Journal of Neurochemistry
Raven Press, Ltd., New York

Sequestration of Muscarinic Cholinergic Receptors in Permeabilized Neuroblastoma Cells

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Abstract: The feasibility of using a permeabilized preparation of human SH-SY-5Y neuroblastoma cells for studies of muscarinic acetylcholine receptor (mAChR) sequestration has been evaluated. Exposure of cells permeabilized with digitonin, streptolysin-O, or the α-toxin from *Staphylococcus aureus* to oxotremorine-M (Oxo-M) for 30 min resulted in a 25–30% reduction in the number of cell surface mAChRs, as monitored by the loss of *N*[^{3}H]methylscopolamine ([^{3}H]NMS) binding sites. The corresponding value for intact cells was 40%. For cells permeabilized with 20 μ*M* digitonin, the Oxo-M-mediated reduction in [^{3}H]NMS binding was time ($t_{1/2}$ ~ 5 min) and concentration (EC_{50} ~ 10 μ*M*) dependent and was agonist specific (Oxo-M > bethanechol = arecoline = pilocarpine). In contrast, no reduction in total mAChR number, as monitored by the binding of [^{3}H]quinuclidinyl benzilate, occurred following Oxo-M treatment. The loss of [^{3}H]NMS sites observed in the presence of Oxo-M was unaffected by omission of either ATP or Ca^{2+}, both of which are required for stimulated phosphoinositide hydrolysis, but could be inhibited by the inclusion of guanosine 5′-*O*-(2-thiodiphosphate). mAChRs sequestered in response to Oxo-M addition were unmasked when the cells were permeabilized in the presence of higher concentrations of digitonin (80 μ*M*). The results indicate (a) that permeabilized SH-SY-5Y cells support an agonist-induced sequestration of mAChRs, the magnitude of which is ~65–70% of that observed for intact cells, (b) that when internalized, mAChRs are located in a cellular compartment to which [^{3}H]NMS has only a limited access despite the removal of the plasma membrane barrier, and (c) that the production of phosphoinositide-derived second messengers is not a prerequisite for mAChR sequestration. **Key Words:** Human SH-SY-5Y neuroblastoma—Muscarinic cholinergic receptor internalization—Phosphoinositide hydrolysis—Digitonin permeabilization.
J. Neurochem. **62**, 1795–1803 (1994).

Agonist occupancy of neural muscarinic acetylcholine receptors (mAChRs) elicits not only a rapid modulation of either adenylyl cyclase or phosphoinositidase C (PIC) activities, but also a slower adaptive response in which the cell surface receptors are internalized into a more hydrophobic cell compartment. The latter process, termed receptor sequestration, can be monitored following cell fractionation as a redistribution of cell surface receptors into a membrane fraction that exhibits a lower density than that of the plasma membrane (Harden et al., 1985; Fraeyman and Buyse, 1989). However, it is more routinely detected as an agonist-induced reduction in the number of binding sites for hydrophilic ligands in intact cells (Fiegenbaum and El-Fakahany, 1985; Baumgold et al., 1989; Cioffi and El-Fakahany, 1989; Thompson and Fisher, 1990). Two lines of evidence suggest that the sequestration of mAChRs is of physiological importance. First, once the receptor becomes internalized, its ability to couple to its intracellular effector enzyme, e.g., PIC, may be lost (Thompson and Fisher, 1991), and second, the extent of agonist-induced mAChR sequestration is predictive of the degree of subsequent receptor down-regulation (Thompson and Fisher, 1990).

Despite frequent documentation of the agonist-induced sequestration of mAChRs, the molecular mechanism(s) underlying receptor internalization remain largely unknown. One factor that may account for this paucity of information is the routine use of intact cells, for which manipulations of intracellular milieu are not readily attained. Although the use of permeabilized cells could potentially circumvent this problem, no systematic assessment of this preparation for studies of cell surface receptor internalization has yet appeared. It is possible that receptor sequestration would be compromised when the plasma membrane barrier is disrupted. Furthermore, even if receptor se-

Received July 14, 1993; revised manuscript received September 21, 1993; accepted September 21, 1993.

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Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); LDH, lactate dehydrogenase; mAChR, muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine; Oxo-M, oxotremorine-M; PIC, phosphoinositidase C; QNB, quinuclidinyl benzilate; SLO, streptolysin-O.

questration persists in permeabilized cells, it may not be readily detectable because the internalized receptors could be more accessible to hydrophilic radioligands than in intact cells. The latter factor may account for previous unsuccessful attempts to monitor the sequestration of β_2-adrenergic receptors in permeabilized cell preparations when assessed by means of radioligand binding (Fratelli et al., 1989; Lohse et al., 1990). In a previous study (Thompson et al., 1991), preliminary data were obtained to suggest that digitonin-permeabilized SK-N-SH cells were capable of mAChR sequestration. To explore this possibility further, in the present study we have evaluated the feasibility of using a permeabilized preparation of human SH-SY-5Y neuroblastoma cells for studies of mAChR sequestration, as monitored by a loss of cell-surface *N*-methylscopolamine (NMS) binding sites. These cells possess a large number of mAChRs, predominantly of the m_3 subtype, as defined from immunological, biochemical, and pharmacological criteria, that are linked to phosphoinositide hydrolysis and Ca^{2+} signaling (Lambert et al., 1989; Cioffi and Fisher, 1990; Lambert and Nahorski, 1990; Wall et al., 1991). We report here that an agonist-induced loss of [^{3}H]NMS sites is retained in SH-SY-5Y cells even after the plasma membrane barrier has been permeabilized by addition of either digitonin, a nonionic detergent, or two bacterial toxins, namely, streptolysin-O (SLO) and the α-toxin from *Staphylococcus aureus*. The characteristics of receptor sequestration in permeabilized cells resemble those of intact cells in terms of kinetics, agonist concentration dependence, and muscarinic agonist specificity. mAChR sequestration in permeabilized cells is largely independent of intracellular Ca^{2+} or ATP, assay components required for stimulated phosphoinositide hydrolysis, but can be inhibited by guanosine 5′-*O*-(2-dithiophosphate) (GDPβS). For digitonin-permeabilized cells, mAChR sites that are internalized following agonist addition become increasingly accessible to [^{3}H]NMS when detergent concentrations are used that are higher than those required for permeabilization of the plasma membrane alone. The results suggest that, under defined conditions, a permeabilized cell preparation may be used to advantage in studies of mAChR sequestration. A preliminary account of part of this study has appeared elsewhere (Slowiejko and Fisher, 1993).

