Chronic Muscarinic Stimulation of SH-SY5Y Neuroblastoma Cells Suppresses Inositol 1,4,5-Trisphosphate Action

PARALLEL INHIBITION OF INOSITOL 1,4,5-TRISPHOSPHATE-INDUCED Ca++ MOBILIZATION AND INOSITOL 1,4,5-TRISPHOSPHATE BINDING*

(Received for publication, April 30, 1991)

Richard J. H. Wojcikiewicz and Stefan R. Nahorski
From the Department of Pharmacology and Therapeutics, University of Leicester, P. O. Box 138, University Road, Leicester LE1 9HN, United Kingdom

The possibility that chronic activation of the phosphoinositide-mediated signaling pathway modifies the Ca²⁺-mobilizing action of inositol 1,4,5-trisphosphate (InsP₃) was examined. SH-SY5Y human neuroblastoma cells were exposed to carbachol, permeabilized electrically, loaded with ⁴⁶Ca²⁺, and ⁴⁶Ca²⁺ mobilization in response to exogenous InsP₃ was assessed. In control permeabilized cells, InsP₃ released 65 ± 2% of sequestered ⁴⁶Ca²⁺ (EC₅₀ = 0.32 ± 0.05 μM). Pre-treatment with carbachol reduced both maximal InsP₃-induced ⁴⁶Ca²⁺ release (to 34 ± 3%, with half-maximal and maximal inhibition at ~3 and 6 h, respectively) and the potency of InsP₃ (EC₅₀ = 0.92 ± 0.13 μM). This inhibitory effect of carbachol was half-maximal at ~5 μM, was mediated by muscarinic receptors, and was reversible following withdrawal of agonist. Pretreatment with phorbol 12,13-dibutyrate did not alter the maximal effect of InsP₃ but doubled its EC₅₀.

Evidence suggesting that the inhibitory effects of carbachol pretreatment resulted from altered Ca²⁺ homeostasis was not forthcoming; both ⁴⁶Ca²⁺ uptake and release induced by ionomycin and thapsigargin were identical in control and pretreated permeabilized cells, as were the characteristics of reuptake of released Ca²⁺. In contrast, carbachol pretreatment, without altering the affinity of InsP₃ (Kᵣ = 64 ± 7 nM), reduced the density of [³²P]InsP₃-binding sites from 2.0 ± 1.0 to 0.1 ± 0.1 pmol/mg protein with a time course essentially identical to that for the reduction in responsiveness to InsP₃. This effect was not mimicked by pretreatment of cells with phorbol 12,13-dibutyrate.

These data indicate that chronic activation of phosphoinositide hydrolysis can reduce the abundance of InsP₃ receptors and that this causes a reduction in size of the InsP₃-sensitive Ca²⁺ store. This modification, possibly in conjunction with a protein kinase C-mediated event, appears to account for the carbachol-induced suppression of InsP₃ action. As intracellular InsP₃ mass remained elevated above basal for at least 24 h after addition of carbachol, suppression of the Ca²⁺-mobilizing activity of InsP₃ represents an important response to cell stimulation that can limit the extent to which intracellular Ca²⁺ is mobilized.

Inositol 1,4,5-trisphosphate (InsP₃) is now recognized widely as a Ca²⁺-mobilizing intracellular messenger (1, 2). It is generated from phosphatidylinositol 4,5-bisphosphate (PIP₂) as a consequence of receptor-mediated activation of phosphoinositidase C (1) and interacts with specific intracellular receptors (3-6) to cause mobilization of sequestered intracellular Ca²⁺ (7-9). The precise nature of the Ca²⁺ store released is unknown (2, 9), although the sequestered Ca²⁺ is clearly intravesicular and non-mitochondrial and may be contained within an endoplasmic reticulum-like compartment (2, 7, 9). In spite of these uncertainties, it can be predicted that the InsP₃-sensitive store will contain a high affinity ATP-dependent uptake mechanism responsible for Ca²⁺ accumulation, an InsP₃-regulated Ca²⁺ channel that allows Ca²⁺ release, and a low affinity high capacity intraluminal binding protein that sequesters Ca²⁺ (9).

In contrast, the InsP₃ receptor has been purified (10, 11) and its gene has been cloned, sequenced, and expressed in mammalian cells (12, 13). The brain receptor (Mᵢ = 313,000) binds InsP₃ and appears to contain several transmembrane domains and forms homotetramers (12-14). Intrinsic to the pure reconstituted receptor protein is the ability to both bind InsP₃ with high affinity and to form InsP₃-gated Ca²⁺ channels (15).

It has recently become apparent, however, that binding of InsP₃ to cell membrane preparations can be complex (16, 17) and that the InsP₃ receptor can exhibit a degree of plasticity following cell stimulation (16). For example, in rat liver membranes, InsP₃ binds to high and low affinity sites and acute hormonal pretreatment increases the proportion of the former (16). These data raise the possibility that the InsP₃ receptor, like cell-surface hormone and neurotransmitter receptors (18), represents a site at which a cell can adapt its response to an extracellular agonist.

