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Recognition of Muscarinic Cholinergic Receptors in Human SK-N-SH Neuroblastoma Cells by Quaternary and Tertiary Ligands Is Dependent Upon Temperature, Cell Integrity, and the Presence of Agonists

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SUMMARY

The recognition of muscarinic cholinergic receptors (mAChRs) in human SK-N-SH neuroblastoma cells by hydrophilic (quaternary) and lipophilic (tertiary) ligands has been examined. When quiescent cells were incubated at 37°, the same maximum number of mAChRs was revealed by antagonists that possessed either a quaternary nitrogen, e.g., *N*-methylscopolamine (NMS) and *N*-methylquinuclidinyl benzilate, or a tertiary nitrogen, e.g., scopolamine and quinuclidinyl benzilate (QNB). If cells were incubated at 0°, the quaternary [^{3}H]NMS labeled 15–20% fewer sites than the tertiary [^{3}H]scopolamine; but upon warming to 37°, these inaccessible sites also became labeled. This suggests that mAChRs are present at both cell surface and sequestered sites in this cell, and that an equilibrium exists between the two cellular compartments. In competition studies at 37°, NMS detected a population of [^{3}H]QNB-binding sites which exhibited a very low affinity for the quaternary antagonist. However, the sites were not evident when mAChRs were labeled with [^{3}H]scopolamine, suggesting that factors other than the lipophilic nature of the probe are involved. Although mAChRs were equally accessible to charged and uncharged antagonists at 37°, the quaternary agonist carbamoylcholine competed for the sites labeled by quaternary antagonists with a 10- to 29-fold higher affinity than those labeled by tertiary antagonists, whereas the tertiary agonist OXO-2 displaced all sites with an equal affinity. However, carbamoylcholine competed equally well for [^{3}H]scopolamine- and [^{3}H]NMS-binding sites in either hypotonic cell lysates at 37° or in intact cells maintained at 0°. These results suggest that, at 37°, agonists induce the sequestration of cell surface receptors into a lipophilic environment in which receptors become inaccessible to quaternary, but not tertiary, ligands. Addition of NMS inhibited the stimulation of phosphoinositide hydrolysis elicited by either carbamoylcholine or OXO-2. The K_i values were similar for both agonists. It is concluded that mAChRs in SK-N-SH cells cycle between cell surface and sequestered sites. At 37°, this cycling is rapid and all receptors have access to the cell surface compartment, whereas at 0°, receptor translocation is prevented and a population of sequestered mAChRs is detected. When cells are exposed to an agonist at 37°, the equilibrium shifts such that more mAChRs are found in a sequestered cell compartment that is inaccessible to quaternary ligands. Although mAChRs are present at more than one location, only those at the cell surface which exhibit a high affinity for NMS appear to functionally couple to phosphoinositide turnover.

Studies of the binding of selective agents to mAChRs continue to manifest the complexities of the ligand-receptor interaction (for review, see Ref. 1). Thus, multiple forms of the mAChR with different agonist affinities (super-high, high, and low) have been identified in radioligand binding studies on the basis of both direct agonist binding and the displacement of labeled antagonists by unlabeled agonists (2, 3). Furthermore, the existence of subtypes of the mAChR has been proposed from the binding of agents such as the M_1-selective agent pirenzepine (4) or, more recently, by agents selective for M_2 or M_3 mAChRs such as AF-DX 116 (5, 6), dicyclomine (6), and 4-diphenylacetoxymethyl piperidine methiodide (6–8). An additional complexity that has recently emerged is the observation that, in some studies, the quaternary antagonists, NMS and NMeQNB have been found to label significantly fewer sites than the tertiary antagonist, QNB (9–12). In these tissues NMS displays a very low affinity for a subpopulation of mAChR sites labeled by [^{3}H]QNB. Similarly, in intact chick heart cells the quaternary agonist carbamoylcholine can detect populations of

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine; NMeQNB, *N*-methylquinuclidinyl benzilate; QNB, quinuclidinyl benzilate; PrBCM, propylbenzilylcholine mustard; PPI, phosphoinositide (phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OXO-2, 2-pyrollidinone 1-[4-(dimethylamino)-2-butynyl]-2-butene-dioate.

[^{3}H]QNB-binding sites which exhibit either high or low affinity for the agonist (12). From these observations it has been concluded that mAChRs are present both at the cell surface, where they readily interact with hydrophilic ligands such as NMS or carbamoylcholine, and in a hydrophobic domain within the cell membrane, where they are detected by the more lipophilic [^{3}H]QNB. However, there is still some uncertainty over this conclusion since, in other studies, the number of binding sites labeled by [^{3}H]QNB and [^{3}H]NMS is the same, suggesting that the two ligands recognize identical populations of receptors (13–16). Similarly, in a recent study, we demonstrated that, following alkylation of mAChRs in human SK-N-SH neuroblastoma cells with the irreversible quaternary antagonist, PrBCM, there was a similar reduction in the number of [^{3}H]NMS- and [^{3}H]QNB-binding sites (17). This result suggests that in SK-N-SH cells mAChRs may be recognized equally well by lipophilic and hydrophilic antagonists.

In the present study, the distribution of mAChR sites in SK-N-SH cells has been examined by monitoring the binding of radiolabeled quaternary and tertiary antagonists to intact cells. These cells are known to possess a high density of mAChRs that can couple to phosphoinositide turnover (17) and, thus, are well suited to studies which address the questions of receptor compartmentation and functional coupling. The results demonstrate that, in unstimulated cells, 80–85% of mAChRs exist at the cell surface, whereas 15–20% are sequestered. These two pools of receptors rapidly equilibrate such that at 37°, all mAChRs can be fully labeled by either quaternary or tertiary antagonists. If cells are incubated at 0°, no cycling of the receptors occurs and a population of mAChRs inaccessible to quaternary ligands is detected. Exposure of intact cells to agonists at 37° results in the sequestration of some of the cell surface receptors into a more lipophilic environment and a reduction in the ability of quaternary probes to compete for sites labeled by tertiary amine antagonists. Although mAChRs can be demonstrated at both cell surface and sequestered locations, only those at the cell surface appear to functionally couple to stimulated phosphoinositide turnover.

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) was obtained either from New England Nuclear (Boston, MA) or from Amersham Corp. (Arlington Heights, IL) at a specific activity of 72 Ci/mmol. [^{3}H]QNB was obtained either from New England Nuclear at a specific activity of 46 Ci/mmol or from Amersham Corp. at a specific activity of 30 Ci/mmol. [^{3}H]NMeQNB (70 Ci/mmol) was obtained from New England Nuclear. [^{3}H]Scopolamine (90 Ci/mmol) was custom synthesized by Amersham Corp. and adjusted to a specific activity of 30 Ci/mmol by the addition of unlabeled scopolamine. Purity of [^{3}H]scopolamine (>95%) was assessed by thin layer chromatography in the solvent system tetrahydrofuran/diisopropylethylamine (95:5). Dowex-1 (100-200 mesh; ×8 in the formate form) was obtained from Bio-Rad (Rockville Center, 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) NMS, atropine, and carbamoylcholine were obtained from Sigma Chemical Co. (St. Louis, MO). The source of SK-N-SH neuroblastoma cells was as previously described (17).