MATERIALS AND METHODS

[^{3}H]NMS (79.5 Ci/mmol), [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB; 45.4 Ci/mmol), and [^{3}H]oxotremorine-M ([^{3}H]-Oxo-M; 87.5 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]Scopolamine (74.9 Ci/mmol) was custom-synthesized by Amersham Corp. Arecoline, bethanechol, pilocarpine, atropine, cholic acid, NADH, and ATP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Digitonin, α-toxin from *S. aureus*, and thapsigargin were from Calbiochem Corp. (La Jolla, CA, U.S.A.). SLO was from Wellcome Diagnostics, U.K. Oxo-M was purchased from Research Biochemicals, Inc. (Natick, MA, U.S.A.). GDPβS was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Fura-2 acetoxymethyl ester and fura-2 (free acid) were obtained from Molecular Probes (Eugene, OR, U.S.A.). Tissue culture supplies were purchased from Corning Glass Works (Corning, NY, U.S.A.). Powdered Dulbecco's modified Eagle's medium and fetal calf serum were obtained from GIBCO (Grand Island, NY, U.S.A.). SH-SY-5Y neuroblastoma cells were obtained from Dr. June Biedler (Sloan Kettering Institute, New York, NY, U.S.A.). The source of SK-N-SH cells was as previously described (Fisher and Snider, 1987). Antibodies to the mAChR subtypes were raised against muscarinic i_3 loop fusion proteins, as previously described (Levey et al., 1990, 1991).

Cell culture conditions

Human SH-SY-5Y or SK-N-SH cells were grown in tissue culture flasks (75-cm^2/250 ml) in 20 ml of Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (medium I). Cells were grown for 10–20 days at 37°C in an atmosphere consisting of 10% CO_2 and 90% humidified air. Cells were detached by aspirating medium I and isolating the cells after incubation with a modified Pucks D_1 solution (Fisher and Snider, 1987).

Immunoprecipitation of mAChR subtypes

The procedure of Levey et al. (1990, 1991) was adopted, with minor modifications. A crude membrane preparation was obtained by Dounce homogenization of SH-SY-5Y or SK-N-SH cells (2 mg of protein/ml) in 10 m*M* Tris and 1 m*M* EDTA buffer (pH 7.5) that contained protease inhibitors (100 μg/ml of phenylmethylsulfonyl fluoride, 1.5 μg/ml of leupeptin, and 1.5 μg/ml of aprotinin) followed by centrifugation at 20,000 *g* for 10 min at 4°C. To solubilize mAChRs, membranes were first resuspended in Tris-EDTA buffer containing 0.4% digitonin (Wako BioProducts, Richmond, VA, U.S.A.) and 0.04% cholic acid at a concentration of 1 mg of protein/ml of buffer and then homogenized and left on ice for 1 h. Membranes were then pelleted (12,000 *g* for 30 min), and the supernatants were retained on ice in the presence of 6 n*M* [^{3}H]NMS for 30 min. [^{3}H]-NMS-labeled receptors were then incubated with antisera (diluted 1:40) for 4 h at 4°C in microcentrifuge tubes. Goat anti-rabbit IgG (Pel-Freez) was then added to a final dilution of 1:8. After overnight incubation at 4°C, samples were centrifuged at 2,000 *g* for 10 min. Immunoprecipitates were then washed twice with Tris-EDTA buffer containing 0.1% digitonin and 0.01% cholic acid and resuspended in 1% sodium dodecyl sulfate, and radioactivity was determined after addition of 5 ml of Universol scintillation fluid. Controls that used nonimmune rabbit antisera to determine nonspecific trapping of receptor in immunoprecipitates (<10% of total radioactivity) were subtracted from experimental values. Approximately 100% of radioactivity associated with solubilized receptors was recovered in the immunoprecipitates. However, the efficiency of mAChR solubilization ranged from 20 to 40%, a value comparable to that previously obtained (Levey et al., 1991; Wall et al., 1991).

Radioligand binding

The binding of [^{3}H]NMS to mAChRs on intact SH-SY-5Y cells was as previously described (Fisher, 1988). In brief,

cells were incubated with [^{3}H]NMS in 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, pH 7.4) for 18 h at 4°C. Permeabilized cells were incubated in KGEH buffer (139 mM potassium glutamate, 2 mM ATP, 4 mM $MgCl_2$, 10 mM LiCl, 10 mM EGTA, 2.2 mM $CaCl_2$ (free [Ca^{2+}] ~ 60nM) and 30 mM HEPES, pH 7.4) with either [^{3}H]NMS (or [^{3}H]scopolamine) for 18 h at 4°C or [^{3}H]QNB for 60 min at 37°C. For both intact and permeabilized cells, reactions were rapidly terminated by filtration through Whatman GF/B glass fiber filters, and radioactivity was determined after addition of 5 ml of Universol scintillation fluid. As previously noted for SK-N-SH cells (Fisher, 1988), a day-to-day variation in mAChR densities in SH-SY-5Y cells was observed (see Table 1).

Cell permeabilization

Unless indicated otherwise, cells were permeabilized with 20 μM digitonin in KGEH buffer (−Ca^{2+}) for 5 min at 37°C at a protein concentration of ~2–3 mg/ml. This ratio of digitonin:cell protein was maintained throughout the study. Failure to exclude trypan blue dye (0.05% wt/vol) was used to assess permeabilization. Alternatively, cells (2–3 mg of protein/ml) were permeabilized by addition of 0.5 IU/ml of SLO for 10 min or 200 U/ml of α-toxin from *S. aureus* for 30 min. Permeabilized cells were then centrifuged, washed with an equal volume of KGEH buffer (minus permeabilizing agent), and resuspended in the same buffer with and without agonist. Incubations (free [Ca^{2+}] ~ 60 nM unless otherwise indicated) were then allowed to proceed at 37°C for the time(s) indicated. Reactions were terminated by addition of 4 volumes of ice-cold KGEH buffer followed by centrifugation at 300 *g* for 3.5 min at 4°C. Cells were then resuspended in KGEH buffer and assayed for radioligand binding as described. Although the 4°C temperature was routinely used for determination of [^{3}H]NMS binding, similar results were obtained when the cells were incubated at 15°C for 4 h. Protein content was measured by the method of Geiger and Bessman (1972).

