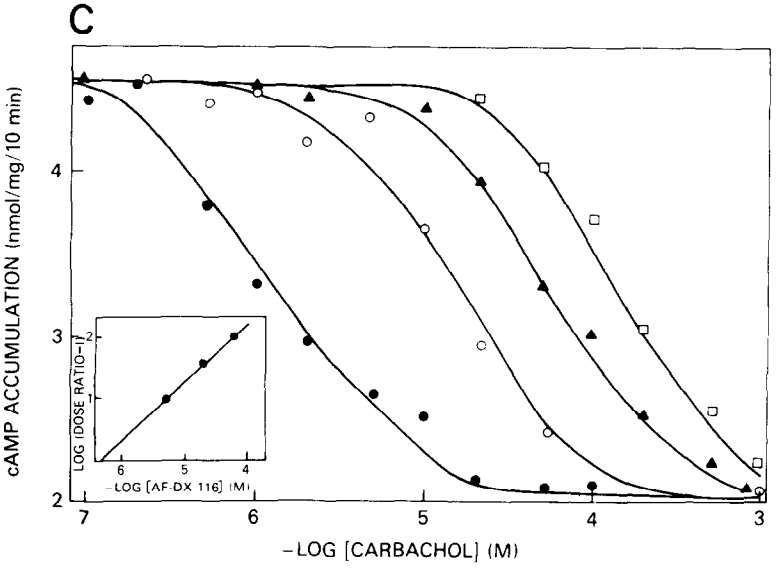
Muscarinic receptor activation of SK-N-SH cells resulted in a response that was similar, in some respects, to that of 1321N1 astrocytoma cells [23, 26]. In both of these cell lines, muscarinic receptor stimulation results in phosphoinositide hydrolysis, with no pertussis toxin-sensitive inhibition of cAMP formation [23, 26]. However, activation of muscarinic receptors in 1321N1 astrocytoma cells results in the stimulation of a calcium-dependent phosphodiesterase which causes a decrease in cAMP levels [27]. In contrast, no such stimulation of calcium-dependent phosphodiesterase activity was observed in SK-N-SH cells. Carbachol stimulation of SK-N-SH cells resulted in increased cAMP levels. This unusual response has pharmacological characteristics of being mediated through muscarinic receptors: it is dose-dependent and inhibitable by atropine and pirenzepine [22]. Thus, SK-N-SH human neuroblastoma cells represent a third example, along with NG108-15 and 1321N1 cells, of a cell line with muscarinic receptors coupled to a single and unique signal transduction pathway. These cells lines will be useful in future studies on the differential coupling of muscarinic receptors.

Two possible explanations could account for the absence of carbachol-mediated phosphoinositide turnover in NG108-15 cells. Either these cells lack the proper coupling proteins and enzymes for phosphoinositide turnover, or these cells express a particular subtype of muscarinic receptor that is unable to couple to this signal transduction system. Since bradykinin has been shown to stimulate IP_3 release in these cells [28], NG108-15 cells have the phospholipase C necessary to mediate receptor-coupled phosphoinositide turnover. It is not clear, however, whether these cells express the G protein necessary to couple muscarinic receptors to phosphoinositide turnover. This G protein may or may not be the same as that which couples bradykinin receptors to phosphoinositide turnover. Therefore, it is still not clear whether the muscarinic receptors expressed in NG108-15 cells are unable to couple the phosphoinositide turnover, or whether a specific G protein is lacking in these cells.