Cell culture conditions. Human SK-N-SH neuroblastoma cells were cultured under conditions that have been described previously (17). Cells were grown for 7–30 days and, in the majority of experiments, 10- to 20-day-old cells were utilized. Cells were detached from the tissue culture dishes by aspiration of the tissue culture medium, and addition of 10 ml of Puck's D_1 solution (18), as previously described (17). Cells were collected by centrifugation at 300 × *g* for 1 min. Cells were then resuspended in buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM Na_2HCO_3, 1 mM $MgCl_2$, 5.6 mM D-glucose, and 30 mM HEPES-N^+ buffer, pH 7.4) prior to either radioligand binding or inositol lipid turnover assays.

Radioligand binding assays. Intact cells (approximately 200–400 μg of protein) were routinely incubated in 2 ml of buffer A at 37° containing the radioligand ([^{3}H]NMS, [^{3}H]scopolamine, [^{3}H]QNB or [^{3}H]NMeQNB) ± displacer as indicated. An optimum ratio of specific/nonspecific binding was obtained with concentrations of radioligand that occupied 50–70% of the available mAChRs. Under these conditions 2–7% of added [^{3}H]NMS, [^{3}H]NMeQNB, and [^{3}H]scopolamine was bound (specific plus nonspecific), whereas comparable values for [^{3}H]QNB were 4–12%. Reactions were initiated by the addition of SK-N-SH cells. Unless indicated otherwise, reactions were terminated after 120 min by vacuum filtration through Whatman GF/B filters and filters were washed three times with 5 ml of 0.9% NaCl. In some experiments, a centrifugation assay was employed to obtain binding parameters for [^{3}H]QNB and [^{3}H]NMS. On those occasions, reactions were terminated by centrifugation at 1000 × *g* for 10 min. The pellets were then surface washed with buffer A and solubilized by the addition of 1% Triton X-100, then, radioactivity was determined after the addition of 5 ml of ACS scintillation fluid. Using this alternative procedure, K_d and B_{max} values were obtained for both ligands that fell within the same range as those obtained using the filtration assay. Nonspecific binding was determined as that unaffected by inclusion of 50 μM atropine. Protein was determined by the method of Geiger and Bessman (19).

Assay for release of labeled inositol phosphates: product identification. SK-N-SH cells were resuspended in buffer A containing [^{3}H]inositol (approximately 10 μCi/ml) at a protein concentration of 1–2 mg/ml and allowed to prelabel for 60 min at 37°. After 55 min, lithium chloride was added to achieve a final concentration of 12.5 mM. Aliquots (450 μl) of labeled cells were then added to tubes containing agonist ± antagonist and reactions were allowed to continue for an addition 45 min. Reactions were terminated by the addition of 1.5 ml of chloroform/methanol (1:2 by vol) and a water-soluble inositol phosphate fraction isolated (17). Since measurement of inositol lipid turnover served primarily as an index of mAChR activation, this prolonged incubation time was used to maximize the sensitivity of the assay. Under these conditions, the increased release of [^{3}H]inositol phosphates observed in the presence of agonist represents predominantly an increased formation of [^{3}H]inositol monophosphate, as reported previously (17). When cells were labeled for 48 hr in the presence of [^{3}H]inositol and then challenged with agonists at very short times (5–15 sec), the initial products detected were inositol trisphosphate and inositol tetrakisphosphate followed shortly thereafter by inositol bis- and monophosphates. In the presence of Li^+, the latter accumulated linearly with time.

Data analysis. Apparent K_i values for radioligand binding studies were calculated from the Cheng and Prusoff equation (20). K_i values for inhibition of stimulated PPI turnover were obtained from Schild analysis. Competition curves were analyzed by the LIGAND program (21).

Results

Binding of quaternary and tertiary antagonists at 37°. When intact SK-N-SH cells were incubated at 37° in buffer A that contained either [^{3}H]QNB or [^{3}H]NMS, specific binding reached a plateau after approximately 40 min and thereafter remained constant (Fig. 1). An incubation time of 120 min was routinely employed to ensure that equilibrium had been fully attained. Nonspecific binding of each radioligand increased in

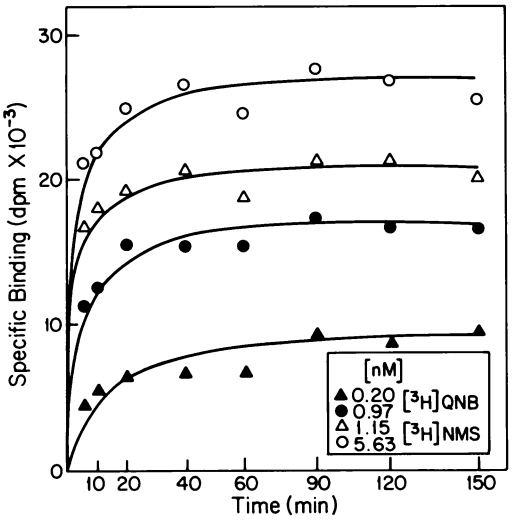

Fig. 1. Time course of specific [^{3}H]QNB and [^{3}H]NMS binding to intact SK-N-SH cells at 37°. SK-N-SH cells (400 μg of protein) were incubated with either [^{3}H]QNB or [^{3}H]NMS for the times indicated. Nonspecific binding was assessed at each time point.

a biphasic manner—an initial rapid increase which occurred within 2–5 min was followed by a slower phase of binding which increased up to 120 min. Specific binding of both ligands was saturable with concentration, although the ratio of specific to nonspecific binding was more favorable for [^{3}H]NMS (Fig. 2). Scatchard analysis of the saturation isotherms revealed that the quaternary ligand [^{3}H]NMS and the tertiary ligand [^{3}H]QNB labeled an equal number of mAChR-binding sites. Binding of [^{3}H]NMeQNB, a quaternary analog of QNB, and of [^{3}H]scopolamine, the tertiary analog of NMS, was also examined. All four ligands revealed the same maximum number of binding sites (Fig. 3). A summary of the B_{max} and K_d data obtained from 4–19 separate experiments is shown in Table 1. It is apparent that, at 37°, both hydrophilic (quaternary) and lipophilic (tertiary) antagonists detect an equal number of mAChR sites in SK-N-SH cells.