Measurement of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ values in SH-SY-5Y cells were determined by monitoring fura-2 fluorescence in a Shimadzu model RF-5000 spectrofluorophotometer using the dual wavelength method as previously described (Thompson et al., 1991). Concentrations of free Ca^{2+} in Ca^{2+}/EGTA buffers were measured directly after addition of fura-2 (free acid).

Lactate dehydrogenase (LDH)

LDH was quantified essentially as described by Wroblewski and LaDue (1955).

Data analysis

Data are mean ± SEM (or range where n = 2) values for the number of separate experiments performed. Student's two-tailed *t* tests were used to evaluate the statistical differences of the means of paired or unpaired sets of data.

RESULTS

Expression of m_1–m_5 muscarinic receptor subtypes in SK-N-SH and SH-SY-5Y cells

Previous studies of mAChR sequestration in this laboratory have used the SK-N-SH human neuroblastoma, a cell line that possesses mAChRs of the M_3 subtype, as determined from biochemical and pharmacological criteria (Fisher and Heacock, 1988). Because of the possibility that the characteristics of mAChR sequestration might differ for individual receptor subtypes, in preliminary experiments we determined the proportion of mAChR subtypes present on both SK-N-SH neuroblastoma and its stable neuroblast subclone, SH-SY-5Y, by means of m_1–m_5 subtype-selective antisera (Fig. 1). Immunoprecipitation of solubilized mAChR proteins confirmed that the m_3 mAChR is the major subtype present on both SK-N-SH and SH-SY-5Y cells. However, SH-SY-5Y cells consistently (over an 18-month period) exhibited a greater proportion of m_3 receptors than the parent SK-N-SH cell line (81 ± 2 vs. 57 ± 4%; $p < 0.001$). Although a previous study pointed to the predominance of the m_1 mAChR in these cells (Serra et al. 1988), <10% of immunoreactivity was associated with m_1, m_2, m_4, or m_5 subtypes in SH-SY-5Y cells (Fig. 1). Thus, SH-SY-5Y cells, which express a relatively homogeneous population of m_3 mAChRs, were used for all subsequent experiments.

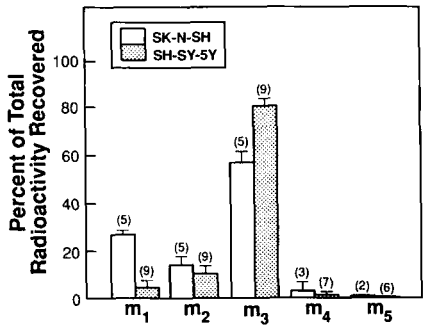

FIG. 1. Immunoprecipitation of mAChR subtypes present in SK-N-SH and SH-SY-5Y cells. mAChRs present in crude membrane preparations were first solubilized by addition of 0.4% digitonin and 0.04% cholic acid and then labeled by addition of 6 nM [^{3}H]-NMS. Labeled receptors were then incubated with antisera to each of the mAChR subtypes, and radioactivity recovered in the immunoprecipitates was determined. Results are expressed as radioactivity recovered in each immunoprecipitate as a function of the total recovered. Data are mean ± SEM (bars) values for the number of separate experiments indicated in parentheses.

Ligand binding characteristics of mAChRs on intact and permeabilized SH-SY-5Y cells

Scatchard analysis of [^{3}H]NMS binding to intact SH-SY-5Y cells revealed a mean B_{max} value of 366 fmol/mg of protein and a K_D value of 1.5 nM (Table 1). When SH-SY-5Y cells were incubated with 20 μM digitonin for 5 min at 37°C, 84 ± 1% (n = 40) of cells were permeabilized, as assessed by failure to exclude trypan blue (mol wt = 960). Under these conditions, 49 ± 6% (n = 3) of cellular lactate dehydrogenase activity was released. When compared with intact cells, permeabilized cells exhibited an increased B_{max} value for [^{3}H]NMS (528 fmol/mg of protein) and a reduced

TABLE 1. *Ligand binding characteristics of intact and digitonin-permeabilized SH-SY-5Y cells*

	Intact	Permeabilized
B_{max} (fmol/mg of protein)	366 ± 75 (4)	528 ± 94 (7)
Range	189–518	339–986
K_D (n*M*)	1.5 ± 0.4 (6)	0.5 ± 0.2 (7)[a]
Range	0.84–2.56	0.14–1.74
mAChR number/cell	21 ± 2 × 10^3 (7)	24 ± 1 × 10^3 (7)
Range	16 – 28 × 10^3	19 – 29 × 10^3

The binding of [^{3}H]NMS to intact or digitonin-permeabilized cells was analyzed by Scatchard analysis. The mAChR number per cell was assessed at 6 n*M* [^{3}H]NMS, a concentration at which 80–92% of mAChR sites are occupied. Data are mean ± SEM values for the number of experiments shown in parentheses. The range of values obtained is also shown.

[a] $p < 0.05$.

K_D (0.5 n*M*). However, only the change in K_D value was statistically significant (Table 1). Because the trend toward an increased B_{max} value for [^{3}H]NMS binding could conceivably be attributed to a loss of cytosolic protein from permeabilized cells (20–45% of total), we also calculated mAChR densities in the two preparations in terms of receptor number per cell. Both intact and permeabilized SH-SY-5Y cells expressed ~21,000–24,000 receptors per cell (Table 1). Thus, whereas digitonin permeabilization results in a small increase in the affinity of mAChRs for [^{3}H]-NMS, it has no effect on receptor density.