Similarly, the absence of a carbachol-mediated inhibition of adenylate cyclase in SK-N-SH cells may be accounted for by the same two possibilities. We have shown that these cells have a 41,000 dalton substrate for pertussis-toxin catalyzed ADP-ribosylation that, presumably, is the α subunit of G_i or G_o (Fig. 1). Furthermore, it has been reported [29], and we have confirmed [22] that these cells have opiate receptors which are coupled negatively to adenylate cyclase, and that this coupling is sensitive


A
100
50
0
^{3}H-NMS BOUND (% of control)
−10
−8
−6
−4
LOG [AF-DX 116] (M)


B
3H-INOSITOL PHOSPHATE (cpm x 10-3)
4
3
2
1
6
5
4
3
2
-LOG [CARBACHOL] (M)
LOG (DOSE RATIO-1)
2
1
0
6
5
4
LOG [AF DX 116] (M)


C
cAMP ACCUMULATION (nmol/mg/10 min)
4
3
2
7
6
5
4
3
-LOG [CARBACHOL] (M)
LOG (DOSE RATIO-1)
2
1
6
5
4
-LOG [AF-DX 116] (M)

to pertussis toxin [22]. Thus, these cells appear to have the necessary components for coupling inhibitory receptors to adenylate cyclase, yet we found no evidence that muscarinic receptors couple to adenylate cyclase in these cells. Whether this is due to the absence of a particular subtype of G_i [13] that may be required for muscarinic receptor coupling to adenylate cyclase, or whether the particular muscarinic receptor subtype expressed in these cells is unable to couple to G_i, is still not known.

Pirenzepine inhibition of the appropriate responses from NG108-15 and SK-N-SH cells (Fig. 3) revealed that both responses were mediated by receptors of low affinity for pirenzepine (M_2 receptors). In contrast to these findings, Akiyama *et al.* [30] found that pirenzepine inhibition of [^{3}H]quinuclidinyl benzilate binding to homogenates of NG108-15 cells is best fit by a two-site model, in which approximately three quarters of the sites are of high affinity for pirenzepine. The reason for the apparent discrepancy between the current work and that of Akiyama *et al.* [30] could be due to a difference in experimental conditions or to differences in the labeled ligand used. Evans *et al.* [23] also found a Hill coefficient for pirenzepine inhibition of [^{3}H]quinuclidinyl benzilate binding to membranes from NG108-15 cells of close to one, indicative of a single site. Their K_i value was slightly lower than that reported in the current work. Muscarinic receptors have been further subclassified on the basis of their affinity for the antagonists AF-DX 116 [5] and 4-DAMP [6]. As shown in the current work, neither of these drugs exhibited substantial selectivity for the differentially coupled receptors.

In contrast to the lack of selectivity exhibited by antagonists, certain muscarinic agonists were very selective for muscarinic receptors coupled to phosphoinositide turnover, compared to those coupled to adenylate cyclase inhibition. In accord with previous work on chick heart slices [31], we found a large difference in the half-maximal concentration of carbachol needed to elicit the two biochemical responses. The half-maximal concentration of carbachol needed to inhibit cAMP formation in NG108-15 cells was 1.3 μM, whereas that needed to stimulate phosphoinositide turnover in SK-N-SH cells was 25 μM (see Figs. 4 and 5), values that are similar to those from previous work with NG108-15 and with astrocytoma cells [23, 26]. Two possibilities have been proposed to account for this difference in the potency of carbachol: either it could be due to differences in receptor reserve in the coupling of the receptor to the two biochemical systems, or two different receptors each with different molecular and pharmacological properties could couple to each biochemical system [14, 32]. Since we detected large differences in the half-maximal concentration of carbachol needed to inhibit [^{3}H]NMS binding in the two cell systems, and since binding studies do not detect receptor reserve, the alternative explanation that these two cell lines have different receptors is the more likely.

The data in the current work show that there are detectable pharmacological differences between muscarinic receptors coupled to adenylate cyclase and those coupled to phosphoinositide turnover. In the cells studied in this work, these pharmacological differences could be detected with certain agonists, but not with antagonists. It is interesting to note that Evans *et al.* [23] also found that carbachol bound with higher affinity to membranes from NG108-15 cells, compared to those from 1321N1 cells, a cell line that has muscarinic receptors coupled only to phosphoinositide turnover. Furthermore, Brann *et al.* [32] recently showed that carbachol binds with higher affinity to COS-7 cells that have been transfected with cDNAs for m_4 receptors, compared to COS-7 cells transfected with m_1 or m_3 receptors. NG108-15 cells have been shown to express the m_4 receptor subtype [33], whereas SK-N-SH cells express a putative M_3 receptor subtype [34] that couples only to phosphoinositide turnover.