Binding of [^{3}H]scopolamine and [^{3}H]NMS at 0°. The binding data obtained at 37° suggested that all of the mAChRs in SK-N-SH cells are present at a location that is accessible to charged hydrophilic ligands at or near the cell surface. However, there remained the possibility that a population of sequestered receptors was present in the cells, but that at physiological temperatures these receptors would rapidly equilibrate with those at the cell surface. To investigate this possibility, radioligand binding assays were performed at 0°—a temperature at which receptor translocation would be minimal. The ligands chosen were [^{3}H]scopolamine and [^{3}H]NMS, since neither [^{3}H]QNB nor [^{3}H]NMeQNB binding attained equilibrium within 24 hr at 0°. Specific binding of [^{3}H]scopolamine reached equilibrium after 14 hr at 0° and thereafter remained constant up to 24 hr of incubation. When SK-N-SH cells were incubated for 24 hr at 0°, the number of mAChRs detected by [^{3}H]scopolamine was 14 ± 1% less (mean ± SE, n = 3) than that detected following a 2-hr incubation at 37° ($p < 0.01$; Fig. 4).

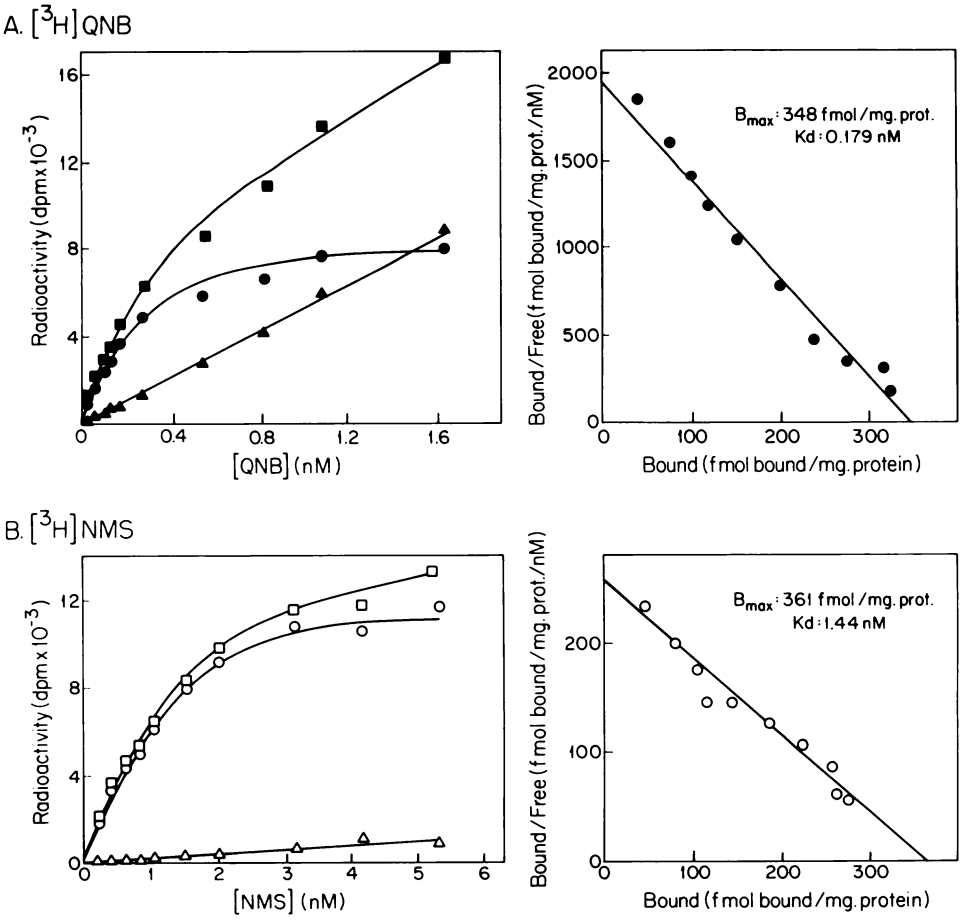

Fig. 2. Saturation isotherms for either [^{3}H]QNB binding (A) or [^{3}H]NMS binding (B). SK-N-SH cells (240 μg of protein) were incubated for 120 min at 37° in the presence of increasing concentrations of each radioligand. At each concentration, values for total binding (■, □), specific binding (●, ○), and nonspecific binding (▲, △) were determined. These values are plotted as a function of the concentration of added ligand (*left panels*). Scatchard analyses of the saturation isotherms were linear with Hill coefficients of 1.03 and 1.05 for [^{3}H]QNB and [^{3}H]NMS, respectively (*right panels*).

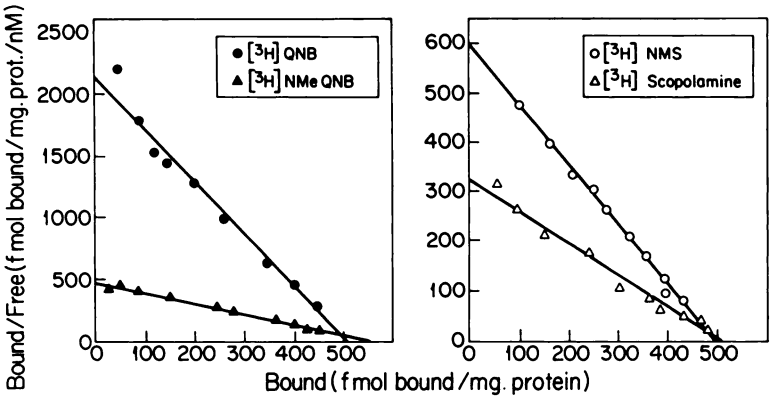

Fig. 3. Scatchard analysis of the binding of four radiolabeled muscarinic antagonists to intact SK-N-SH cells. The B_{max} values for the quaternary ligands [^{3}H]NMS and [^{3}H]NMeQNB were 487 and 536 fmol/mg of protein. The corresponding values for the tertiary ligands [^{3}H]scopolamine and [^{3}H]QNB were 503 and 506 fmol/mg of protein, respectively. The K_d values for [^{3}H]NMS, [^{3}H]NMeQNB, [^{3}H]scopolamine, and [^{3}H]QNB were 0.82, 1.09, 1.53, and 0.23 nM, respectively.

TABLE 1

Ligand binding to intact SK-N-SH neuroblastoma cells at 37°: maximum binding (B_{max}) and equilibrium dissociation constants (K_d)

Values shown are means ± standard errors for the number (n) of separate experiments as indicated. Range of values is shown in parentheses.