Characteristics of agonist-induced loss of [^{3}H]NMS sites in digitonin-permeabilized cells

When digitonin-permeabilized cells were incubated in the presence of 1 m*M* Oxo-M, a time-dependent ($t_{1/2}$ ~ 5 min) loss of cell surface [^{3}H]NMS binding sites was observed, which, after 30 min of agonist exposure, amounted to ~25–30% of those sites originally present. In contrast, addition of Oxo-M had little or no effect on total mAChR number as monitored by the binding of [^{3}H]QNB, a lipophilic antagonist (Fig. 2). However, a small reduction in the binding of [^{3}H]scopolamine, another lipophilic antagonist, was observed after 30 min of Oxo-M addition (14 ± 5%, n = 4). The nonequivalence of [^{3}H]scopolamine and [^{3}H]QNB binding data may reflect the greater degree of lipophilicity of QNB (Frey, 1984). Thus, [^{3}H]QNB appears to recognize a greater fraction of available mAChRs than does [^{3}H]scopolamine, and, consequently, the use of [^{3}H]scopolamine overestimates total mAChR loss. Scatchard analysis of [^{3}H]NMS binding to control and Oxo-M-treated cells demonstrated that the loss of binding sites was due to a reduction in the number of receptor sites without a change in ligand affinity (Fig. 3). The loss of cell surface [^{3}H]-NMS binding sites was concentration dependent (EC_{50} for Oxo-M ~ 10 μ*M*; data not shown) and agonist specific (Fig. 4). Thus, as previously noted for intact SK-N-SH cells (Thompson and Fisher, 1990),

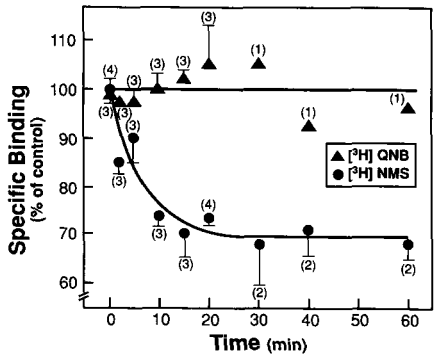

FIG. 2. Oxo-M addition results in a time-dependent loss of [^{3}H]-NMS sites from digitonin-permeabilized SH-SY-5Y neuroblastoma. Cells were first permeabilized for 5 min in the presence of 20 μ*M* digitonin, washed free of detergent, and incubated for the times indicated in KGEH buffer in the presence or absence of 1 m*M* Oxo-M. Cells were then washed free of agonist at 4°C, and aliquots were taken for either [^{3}H]NMS or [^{3}H]QNB radioligand binding. Results are expressed as specific binding relative to controls that were incubated with KGEH alone. Data (for n ≥ 3) are mean ± SEM (bars) (or range where n = 2) values for the number of separate experiments indicated in parentheses. The binding of [^{3}H]NMS to control cells was unchanged over a 60-min incubation period.

addition of bethanechol, pilocarpine, and arecoline, partial agonists for stimulated phosphoinositide turnover, elicits little or no mAChR sequestration in permeabilized SH-SY-5Y cells.

mAChR sequestration and phosphoinositide hydrolysis

In a previous study with intact SK-N-SH cells, mAChR sequestration was unaffected by chelation of extracellular Ca^{2+}, a condition that resulted in a partial inhibition of stimulated phosphoinositide hydroly-

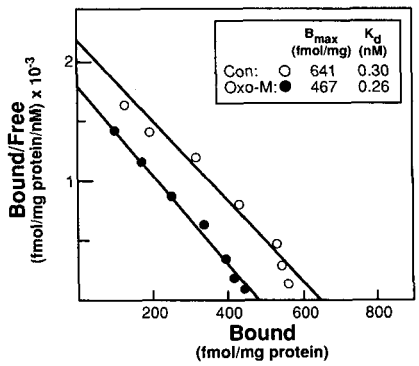

FIG. 3. Scatchard analysis of [^{3}H]NMS binding to digitonin-permeabilized cells that had been incubated in either the absence [control (Con; ○)] or presence (●) of 1 m*M* Oxo-M for 30 min. Cells were washed to remove the agonist, and aliquots were taken for radioligand binding at 4°C for 18 h. Data shown are from one of five experiments. The mean ± SEM values for the B_{max} from the five experiments were 473 ± 73 and 362 ± 45 fmol/mg of protein for control and Oxo-M-treated cells, respectively ($p < 0.05$, matched-pair). The corresponding K_D values were 0.23 ± 0.05 and 0.21 ± 0.06 n*M*.

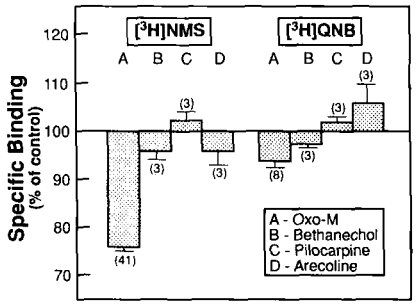

FIG. 4. Muscarinic agonists differ in their ability to induce the loss of [^{3}H]NMS sites from digitonin-permeabilized SH-SY-5Y neuroblastoma cells. Permeabilized cells were incubated with 1 m*M* Oxo-M, 1 m*M* pilocarpine, 1 m*M* arecoline, or 10 m*M* bethanechol for 30 min at 37°C. Cells were washed at 4°C to remove agonist, and then aliquots were taken for either [^{3}H]NMS or [^{3}H]QNB binding. Results are expressed as specific binding relative to controls that were incubated in the absence of agonist. Data are mean ± SEM (bars) values for the number of separate experiments indicated in parentheses.

sis (Thompson et al., 1991). The ability to monitor mAChR sequestration in a permeabilized cell preparation has permitted a more precise definition of the relationship, if any, between receptor internalization and the production of phosphoinositide-derived second messengers (Fig. 5). When ATP was omitted [conditions under which mAChR-stimulated inositol phosphate production is inhibited by 70–90% (see Fisher et al., 1990)], the extent of mAChR internalization remained unchanged. Furthermore, the magnitude of mAChR sequestration was little changed when the ATP concentration was increased from 2 to 4 m*M*. Inclusion of 50 μM guanosine 5′-*O*-(3-thiotri-

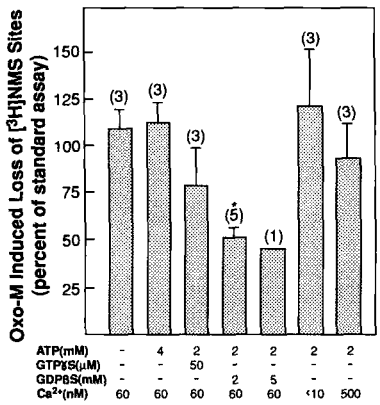

FIG. 5. Regulation of mAChR sequestration by ATP, guanine nucleotides, and Ca^{2+}. Digitonin-permeabilized cells were incubated in the absence or presence of 1 m*M* Oxo-M for 30 min with ATP, GTPγS, GDPβS, or Ca^{2+} at the concentrations indicated. Results are expressed as percent loss of [^{3}H]NMS sites, relative to that observed under standard assay conditions (KGEH buffer that contained 2 m*M* ATP and 60 n*M* Ca^{2+}; see Materials and Methods). Under standard assay conditions 20–25% of [^{3}H]NMS sites were lost in the presence of Oxo-M. Data are mean ± SEM (bars) (or range, where n = 2) values for the number of separate experiments indicated in parentheses. *Different from 100%, $p < 0.05$.