Fig. 4. AF-DX 116 inhibition of [^{3}H]NMS binding and of carbachol-stimulated responses in SK-N-SH and in NG108-15 cells. (A) AF-DX 116 inhibition of [^{3}H]NMS binding to SK-N-SH (●) and NG108-15 (○) cells. Cells were incubated with the indicated concentrations of AF-DX 116 and 0.5 nM [^{3}H]NMS for 60 min at 37°. Control values: 1000 cpm/250,000 cells for SK-N-SH cells and 1100 cpm/375,000 cells for NG108-15 cells. (B) Schild analysis of AF-DX 116 inhibition of carbachol-activated phosphoinositide turnover in SK-N-SH cells. Cells were incubated with 10 mM LiCl in either the absence (●) or the presence of 5 μM (○), 15 μM (▲), or 60 μM (□) AF-DX 116 for 15 min at 37°. Carbachol was then added at the indicated concentrations and the cells were incubated for another 30 min at 37°. Values shown are the averages of duplicate determinations from a single experiment that was repeated three times with similar results (see text for K_i values). (C) Schild analysis of AF-DX 116 inhibition of the carbachol effect on cAMP accumulation in NG108-15 cells. Cells were incubated in 1 mM IBMX and either no (●), 5 μM (○), 20 μM (▲), or 60 (□) μM AF-DX 116 for 15 min at 37°. Carbachol was then added at the indicated concentrations, and the cells were incubated for another 5 min at 37°. PGE_1 was then added to a final concentration of 5 μM, and the cells were incubated for 10 min at 37°. Values shown are the averages of duplicate determinations from a single experiment that was repeated three times with similar results (see text for K_i values).