Ligand	n	B_{max}	K_d
		fmol/mg protein	*nM*
[^{3}H]QNB	19	417 ± 17 (289–560)	0.23 ± 0.02 (0.13–0.39)
[^{3}H]NMS	12	414 ± 26 (273–631)	1.20 ± 0.12 (0.69–2.06)
[^{3}H]Scopolamine	9	480 ± 34 (359–652)	1.60 ± 0.31 (0.69–2.85)
[^{3}H]NMeQNB	4	455 ± 45 (353–536)	1.31 ± 0.10 (1.09–1.56)

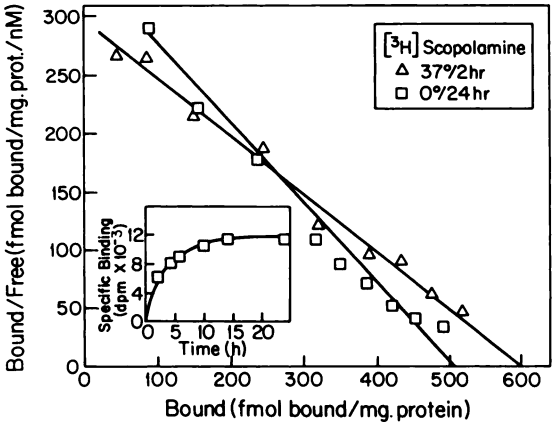

Fig. 4. Scatchard analysis of the binding of [^{3}H]scopolamine to intact SK-N-SH cells either at 0° for 24 hr or at 37° for 2 hr. The B_{max} values were 596 fmol/mg of protein at 37°, whereas that at 0° was 15% lower (506 fmol/mg of protein). The K_d values for [^{3}H]scopolamine were 1.99 nM at 37° and 1.10 nM at 0°. *Inset*: Time course for the binding of [^{3}H]scopolamine at 0°. Equilibrium was achieved after a 14-hr incubation.

Comparable experiments employing [^{3}H]NMS revealed that equilibrium conditions were attained following a 6- to 8-hr incubation at 0°, and that specific binding remained stable for up to 24 hr. At 0°, [^{3}H]NMS labeled significantly fewer mAChR sites than at 37° (Fig. 5). In five separate experiments at time points ranging from 6 to 24 hr, 35 ± 2% fewer sites were detected at 0°. To determine whether the mAChRs that were not detected by [^{3}H]NMS at 0° were still present in the cells, experiments were performed in which SK-N-SH cells were first incubated at 0° and then transferred to a 37° water bath for 2 hr before termination of the reaction (Fig. 6). At each time

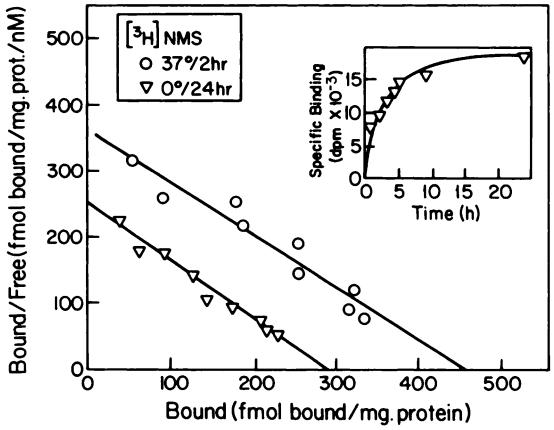

Fig. 5. Scatchard analysis of the binding of [^{3}H]NMS to intact SK-N-SH cells either at 0° for 24 hr or at 37° for 2 hr. The B_{max} values were 451 fmol/mg of protein at 37°, while that obtained at 0° was 37% lower (286 fmol/mg of protein). The K_d values for [^{3}H]NMS were 1.25 nM at 37° and 1.15 nM at 0°. *Inset*: Time course for the binding of [^{3}H]NMS at 0°. In this experiment, equilibrium was achieved after approximately 8 hr. In other experiments, equilibrium was reached after 4–6 hr of incubation.

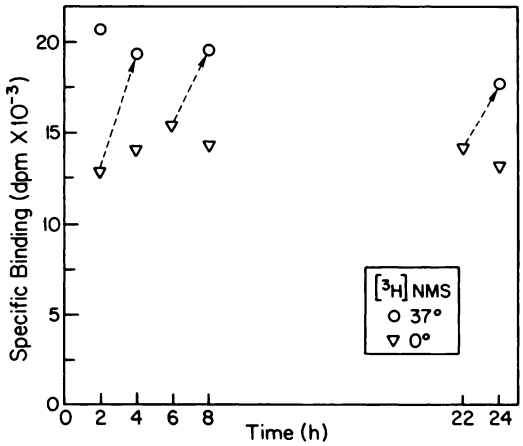

Fig. 6. Transfer of SK-N-SH cells at 0–37° increases the specific binding of [^{3}H]NMS. Cells were allowed to incubate for various times at 0° and then transferred to a 37° water bath for 2 hr before termination of the reaction. At 4, 8, and 24 hr there were 27, 24, and 29% increases in specific binding in comparison to cells maintained at 0°.

point chosen (4, 6, and 24 hr), there was a 25–35% increase in specific binding following the transfer of the tubes to 37° over comparable incubations maintained at 0°. In four separate experiments, the increase in specific binding observed at 24 hr was 22 ± 3%. In absolute terms, the specific binding of [^{3}H]

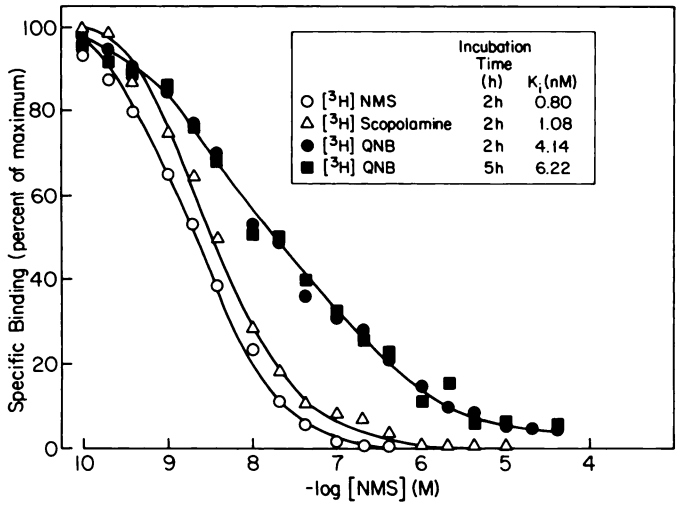

Fig. 7. Inhibition of specific [^{3}H]NMS, [^{3}H]scopolamine, and [^{3}H]QNB binding to SK-N-SH cells by the addition of NMS. Cells were incubated for either 2 hr ([^{3}H]scopolamine, [^{3}H]NMS, and [^{3}H]QNB) or for 5 hr ([^{3}H]QNB) in the presence of NMS at the concentrations indicated. The Hill coefficients for displacement of [^{3}H]scopolamine and [^{3}H]NMS binding were 1.11 and 0.83, respectively, while those for [^{3}H]QNB were 0.50 and 0.53 (2- and 5- hr incubations, respectively).