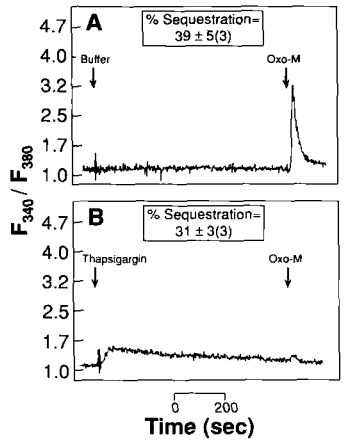

FIG. 6. An agonist-induced rise in $[Ca^{2+}]_i$ is not required for mAChR sequestration. Intact cells were first loaded with fura-2 and then incubated in a modified buffer A (−Ca^{2+}, 0.2 m*M* EGTA). After 60 s, either modified buffer A (**A**) or 1 μM thapsigargin (**B**) was added, and cells were allowed to preincubate for 10 min at 37°C before addition of 1 m*M* Oxo-M. In A, addition of Oxo-M resulted in a rise in $[Ca^{2+}]_i$ from <10 to 281 n*M*. In B, little or no increase in $[Ca^{2+}]_i$ occurred in the presence of the agonist. Thapsigargin increased $[Ca^{2+}]_i$ from <10 to 42 n*M*. Results shown are from one of three experiments that gave similar results. The ability of Oxo-M to induce a loss of [^{3}H]NMS sites under conditions A and B is given.

phosphate) (GTPγS), which results in the activation of PIC (Fisher et al., 1989; Cioffi and Fisher, 1990), had no effect on receptor internalization when added alone. In addition, it failed to enhance the extent of agonist-induced mAChR sequestration. In contrast, inclusion of 2 or 5 m*M* GDPβS resulted in a 48–53% inhibition of mAChR sequestration, values comparable to those obtained for inhibition of stimulated phosphoinositide hydrolysis in permeabilized SK-N-SH or SH-SY-5Y cells (Cioffi and Fisher, 1990; Thompson et al., 1991). Because the activation of mAChRs in SH-SY-5Y cells results in a rapid transient rise in $[Ca^{2+}]_i$, which itself may serve further to activate PIC, we also determined the effect of $[Ca^{2+}]_i$ on mAChR sequestration. The extent of Oxo-M-induced sequestration was unaltered when $[Ca^{2+}]_i$ was buffered at concentrations that either do not support PIC activity [<10 n*M* (Fisher et al., 1989)] or approximate the peak $[Ca^{2+}]_i$ achieved during the first 5–10 s of agonist addition (~500 n*M*). Further evidence that changes in $[Ca^{2+}]_i$ do not modulate the extent of sequestration was obtained from parallel experiments in which intact cells were preincubated with thapsigargin, an inhibitor of the microsomal Ca^{2+}-ATPase pump. Whereas pretreatment of SH-SY-5Y cells with 1 μM thapsigargin abolished the ability of Oxo-M to induce a rise in $[Ca^{2+}]_i$, it had little effect on the extent of mAChR sequestration (Fig. 6).

Comparison of the extent of mAChR sequestration in intact and permeabilized cells

The magnitude of the Oxo-M-induced loss of [^{3}H]-NMS sites observed for intact cells (~40%) was con-

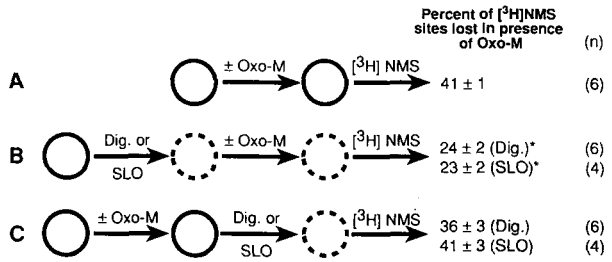

FIG. 7. Fewer [^{3}H]NMS sites are lost from cells that are permeabilized before agonist addition than from cells that are first exposed to agonist and then permeabilized. Three separate protocols were used. **A:** Intact cells were first incubated in the presence or absence of 1 m*M* Oxo-M for 30 min. Cells were then washed free of agonist, and aliquots were then taken for [^{3}H]-NMS binding. **B:** Cells were first permeabilized in the presence of either 20 μM digitonin (Dig.) or 0.5 IU/ml of SLO (as indicated by the breached plasma membrane), the cells were washed to remove these agents, and then the permeabilized cells were incubated in the absence or presence of 1 m*M* Oxo-M for 30 min. After removal of the agonist by washing, aliquots of the cells were taken for [^{3}H]NMS binding. **C:** Intact cells were first incubated with or without 1 m*M* Oxo-M for 30 min and then permeabilized with digitonin or SLO (in the continuing presence of the agonist). Cells were then washed with KGEH buffer, and aliquots were taken for [^{3}H]NMS binding. For all three protocols, results are expressed as percentages of [^{3}H]NMS sites lost in the presence of agonist, relative to the controls. Data are mean ± SEM (bars) values for the number of separate experiments shown in parentheses. *Different from protocols A or C, $p < 0.05$.