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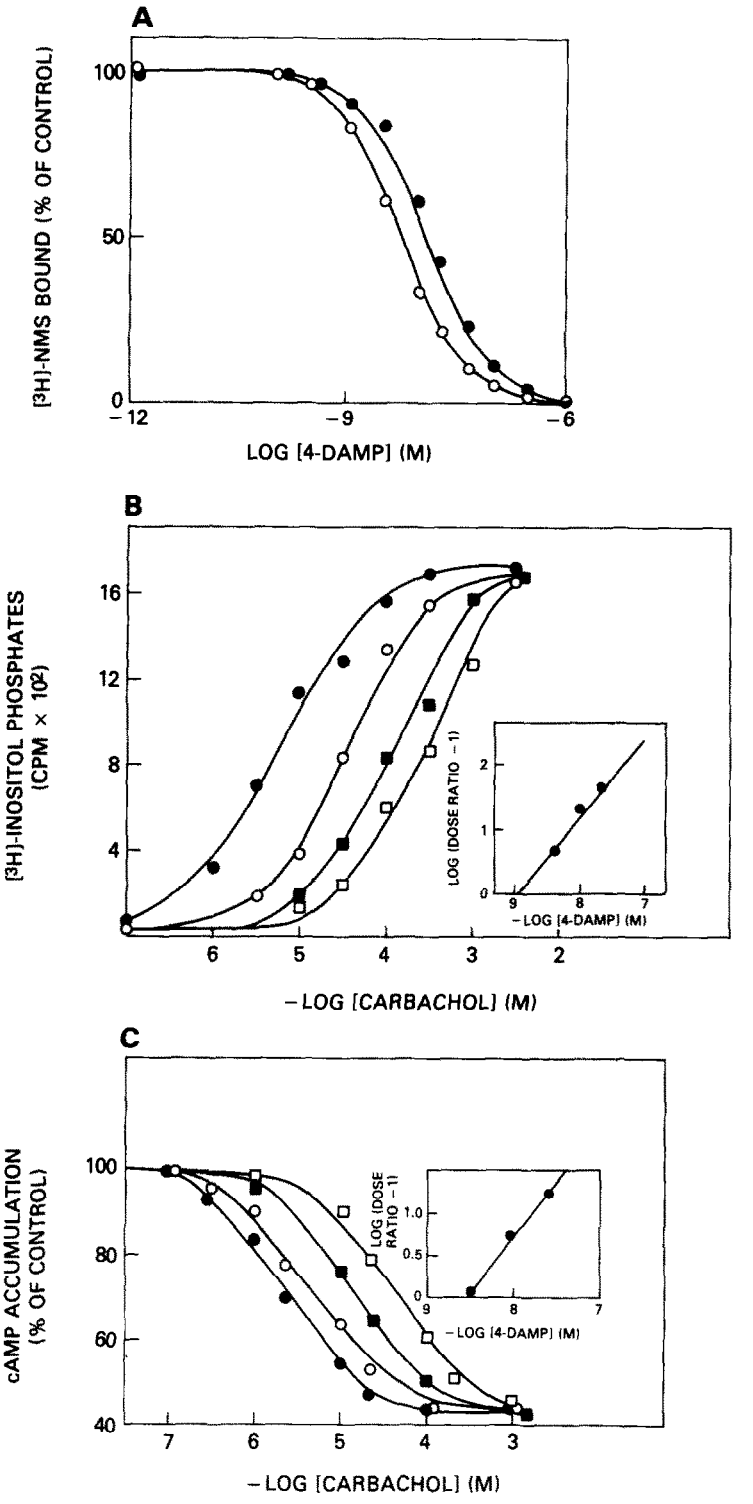
A
[3H]-NMS BOUND (% OF CONTROL)
100
50
0
−12
−9
−6
LOG [4-DAMP] (M)
B
[3H]-INOSITOL PHOSPHATES (CPM × 10²)
16
12
8
4
6
5
4
3
2
−LOG [CARBACHOL] (M)
LOG (DOSE RATIO − 1)
2
1
0
9
8
7
−LOG [4-DAMP] (M)
C
cAMP ACCUMULATION (% OF CONTROL)
100
80
60
40
7
6
5
4
3
−LOG [CARBACHOL] (M)
LOG (DOSE RATIO − 1)
1.0
0.5
0
9
8
7
−LOG [4-DAMP] (M)

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Fig. 5. 4-DAMP inhibition of [^{3}H]NMS binding and of carbachol-activated responses in SK-N-SH and NG108-15 cells. (A) 4-DAMP inhibition of [^{3}]HNMS binding to SK-N-SH (●) and to NG108-15 cells (○) cells. Cells were incubated with the indicated concentrations of 4-DAMP and 0.5 nM [^{3}H]NMS for 60 min at 37°. Control values: 1000 cpm/250,000 cells for SK-N-SH cells and 1100 cpm/375,000 cells for NG108-15 cells. (B) Schild analysis of 4-DAMP inhibition of carbachol-activated PI turnover in SK-N-SH cells. Cells were incubated in 10 mM LiCl in either the absence (●) or the presence of 4 nM (○), 10 nM (■), or 30 nM (□) 4-DAMP for 15 min at 37°. Carbachol was then added at the indicated concentrations and the cells were incubated for another 30 min at 37°. Values shown are the averages of duplicate determinations from one experiment that was repeated three times (see text for K_i values). (C) Schild analysis of 4-DAMP inhibition of the carbachol effect on cAMP accumulation in NG108-15 cells. Cells were incubated in the absence (●) or the presence of 3 nM (○), 9 nM (■), or 25 nM (□) 4-DAMP for 15 min at 37°. Carbachol was then added at the indicated concentrations and the cells were incubated for another 5 min at 37°. PGE_1 was then added to a final concentration of 5 μM, and the cells were incubated for another 10 min at 37°. Values shown are the averages of duplicate determinations from a single experiment that was repeated three times with similar results (see text for K_i values). Control value: 4230 pmol/mg protein/10 min.

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