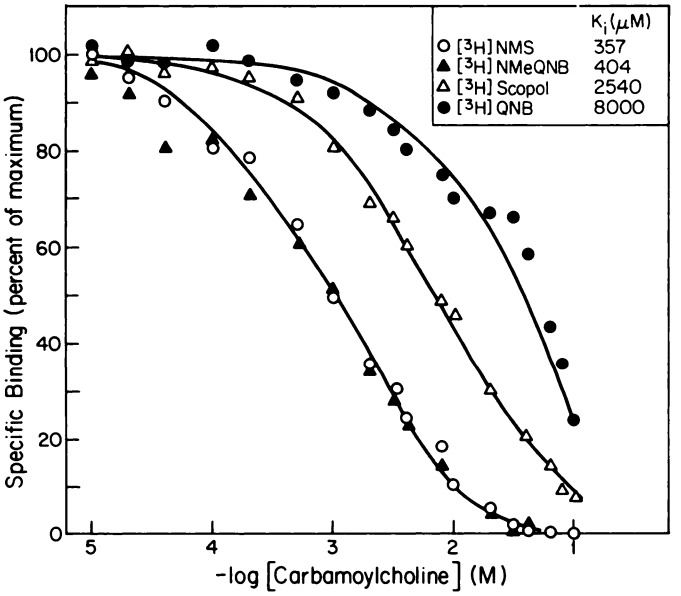

Fig. 8. Inhibition of specific [^{3}H]NMS, [^{3}H]NMeQNB, [^{3}H]scopolamine, and [^{3}H]QNB binding to SK-N-SH cells by the addition of the quaternary agonist carbamoylcholine. Cells were incubated for 120 min at 37° in the presence of carbamoylcholine at the concentrations indicated. The Hill coefficients were 0.85, 0.78, 0.77, and 0.63 for [^{3}H]NMS, [^{3}H]NMeQNB, [^{3}H]scopolamine, and [^{3}H]QNB, respectively. For the sake of clarity, a single line is drawn for [^{3}H]NMS and [^{3}H]NMeQNB displacement.

NMS was 19 ± 1% lower than that obtained for cells incubated for 2 hr at 37°, indicating that a small amount of receptor degradation occurs during the 24-hr incubation period. When this is taken into account, the results indicate that [^{3}H]scopolamine can fully label the mAChR complement at 0°, while [^{3}H]NMS can label approximately 80–85% of the receptors present. The latter would appear to represent receptors at the cell surface, whereas the remaining 15–20% are sequestered.

Displacement of [^{3}H]QNB, [^{3}H]NMS, and [^{3}H]scopolamine by unlabeled NMS. Atropine displaced [^{3}H]QNB and

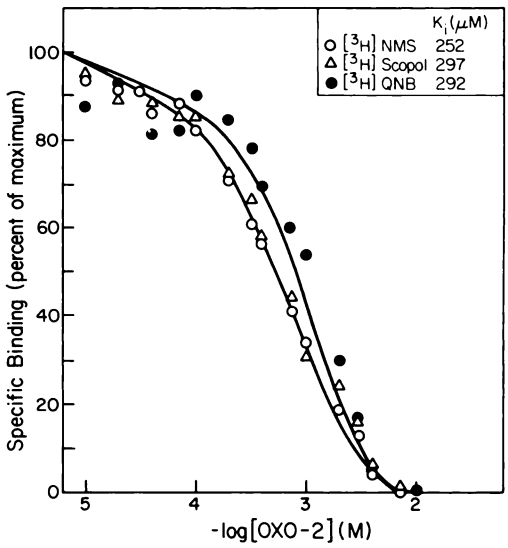

Fig. 9. Inhibition of specific [^{3}H]NMS, [^{3}H]scopolamine, and [^{3}H]QNB binding to SK-N-SH cells by addition of the tertiary agonist OXO-2. Cells were incubated for 120 min at 37° in the presence of OXO-2 at the concentrations indicated. The Hill coefficients were 0.99, 0.97, and 1.08 for [^{3}H]NMS, [^{3}H]scopolamine, and [^{3}H]QNB, respectively. For the sake of clarity, a single line is drawn for [^{3}H]NMS and [^{3}H]scopolamine displacement.

[^{3}H]NMS bound to intact cells at 37° according to the law of mass action, with K_i values of 2–5 nM. In contrast, whereas NMS readily displaced [^{3}H]NMS, its ability to displace [^{3}H]QNB was more limited (Fig. 7). NMS competed for [^{3}H]QNB-binding sites in a complex manner, with Hill coefficients (0.48 ± 0.02, mean ± SE, n = 9) that were significantly less than unity ($p < 0.01$). However, the displacement data were only successfully fitted by LIGAND to a two-site model in four of these experiments. On those occasions when a two-site fit was indicated, 72 ± 4% sites had a high affinity for NMS (K_H = 3.1 ± 0.7 nM), whereas the remaining 28% of sites exhibited a very low affinity for the quaternary antagonist (K_L = 437 ± 194 nM). The complex interaction of NMS with [^{3}H]QNB sites was observed at either 2 or 5 hr of incubation at 37°, and also following preincubation of the tissue with NMS prior to the addition of [^{3}H]QNB. In contrast, NMS was able to readily displace the tertiary ligand [^{3}H]scopolamine with an apparent K_i of 0.97 ± 0.15 nM (mean ± SE, n = 3), a value similar to the K_d for binding of [^{3}H]NMS (1.2 nM; see Table 1). The Hill coefficient for [^{3}H]scopolamine binding was 0.94 ± 0.11 (n = 3), a value not significantly less than 1.

Binding of quaternary and tertiary agonists at 37°. Although mAChRs on SK-N-SH cells are readily accessible to both charged and uncharged radiolabeled antagonists at 37°, there are marked differences in the ability of the quaternary agonist, carbamoylcholine, to displace these ligands. Thus, under equilibrium conditions, carbamoylcholine displayed a 10- to 29-fold higher affinity for [^{3}H]NMS- and [^{3}H]NMeQNB-binding sites than for the sites labeled by either [^{3}H]scopolamine or [^{3}H]QNB (Fig. 8). The Hill coefficients for all four ligands were significantly less than 1, indicating receptor heterogeneity. It remains possible that the high concentrations of carbamoylcholine necessary to displace the tertiary radioligands (and hence the increase in ionic strength) may complicate the calculation of the Hill coefficients. In contrast, addition of the oxotremorine analog, OXO-2, a lipophilic molecule (22, 23),

TABLE 2

Carbamoylcholine and OXO-2 competition for sites labeled by tertiary and quaternary antagonists at 37°: inhibition constants (K_i) and Hill coefficients (n_H)

Values shown are means ± standard errors (or range where $n = 2$) for the number of separate experiments as indicated.