sistently found to be greater than that obtained for digitonin-permeabilized cells (~25%; Fig. 7A and B). To determine whether this differential loss of [^{3}H]-NMS sites was specific to the digitonin treatment, cells were also rendered permeable by addition of SLO (Ahnert-Hilger et al., 1989). Under these conditions, 85% of the cells failed to exclude trypan blue, and 21 ± 5% (n = 3) of the cellular LDH was released. When 1 m*M* Oxo-M was added to SLO-permeabilized cells, the loss of [^{3}H]NMS sites (~25%) was comparable to that obtained for digitonin-treated cells (Fig. 7B). However, if intact cells were first incubated with Oxo-M and *then* permeabilized with either digitonin or SLO, the loss of [^{3}H]NMS binding sites was comparable to that observed for cells incubated with Oxo-M alone (Fig. 7C). Both digitonin and SLO result in the formation of pores in the plasma membrane that allow the passage of large-molecular-weight compounds such as LDH (135,000). In contrast, the α-toxin from *S. aureus* is reported to result in the formation of pores 1–2 nm in diameter, which allows the entry of trypan blue but not the release of LDH (Ahnert-Hilger et al., 1989). Of the cells permeabilized with the α-toxin, 80–85% failed to exclude trypan blue. When these cells were then incubated with 1 m*M* Oxo-M for 30 min, 28 ± 5% (n = 4) of [^{3}H]NMS sites were lost, a value similar to that obtained for either digitonin- or SLO-treated cells. This loss of [^{3}H]NMS sites in α-toxin-permeabilized cells was less marked than for intact cells, monitored concurrently (42 ± 3% of total, n = 4; $p < 0.05$). In contrast to digitonin or SLO permeabilization, addition of the α-toxin induced release of little or no cellular LDH activity (4 ± 0%, n = 3), a value indistinguishable from LDH release from nominally intact cells (2 ± 0% of total, n = 3).

Sequestered mAChR sites become accessible to [^{3}H]-NMS at higher concentrations of digitonin

Relatively low concentrations of digitonin (20 μM) were required for permeabilization of the majority of SH-SY-5Y cells. However, when higher concentrations of the nonionic detergent were used, an increased accessibility of the sequestered mAChRs to [^{3}H]NMS was observed. Thus, when intact cells were first challenged with 1 m*M* Oxo-M and then permeabilized by addition of 20 μM digitonin, 35–40% of [^{3}H]NMS sites originally present were inaccessible. However, when the concentration of digitonin was increased to 80 μM, only 7% of the sites were lost (Fig. 8). If cells were initially permeabilized with this higher concentration of digitonin before Oxo-M addition, little or no loss of [^{3}H]NMS sites could be observed (data not shown). [^{3}H]NMS binding to control cells was unaffected by digitonin in the 10–80 μM concentration range, as was [^{3}H]QNB binding to either control or agonist-treated cells (Fig. 8). Higher concentrations of digitonin (160 μM) resulted in a substantial loss of [^{3}H]NMS and [^{3}H]QNB sites from both control and Oxo-M-treated cells.

DISCUSSION

In the present study, we have extensively characterized the loss of mAChR sites that occurs following

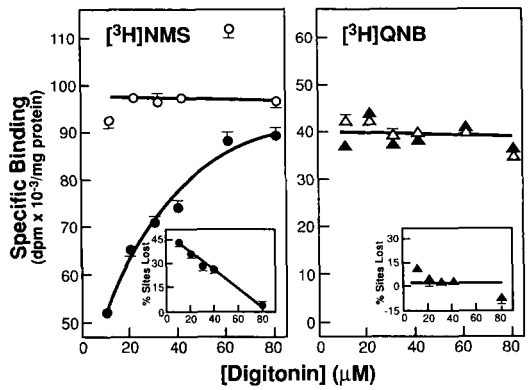

FIG. 8. mAChR sites in digitonin-permeabilized cells that are inaccessible to [^{3}H]NMS can be exposed by use of higher concentrations of the detergent. Intact cells were first incubated in the presence (solid symbols) or absence (open symbols) of 1 m*M* Oxo-M for 30 min and then permeabilized for 5 min with digitonin at the concentrations indicated. Cells were then washed free of detergent and agonist, and aliquots were taken for either [^{3}H]NMS or [^{3}H]QNB binding. Data are mean ± SEM (bars) values for triplicate replicates from one of three experiments that gave similar results. **Insets:** Percentage of mAChR sites that are inaccessible to [^{3}H]NMS, as a function of digitonin concentration. Data are mean ± SEM (bars) values for three separate experiments.

agonist addition and demonstrate that permeabilized preparations of SH-SY-5Y neuroblastoma cells can be readily used for studies of mAChR sequestration. Cells rendered permeable with digitonin, as monitored by trypan blue exclusion and LDH release, possessed a full complement of mAChRs and exhibited characteristics of mAChR sequestration similar to those previously observed for intact cells. Thus, addition of Oxo-M to permeabilized SH-SY-5Y cells resulted in a selective loss of cell surface mAChR sites, i.e., without a loss of total receptor number, as monitored by [^{3}H]QNB, which was concentration dependent and occurred within a time frame of minutes ($t_{1/2}$ ~ 5 min). Although previous studies with electroporated SH-SY-5Y cells indicated that the permeabilization process can result in an altered agonist efficacy profile for activation of PIC (Wojcikewicz et al., 1990*a*), the inability of partial muscarinic agonists (bethanechol, arecoline, or pilocarpine) to induce a significant amount of receptor sequestration in digitonin-permeabilized SH-SY-5Y cells is in agreement with results previously obtained with intact cells (Thompson and Fisher, 1990). The ability of Oxo-M to elicit a loss of cell surface mAChRs was not restricted to cells permeabilized with the nonionic detergent digitonin but was also observed for cells permeabilized with two bacterial exotoxins, SLO and the α-toxin from *S. aureus* (Ahnert-Hilger et al., 1989). Taken collectively, the present results indicate that mAChR sequestration can be initiated even when the structural integrity of the plasma membrane has been compromised by permeabilization. Furthermore, the data obtained with permeabilized cells suggest that when internalized, mAChRs exist in a cellular compartment that remains relatively inaccessible to [^{3}H]-NMS even after the plasma membrane barrier has been removed. This may reflect the presence of the mAChR in an endocytotic vesicle, as has been described for the β_2-adrenergic receptor (Hertel et al., 1983; von Zastrow and Kobilka, 1992).