As part of our investigation of phosphoinositidase C-mediated signal transduction in neuronal cells, we have begun to examine mechanisms of adaptation to agonists that stimulate PIP₂ hydrolysis. For these studies we have utilized SH-SY5Y human neuroblastoma cells which express M₃ muscarinic receptors (19) linked via a GTP-binding regulatory protein to phosphoinositidase C (20) and which possess substantial InsP₃-sensitive Ca²⁺ stores (21, 22). We report here that chronic pretreatment of SH-SY5Y cells with muscarinic agonists suppresses InsP₃-induced Ca²⁺ mobilization and that

---

*This work was supported by the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed.

The abbreviations used are: InsP₃, inositol 1,4,5-trisphosphate; InsP₃S₃, inositol 1,4,5-trisphosphothioate; PIP₂, phosphatidylinositol 4,5-bisphosphate; EGTA, ethylenebis(oxyethylene)nitrilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDBu, phorbol 12,13-dibutyrate; HPLC, high performance liquid chromatography.
this change correlates well with a reduction in the density of InsP<sub>3</sub>-binding sites. This novel form of adaptation to cell-surface receptor activation indicates that InsP<sub>3</sub> receptor number and thus InsP<sub>3</sub> action can be suppressed following cell stimulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Pretreatment**—Monolayers of SH-SY5Y cells in 175-cm<sup>2</sup> flasks (passage number, 60–90) were cultured in minimal essential medium supplemented with 10% newborn calf serum and antibiotics as described (20). When required for experiments, small quantities of either water (vehicle) or a concentrated solution of muscarinic agonist were added directly to the culture medium and the cells were maintained at 37 °C for the time of pretreatment required.

**"Ca<sup>2+</sup> Mobilization and Efflux**—Medium from control or pretreated cells was removed and 20 ml of ice-cold 155 mM NaCl, 10 mM HEPES, pH 7.4 (HBS) plus 0.02% EDTA was added to each flask. This caused cells to detach after several min. Cells were then centrifuged (500 × g for 2 min), resuspended in 20 ml of ice-cold HBS, recentrifuged, resuspended in 5 ml of ice-cold 120 mM KCl, 6 mM MgCl<sub>2</sub>, 5 mM sodium succinate, 20 mM HEPES, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM Na<sub>2</sub>ATP, 5-10 mM Na<sub>2</sub>EGTA, pH 7.0, pCa<sub>2+</sub> 7.5-7.0 (buffer A), centrifuged again, and finally resuspended in 0.8 ml of ice-cold buffer A. Cells were then permeabilized with three discharges of a 3 µF capacitor as described (20), diluted to 4 ml with ice-cold buffer A, centrifuged (500 × g for 2 min), and finally resuspended in 0.5–1.2 ml of buffer A (2.0–3.5 mg of cell protein/ml) supplemented with 1.3–1.6 µCi of [3H]InsP<sub>3</sub>/ml, pCa<sub>2+</sub> 6.4–6.2. The time intervals of removal of culture medium to exposure to [3H]InsP<sub>3</sub> was ~30 min. Cells were then incubated for 15 min at 20 °C to allow uptake of [3H]InsP<sub>3</sub> to reach equilibrium.

Standard experimental incubations for measurement of "Ca<sup>2+</sup>" mobilization were initiated by addition of 50 µl of cell suspension to 10 µl of buffer A plus stimulus (InsP<sub>3</sub>, carbobal or ionomycin). After 2 min at 20 °C, at which point "Ca<sup>2+</sup>" release was maximal and no significant reuptake had occurred (22), incubations were terminated by addition of silicone oil (Dow Corning 550/556, 9:11, v/v) and centrifugation, indicating that the amount of ligand dissociation during filtration was insignificant. More binding was obtained with this method (~7.3%), but a contrast, carbachol-induced 45Ca<sup>2+</sup> release which in permeabilized SH-SY5Y cells is dependent on the channel opening (27), slope factors of the 45Ca<sup>2+</sup> release curves were derived by computer-assisted curve fitting (25). Protein concentrations were determined with Coomassie Blue (26). Combined data from a number of independent experiments (n) are expressed as mean ± S.E. when n ≥ 3. Significance was assessed by unpaired t test. InsP<sub>3</sub> was from Research Biochemicals Inc. [4.5–22]InPS<sub>3</sub> (100–155 Ci/mmol), [3H]InsP<sub>3</sub> (17–20 Ci/mmol), D-inositol 1,4,5-trisphosphorothioate (InsP<sub>3</sub>thioate), and "Ca<sup>2+</sup>" (1000 Ci/mmol) were from Du Pont-New England Nuclear, the former as kind gifts. Thapsigargin was from Sigma.