Antagonist	Carbamoylcholine			OXO-2		
	K_i	n_H	(n)	K_i	n_H	(n)
	μM			μM		
[^{3}H]QNB	8078 ± 1329[a,b]	0.63 ± 0.09[c]	(5)	243 ± 26	1.08 ± 0.07	(4)
[^{3}H]Scopolamine	3194 ± 336[b]	0.77 ± 0.03[c]	(4)	206 ± 46	0.97 ± 0.08	(4)
[^{3}H]NMS	277 ± 48	0.85 ± 0.02[c]	(4)	200 ± 22	0.99 ± 0.04	(4)
[^{3}H]NMeQNB	312 ± 65	0.78 ± 0.04[c]	(4)	163 ± 8	0.95 ± 0.03	(2)

[a] Different from [^{3}H]scopolamine, $p < 0.01$.
[b] Different from either [^{3}H]NMS or [^{3}H]NMeQNB, $p < 0.01$.
[c] Significantly less than 1, $p < 0.01$.

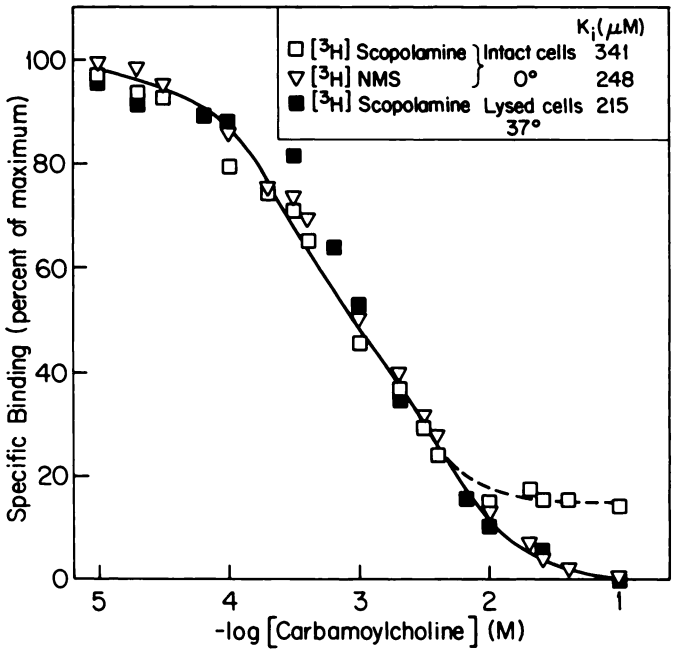

Fig. 10. Inhibition of specific [^{3}H]scopolamine and [^{3}H]NMS binding to intact SK-N-SH cells by carbamoylcholine. Cells were incubated for 24 hr at 0° in the presence of carbamoylcholine at the concentrations indicated. A single line is drawn for the displacement of the radioligands in the interest of clarity. Note that while the binding of [^{3}H]NMS can be completely displaced by carbamoylcholine, approximately 15–20% of [^{3}H]scopolamine-binding sites were refractory to agonist addition (– – –). However, carbamoylcholine is able to fully displace [^{3}H]scopolamine bound to hypotonic cell lysates assayed at 37°. In a second experiment performed under identical conditions, carbamoylcholine competed for [^{3}H]scopolamine sites with K_i values of 400 μM (intact cells, 0°) and 350 μM (hypotonic lysates, 37°).

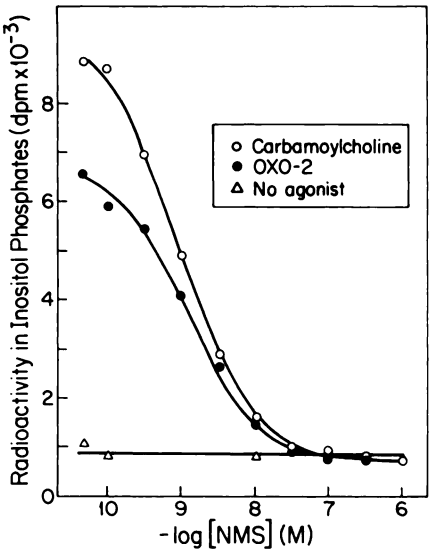

Fig. 11. Inhibition of carbamoylcholine- and OXO-2-stimulated inositol phosphate release by NMS. Cells (approximately 500 μg of protein) were prelabeled for 60 min at 37° with [^{3}H]inositol (5 μCi) and then incubated in the presence of agonist for an additional 45 min.

resulted in the displacement of [^{3}H]NMS, [^{3}H]scopolamine, or [^{3}H]QNB with equal affinity (K_i = 163–243 μM) and with Hill coefficients (n_H) close to unity (Fig. 9). A summary of the K_i values and Hill coefficients obtained for carbamoylcholine and OXO-2 from two to five-separate experiments is shown in Table 2.

Displacement of [^{3}H]scopolamine and [^{3}H]NMS by carbamoylcholine at either 0° in intact cells or in cell lysates at 37°. Following exposure of cells to agonists, the process of receptor sequestration has been shown to occur for several receptors, including the mAChR (24, 25). To evaluate the contribution of receptor sequestration to the results shown in Fig. 8, the ability of carbamoylcholine to displace [^{3}H]scopolamine was determined at 0° to minimize receptor translocation. Under these conditions, carbamoylcholine exhibited a 9-fold higher affinity for [^{3}H]scopolamine sites than at 37° (K_i = 341 μM; Fig. 10). Approximately 15% of the [^{3}H]scopolamine-binding sites were not readily displaced by agonist concentrations as high as 10^{-1} M (Fig. 10). This fraction of mAChRs may represent the sequestered pool which can be labeled by [^{3}H]scopolamine but is inaccessible to charged ligands such as carbamoylcholine (or NMS). In contrast, carbamoylcholine was able to completely displace [^{3}H]NMS bound at 0° with an affinity similar to that observed at 37°. Carbamoylcholine also displaced [^{3}H]scopolamine binding from hypotonic lysates of SK-N-SH cells with high affinity (K_i = 215 μM; Fig. 10), providing further evidence that receptor sequestration accounts for the low affinities observed for this agonist in intact cells at 37°.