Of the three agents used for cell permeabilization, the steroid glycoside digitonin, which targets the plasma membrane owing to its relatively high concentration of cholesterol, has had the most widespread use. However, concentrations of digitonin that selectively permeabilize the plasma membrane, while sparing intracellular membranes, must be used (Sarafian et al., 1987). The majority (85%) of SH-SY-5Y cells were permeabilized following short-term incubation with 20 μM digitonin, and mAChR sequestration was consistently detected in cells thus treated. However, when higher concentrations of digitonin were used, sequestered mAChRs then became accessible to [^{3}H]NMS (Fig. 8). Thus, the concentration of digitonin used appears critical to the successful application of permeabilized cells to studies of mAChR sequestration in SH-SY-5Y neuroblastoma.

mAChR sequestration observed for SH-SY-5Y cells permeabilized before agonist addition, while reliably and reproducibly observed, was consistently 30–35% less than that monitored either for intact cells or for cells permeabilized after agonist addition (see Fig. 7). This attenuation in responsiveness could conceivably reflect a loss of cytosolic components that occurred during the permeabilization process. However, because the Oxo-M-mediated loss of [^{3}H]NMS sites observed following permeabilization with α-toxin (which releases no LDH) is comparable to that obtained following digitonin treatment (which releases 50% of the cellular LDH), it appears unlikely that the leakage of a high-molecular-weight component is a relevant factor. A role for a low-molecular-weight component remains possible, although ATP, Ca^{2+}, or guanine nucleotides do not appear to be likely candidates. Two other possible explanations need to be considered: first, that the permeabilization process itself partially impairs the mAChR sequestration machinery, and second, that [^{3}H]NMS may have greater access to mAChRs present in endocytotic vesicles derived from permeabilized cells than from intact cells.

Although agonist occupancy of mAChRs on SH-SY-5Y cells leads to both the activation of PIC and receptor sequestration, the production of phosphoinositide-derived second messengers appears not to be required for receptor internalization, a finding consistent with previous observations obtained for m_1 mAChRs transfected into U293 cells (Lameh et al., 1992). Thus, in the present study, neither the exclusion of ATP nor the lowering of $[Ca^{2+}]_i$ to <10 nM, both of which strongly inhibit receptor-stimulated inositol lipid hydrolysis (Fisher et al., 1989, 1990), had any effect on mAChR sequestration. Depletion of the inositol trisphosphate-sensitive Ca^{2+} pools with thapsigargin also failed to block receptor internalization. In contrast, inclusion of GDPβS inhibited mAChR sequestration and phosphoinositide hydrolysis (see Cioffi and Fisher, 1990) to the same extent. This is consistent with previous speculation that an event before PIC activation, possibly at the level of a guanine nucleotide binding protein, is linked to mAChR internalization (Thompson et al., 1991). Thus, receptor activation, but not second messenger production, appears to be a prerequisite for mAChR sequestration.

Permeabilized SK-N-SH and SH-SY-5Y neuroblastoma cells have previously been used for studies of mAChR coupling to PIC (Fisher et al., 1989; Cioffi and Fisher, 1990; Wojcikiewicz et al., 1990*a*), mAChR-stimulated Ca^{2+} release (Wojcikiewicz et al., 1990*b*), and metabolism of inositol phosphate isomers generated on receptor activation (Fisher et al., 1990). To this list can now be added mAChR sequestration, a slower adaptive response that results from continuous agonist occupancy of the mAChR (for kinetic comparisons, see Fisher et al., 1994). It is also pertinent to note that the SH-SY-5Y cell line possesses an abundance of a relatively homogeneous population of m_3 receptors. Because it is increasingly evident that receptor subtypes, e.g., β_1, β_2, and β_3-adren-

ergic (see Suzuki et al., 1992; Nantel et al., 1993) differ considerably in their propensity to undergo sequestration, the issue of receptor heterogeneity assumes major importance. Although transfected cells can provide an alternative approach for studying the internalization of a single receptor subtype, the results obtained may be influenced by the host cell type.

In summary, the present results indicate that mAChR sequestration can be successfully monitored in permeabilized neuroblastoma cells. This preparation may facilitate future studies of the interrelationships among receptor occupancy, activation of phosphoinositide hydrolysis, and receptor internalization.

Acknowledgment: The authors wish to thank Dr. Anne M. Heacock for her helpful comments and Jo Ann Kelsch for preparation of the manuscript. This work was supported by grants NS 23831 (to S.K.F.), NS 30454 (to A.I.L.), and NIH 2507RR05383-31 (to University of Michigan Medical School–Biomedical Research Support Grant) from the National Institutes of Health and MH 42652 (to S.K.F.) from the National Institute of Mental Health. D.M.S. was supported by training grant 5T 32 GM 07767 from the National Institutes of Health.

REFERENCES

Ahnert-Hilger G., Mach W., Föhr K. J., and Gratzl M. (1989) Poration by α-toxin and streptolysin O: an approach to analyze intracellular processes. *Methods Cell Biol.* **31,** 63–90.

Baumgold J., Cooperman B. B., and White T. M. (1989) Relationship between desensitization and sequestration of muscarinic cholinergic receptors in two neuronal cell lines. *Neuropharmacology* **28,** 1253–1261.

Cioffi C. L. and El-Fakahany E. E. (1989) Differential sensitivity of phosphoinositide and cyclic GMP responses to short-term regulation by a muscarinic agonist in mouse neuroblastoma cells. *Biochem. Pharmacol.* **38,** 1827–1834.

Cioffi C. L. and Fisher S. K. (1990) Reduction of muscarinic receptor density and of guanine nucleotide-stimulated phosphoinositide hydrolysis in human SH-SY-5Y neuroblastoma cells following long-term treatment with 12-*O*-tetradecanoylphorbol-13-acetate or mezerein. *J. Neurochem.* **54,** 1725–1734.

Feigenbaum P. and El-Fakahany E. E. (1985) Regulation of muscarinic cholinergic receptor density in neuroblastoma cells by brief exposure to agonist: possible involvement in desensitization of receptor function. *J. Pharmacol. Exp. Ther.* **233,** 134–140.

Fisher S. K. (1988) 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. *Mol. Pharmacol.* **33,** 414–422.

Fisher S. K. and Heacock A. M. (1988) A putative M_3 muscarinic cholinergic receptor of high molecular weight couples to phosphoinositide hydrolysis in human SK-N-SH neuroblastoma cells. *J. Neurochem.* **50,** 984–987.

Fisher S. K. and Snider R. M. (1987) Differential receptor occupancy requirements for muscarinic cholinergic stimulation of inositol lipid hydrolysis in brain and in neuroblastomas. *Mol. Pharmacol.* **32,** 81–90.

Fisher S. K., Domask L. M., and Roland R. M. (1989) Muscarinic receptor regulation of cytoplasmic Ca^{2+} concentrations in human SK-N-SH neuroblastoma cells: Ca^{2+} requirements for phospholipase C activation. *Mol. Pharmacol.* **35,** 195–204.