Coupling of cell surface mAChRs in SK-N-SH cells to PPI hydrolysis. Evidence that it is the cell surface rather than the sequestered receptors that are functionally coupled was obtained from measurement of stimulated PPI turnover. In these experiments, the relative inability of either carbamoylcholine or NMS to detect sequestered mAChRs, together with the ability of OXO-2 to recognize receptors in both hydro-

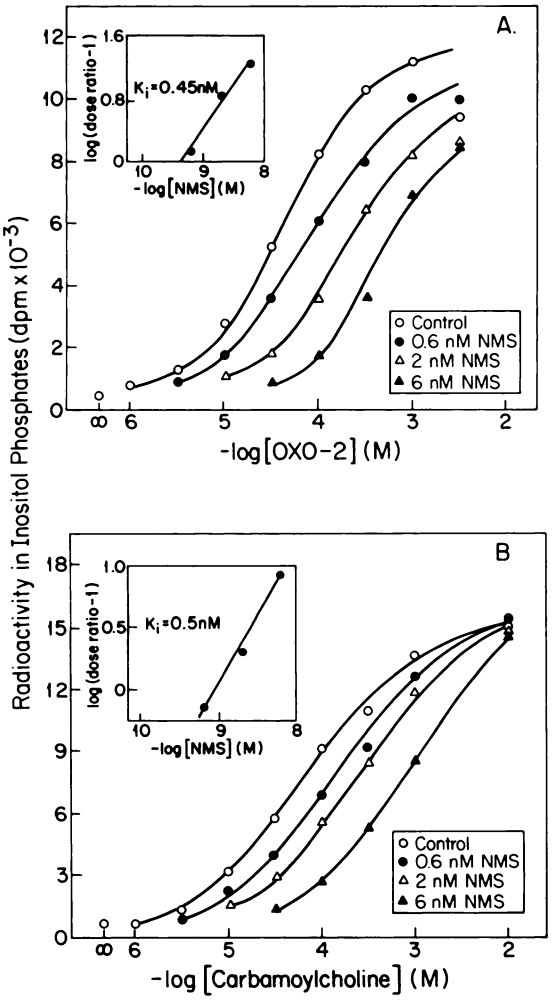

Fig. 12. Schild analysis of NMS inhibition of OXO-2 (A) and carbamoylcholine (B)-stimulated PPI turnover in SK-N-SH cells. Dose response curves for stimulated inositol phosphate release were determined in the absence or presence of NMS at concentrations indicated. Values shown are the means of triplicate replicates from a single experiment. *Insets*: The log of the dose ratio-1 is plotted as a function of NMS concentration. The calculated K_i values are 0.45 nM and 0.50 nM for OXO-2 and carbamoylcholine, respectively. In a second experiment using the same experimental conditions, K_i values of 0.30 and 0.50 nM were calculated for these two agonists.

philic and lipophilic locations, provided the means whereby the participation of each population of receptors could be evaluated. Optimal concentrations of OXO-2 (3 mM) were found to be approximately 80% as effective as carbamoylcholine in the stimulation of PPI turnover, as previously observed (17). Inclusion of OXO-2 attenuated the accumulation of inositol phosphates induced by the addition of carbamoylcholine, indicating that both OXO-2 and carbamoylcholine interact with the same population of mAChRs to elicit an increased lipid turnover. The addition of NMS at nM concentrations could fully block both carbamoylcholine- and OXO-2-stimulated PPI turnover. No indication was obtained that higher concentrations of NMS (required to interact with the sequestered receptor pool) were required to block the OXO-2 stimulation of PPI turnover (Fig. 11). Since we have previously demonstrated that spare receptors exist for PPI turnover in SK-N-SH cells (17), it was necessary to determine the K_i values for NMS inhibition of carbamoylcholine- and OXO-2-stimulated PPI turnover using Schild analysis (Fig. 12). Addition of increasing concentrations of NMS to incubations containing either OXO-2 or carbamoylcholine resulted in a parallel shift in the dose response curves, characteristic of a competitive antagonist. For both agonists, Schild regression analysis of the data yielded a K_i value of approximately 0.5 nM, a value similar to the K_d for NMS (Table 1). The slopes of the Schild plots were close to unity, a further indication that NMS is interacting with a single homogeneous population of high affinity receptors.

Discussion

The results obtained from the radioligand binding studies indicate that, at 37°, mAChRs present in unstimulated SK-N-SH neuroblastoma cells can be recognized equally well by quaternary and tertiary antagonists of either the tropate or benzilate chemical class. The similarity of B_{max} values obtained with antagonists that are either lipophilic or hydrophilic suggests that all of the mAChRs in these cells have access to a hydrophilic environment at or near the cell surface. Although this result might indicate that mAChRs are uniquely present at the cell surface, evidence for the existence of a sequestered population of mAChRs was obtained from experiments performed at 0°. Under these conditions, 15–20% of the sites labeled by the tertiary [^{3}H]scopolamine were not labeled by the quaternary [^{3}H]NMS. The population of mAChRs inaccessible to quaternary ligands can, however, be labeled if the cells are warmed to 37°, suggesting that sequestered receptors can equilibrate with those at the cell surface at higher temperatures. Taken together, these results indicate that 80–85% of mAChRs in SK-N-SH cells are present at the cell surface, whereas 15–20% are sequestered. The equilibration of sequestered mAChRs with those at the cell surface at 37° results in the ability of both quaternary and tertiary antagonists to fully label the mAChR complement. At 0°, when receptor translocation is prevented, a pool of sequestered mAChRs is revealed. The results obtained for the SK-N-SH cell may also be germane to interpretation of muscarinic antagonist binding data obtained in other studies. Thus, the proportion of mAChRs found in sequestered and cell surface sites may depend upon the rate of equilibration of receptors between the two cell compartments, the physiological state of the tissue, and assay conditions employed.

In tissues in which [^{3}H]QNB can label a substantially larger number of mAChR sites than [^{3}H]NMS or [^{3}H]NMeQNB, a consistent observation is that the quaternary antagonists compete for a subpopulation of [^{3}H]QNB sites with a very low affinity (10–12). A logical interpretation of this result is that quaternary antagonists have only limited access to mAChRs in a lipophilic environment. Unexpectedly, a similar inability of NMS to effectively displace [^{3}H]QNB-binding sites was observed for the human neuroblastoma cells incubated at 37°, even though [^{3}H]QNB and [^{3}H]NMS label the same number of mAChR sites under these conditions. Although an explanation for this complex interaction remains uncertain, one possibility is that mAChRs labeled with [^{3}H]QNB are less able to equilibrate between the two cell compartments and that this permits the detection at 37° of a population of [^{3}H]QNB sites which exhibits a low affinity for NMS. In this context, it is noteworthy that there have been previous indications that the interaction of mAChRs with [^{3}H]QNB is complex (15, 26). Regardless of the molecular mechanism(s) involved, a major

conclusion to emerge from the present study is that the inability of NMS to effectively compete for [^{3}H]QNB sites in SK-N-SH cells is not *solely* a reflection of the lipophilic nature of the radioligand. In competition studies, NMS displaces another tertiary antagonist, [^{3}H]scopolamine, with a single affinity constant (1.08 nM) that is in good agreement with the K_d for [^{3}H]NMS obtained from direct binding studies (1.20 nM). Both *in vivo* and *in vitro* experiments have indicated that [^{3}H]scopolamine can label all of the mAChR sites in the brain that are accessible to [^{3}H]QNB (27, 28). Furthermore, from studies of octanol-saline partitioning, the presumed highly lipophilic nature of this molecule has been confirmed (29). It thus seems possible that the complex interaction between NMS- and [^{3}H]QNB-binding sites observed for SK-N-SH cells incubated at physiological temperatures reflects a unique feature of the interaction of the mAChR with this particular radioligand, rather than the inability of a quaternary probe to detect a population of sequestered receptor sites.

In SK-N-SH cells incubated at 37°, the quaternary agonist carbamoylcholine is considerably more effective at displacing [^{3}H]NMS and [^{3}H]NMeQNB than either [^{3}H]scopolamine or [^{3}H]QNB. A similar situation is seen in the intact chick heart cell (12). Although the results obtained with the human neuroblastoma cells could reflect the inability of a quaternary agonist to interact with existing internalized mAChRs within the plasma membrane, a more likely explanation of the data is that exposure to the agonist *induces* the sequestration of receptors originally present at the cell surface into a more lipophilic environment. Three lines of evidence support this conclusion. First, when competition studies are performed at 0°, carbamoylcholine can displace [^{3}H]scopolamine and [^{3}H]NMS with equal affinity, whereas at 37°, the agonist displays an 11-fold lower affinity for sites labeled by the tertiary antagonist. Although all of [^{3}H]NMS binding can be displaced at 0°, 15–20% of specific [^{3}H]scopolamine binding is inaccessible to even very high concentrations of the agonist. These inaccessible sites are presumably those that also cannot be detected by [^{3}H]NMS at 0°. Second, if SK-N-SH cells are first lysed in hypotonic buffer prior to carbamoylcholine competition studies, the agonist competes for [^{3}H]scopolamine sites with a 15-fold higher affinity than in intact cells. Third, when SK-N-SH cells are first exposed to carbamoylcholine at 37°, washed free of agonist, and then incubated at a reduced temperature (0–10°) with radiolabeled antagonists, there is a 40–50% reduction in [^{3}H]NMS and [^{3}H]NMeQNB binding, whereas little or no loss of [^{3}H]scopolamine or [^{3}H]QNB sites occurs.[1] Collectively, these results suggest that upon agonist administration, some of the receptors which predominate at the surface of SK-N-SH cells are sequestered into a more lipophilic environment and then become inaccessible to the quaternary agonist. The greater affinity of carbamoylcholine for [^{3}H]NMS or [^{3}H]NMeQNB sites reflects the ability of the agonist to readily compete for mAChRs that remain at the cell surface and that exhibit a high affinity for quaternary ligands. In contrast, the more lipophilic agonist OXO-2 can readily penetrate the plasma membrane and is able to compete with similar affinities for labeled receptor sites at either surface or sequestered locations. That OXO-2 interacts with an apparently homogeneous population of receptor sites is also indicated by the fact that the Hill coefficients for the displacement of radioligands are very close to unity. In contrast, carbamoylcholine competed for the radiolabeled sites with n_H values of 0.63 to 0.85, which indicates that the agonist is competing for more than a single population of binding sites. However, the binding data were adequately fitted to a one-site model and no improvement in fit was obtained for a two-site model. The very low affinity with which carbamoylcholine binds to mAChRs labeled by [^{3}H]QNB in intact SK-N-SH cells at 37° (K_i = 8.1 mM) is similar to that observed for mouse pituitary cells (30) and chick heart cells (12, 31). In SK-N-SH cells, carbamoylcholine competes for [^{3}H]scopolamine-binding sites with a 2- to 3-fold higher affinity than for [^{3}H]QNB sites (K_i = 3.1 mM; $p < 0.01$), a further indication that these two tertiary radioligands do not interact with mAChRs in an identical manner.

The ability of the tertiary agonist OXO-2 to both interact with the population of sequestered receptors in SK-N-SH cells and to enhance the hydrolysis of inositol lipids (17) permits an assessment to be made of the functional participation of these sites. Three lines of evidence, however, point to the involvement of cell surface rather than these sequestered receptors in stimulated PPI hydrolysis. First, the stimulation of PPI turnover elicited by both carbamoylcholine and OXO-2 could be fully blocked by nM concentrations of NMS. Since OXO-2 (unlike carbamoylcholine) can readily interact with the sequestered receptors, a coupling of these mAChRs to PPI turnover could be indicated by a requirement for higher concentrations of NMS to block stimulated inositol lipid hydrolysis. Second, the K_i values obtained for NMS inhibition were very similar for the two agonists (0.3–0.5 nM) indicating that a population of receptors with high affinity for NMS mediates PPI turnover in these cells. Third, the K_A value obtained for carbamoylcholine-stimulated inositol phosphate release (65 μM, ref. 17) resembles more the affinity with which the agonist binds to the cell surface receptors than to the sequestered sites. The involvement of cell surface rather than sequestered mAChRs sites in stimulated PPI turnover has also been proposed for the chick heart cell (12). In this tissue, alkylation of mAChRs with PrBCM abolishes stimulated PPI turnover and [^{3}H]NMS binding, while the pool of sequestered receptors is largely unaffected. Further evidence to implicate cell-surface mAChRs in stimulated PPI turnover comes from studies in which activation of protein kinase C by phorbol esters can result in the sequestration of cell surface mAChRs (32) and an inhibition of stimulated inositol lipid hydrolysis (33, 34). Taken together, these results suggest that mAChRs at the cell surface are those that are primarily responsible for functional coupling to phosphoinositide hydrolysis.

In summary, the results demonstrate that muscarinic cholinergic receptors in human SK-N-SH cells exist in more than one cellular compartment. The majority (80–85%) of receptors are present at the cell surface, while a smaller number (15–20%) exist in a sequestered membrane domain. Receptors in these two membrane compartments can equilibrate at 37°, such that all mAChRs can be labeled by either hydrophilic or lipophilic ligands. In the presence of agonists some of the cell surface receptors are translocated to sites which can still be recognized by tertiary but not by quaternary amine probes. Finally, only mAChRs present at the surface of SK-N-SH cells appear to participate in stimulated PPI hydrolysis.

[1] A. K. Thompson and S. K. Fisher, unpublished observation.

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