Fisher S. K., Heacock A. M., Seguin E. B., and Agranoff B. W. (1990) Polyphosphoinositides are the major source of inositol phosphates in carbamoylcholine-stimulated SK-N-SH neuroblastoma cells. *Mol. Pharmacol.* **38,** 54–63.

Fisher S. K., Slowiejko D. M., and McEwen E. L. (1994) A rapid attenuation of muscarinic agonist stimulated phosphoinositide hydrolysis precedes receptor sequestration in human SH-SY-5Y neuroblastoma cells. *Neurochem. Res.* **19,** 551–556.

Fraeyman N. H. and Buyse M.-A. (1989) Gradient distribution pattern of muscarinic receptors in N1E 115 mouse neuroblastoma cells. *Experientia* **45,** 742–747.

Fratelli M., Gagliardini V., and De Blasi A. (1989) Low affinity of β-adrenergic receptors for agonists on intact cells is not due to receptor sequestration. *Biochim. Biophys. Acta* **1012,** 178–183.

Frey K. A. (1984) The in vivo determination of ligand binding within the central nervous system [Ph.D. Thesis]. University of Michigan, Ann Arbor.

Geiger P. J. and Bessman S. P. (1972) Protein determination by Lowry's method in the presence of sulfhydryl reagents. *Anal. Biochem.* **49,** 467–474.

Harden T. K., Petch L. A., Traynelis S. F., and Waldo G. L. (1985) Agonist-induced alteration in the membrane form of muscarinic cholinergic receptors. *J. Biol. Chem.* **260,** 13060–13066.

Hertel C., Staehelin M., and Perkins J. P. (1983) Evidence for intravesicular β-adrenergic receptors in membrane fractions from desensitized cells: binding of the hydrophilic ligand CGP-12177 only in the presence of alamethicin. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **9,** 119–128.

Lambert D. G. and Nahorski S. R. (1990) Muscarinic-receptor-mediated changes in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate mass in a human neuroblastoma cell line, SH-SY-5Y. *Biochem. J.* **265,** 555–562.

Lambert D. G., Ghataorre A. S., and Nahorski S. R. (1989) Muscarinic receptor binding characteristics of a human neuroblastoma SK-N-SH and its clones SH-SY-5Y and SH-EP1. *Eur. J. Pharmacol.* **165,** 71–77.

Lameh J., Philip M., Sharma Y. K., Moro O., Ramachandran J., and Sadée W. (1992) Hm1 muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop. *J. Biol. Chem.* **267,** 13406–13412.

Levey A. I., Stormann T. M., and Brann M. R. (1990) Bacterial expression of human muscarinic receptor fusion proteins and generation of subtype-specific antisera. *FEBS Lett.* **275,** 65–69.

Levey A. I., Kitt C. A., Simonds W. F., Price D. L., and Brann M. R. (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain and subtype-specific antibodies. *J. Neurosci.* **11,** 3218–3226.

Lohse M. J., Benovic J. L., Caron M. G., and Lefkowitz R. J. (1990) Multiple pathways of rapid β_2-adrenergic receptor desensitization. *J. Biol. Chem.* **265,** 3203–3209.

Nantel F., Bonin H., Emorine L. J., Zilberfarb V., Strosberg A. D., Bouvier N., and Naurullo S. (1993) The human β_3-adrenergic receptor is resistant to short term agonist-promoted desensitization. *Mol. Pharmacol.* **43,** 548–555.

Sarafian T., Aunis D., and Bader M.-F. (1987) Loss of proteins from digitonin-permeabilized adrenal chromaffin cells essential for exocytosis. *J. Biol. Chem.* **262,** 16671–16676.

Serra M., Mei L., Roeske W. R., Lui G. K., Watson M., and Yamamura H. I. (1988) The intact neuroblastoma cell (SH-SY-5Y) exhibits high-affinity [^{3}H]pirenzepine binding associated with hydrolysis of phosphatidylinositols. *J. Neurochem.* **50,** 1513–1521.

Slowiejko D. M. and Fisher S. K. (1993) Muscarinic agonists induce a loss of hydrophilic binding sites from both intact and permeabilized neuroblastoma. *Trans. Am. Soc. Neurochem.* **24,** 106.

Suzuki T., Nguyen C. T., Nantel F., Bonin H., Valiquette M., Frielle T., and Bouvier M. (1992) Distinct regulation of β_1- and β_2-adrenergic receptors in Chinese hamster fibroblasts. *Mol. Pharmacol.* **41,** 542–548.

Thompson A. K. and Fisher S. K. (1990) Relationship between agonist-induced muscarinic receptor loss and desensitization

of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. *J. Pharmacol. Exp. Ther.* **252,** 744–752.

Thompson A. K. and Fisher S. K. (1991) Preferential coupling of cell surface muscarinic receptors to phosphoinositide hydrolysis in human neuroblastoma cells. *J. Biol. Chem.* **266,** 5004–5010.

Thompson A. K., Mostafapour S. P., Denlinger L. C., Bleasdale J. E., and Fisher S. K. (1991) The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J. Biol. Chem.* **266,** 23856–23862.

von Zastrow M. and Kobilka B. K. (1992) Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267,** 3530–3538.

Wall S. J., Yasuda R. P., Li M., and Wolfe B. B. (1991) Development of an antiserum against m3 muscarinic receptors: distribution of m3 receptors in rat tissues and clonal cell lines. *Mol. Pharmacol.* **40,** 783–789.

Wojcikiewicz R. J. H., Lambert D. G., and Nahorski S. R. (1990*a*) Regulation of muscarinic agonist-induced activation of phosphoinositidase C in electrically permeabilized SH-SY5Y human neuroblastoma cells by guanine nucleotides. *J. Neurochem.* **54,** 676–685.

Wojcikiewicz R. J. H., Safrany S. T., Challiss R. A. J., Strupish J., and Nahorski S. R. (1990*b*) Coupling of muscarinic receptors to the mobilization of intracellular Ca^{2+} stores in permeabilized SH-SY5Y human neuroblastoma cells. *Biochem. J.* **272,** 269–272.

Wroblewski F. and LaDue J. S. (1955) Lactic dehydrogenase activity in blood. *Proc. Soc. Exp. Biol. Med.* **90,** 210–213.