**Miscellaneous**—EC<sub>50</sub> and IC<sub>50</sub> values (concentrations causing half-maximal stimulation and inhibition, respectively) and slope factors were derived by computer-assisted curve fitting (25). Protein concentration was assayed with Coomassie Blue (26). Combined data from a number of independent experiments (n) are expressed as mean ± S.E. when n ≥ 3. Significance was assessed by unpaired t test. InsP<sub>3</sub> was from Research Biochemicals Inc. [4.5–22]InPS<sub>3</sub> (100–155 Ci/mmol), [3H]InsP<sub>3</sub> (17–20 Ci/mmol), D-inositol 1,4,5-trisphosphorothioate (InsP<sub>3</sub>thioate), and "Ca<sup>2+</sup>" (1000 Ci/mmol) were from Du Pont-New England Nuclear, the former as kind gifts. The P<sub>2</sub>Y<sub>1</sub> receptor was from LC Services Corp. Carbobal, muscarine, atropine, ibomycin, phorbol 12,13-dibutyrate (PDBu), forskolin, and retinoic acid were from Sigma.

**RESULTS**

**Effects of Carbachol Pretreatment on "Ca<sup>2+</sup> Mobilization**—The ability of exogenous InsP<sub>3</sub> to mobilize sequestered "Ca<sup>2+</sup>" from permeabilized SH-SY5Y cells is shown in Fig. 1a. In control cells, InsP<sub>3</sub> released maximally 65 ± 2% of "Ca<sup>2+</sup>" with EC<sub>50</sub> = 0.32 ± 0.05 µM (n = 12). Pretreatment with carbachol for 3, 6, or 24 h reduced significantly (p < 0.01) maximal release to 49 ± 2, 36 ± 2, and 34 ± 3%, respectively (n ≥ 5) and increased significantly (p < 0.01) EC<sub>50</sub> values to 0.78 ± 0.18, 1.17 ± 0.29, and 0.92 ± 0.13 µM, respectively (n ≥ 5). Consistent with the requirement for interaction of more than one InsP<sub>3</sub> molecule with the InsP<sub>3</sub> receptor to cause Ca<sup>2+</sup> channel opening (27), slope factors of the "Ca<sup>2+</sup>" release curves were always >1 (1.2–1.7). It is clear from Fig. 1b that half-maximal and maximal reduction of InsP<sub>3</sub>-induced "Ca<sup>2+</sup>" mobilization was achieved after ~3 and 6 h of carbachol-pretreatment, respectively. In contrast, carbachol-induced "Ca<sup>2+</sup>" release (49 ± 5%, n = 9), which in permeabilized SH-SY5Y cells is dependent on the formation of InsP<sub>3</sub> from cellular polyphosphoinositides (21) and will reflect desensitization of muscarinic receptors as well as the reduced ability of InsP<sub>3</sub> to release Ca<sup>2+</sup>", was obliterately complete and more rapidly (Fig. 1b). The Ca<sup>2+</sup> ionophore...
ionomycin (10 μM) released 84 ± 1% of 45Ca2+ in control cells (n = 10); an effect that was unaltered by carbachol pretreatment (Fig. 1b).

Fig. 1c shows that the inhibition of InsP3-induced 45Ca2+ release by carbachol pretreatment is dose dependent with half-maximal inhibition at ~6 μM carbachol. Clearly, the inhibitory effects of carbachol on InsP3 action were mediated by muscarinic receptors, since the effects of carbachol were blocked by 10 μM atropine, a muscarinic antagonist, and were mimicked by muscarine (Fig. 1c).

In order to determine whether the decrease in responsiveness to InsP3 seen in Fig. 1 was reversed after an appropriate recovery period, cells that had been exposed to 1 mM carbachol for 24 h were incubated with fresh carbachol-free medium supplemented with 5 μM atropine for 48 h. The maximal effect and potency of InsP3 in these cells after pretreatment were essentially identical to that in untreated cells, indicating that full recovery of responsiveness had occurred.2

Effects of Carbachol Pretreatment on the Kinetics of Ca2+ Mobilization and InsP3 Metabolism—It was important to establish whether or not the reduced responsiveness to InsP3, seen 2 min after addition of the inositol phosphate (Fig. 1), reflected a change in the kinetics of Ca2+ mobilization. Thus, InsP3-induced Ca2+ release in suspensions of permeabilized cells was monitored with a Ca2+-sensitive electrode (Fig. 2).

It should be noted, however, that because of the response time of the electrode and the time required to achieve mixing (21), this method does not provide an absolute measure of the rate of InsP3-induced Ca2+ mobilization, which stopped-flow experiments have shown to occur with a half-time of <1 s (28, 29). Nevertheless, Fig. 2 shows that although responses to InsP3 were reduced by pretreatment with carbachol, the point at which the increase in Ca2+ concentration was maximal (~1 min after addition of InsP3) was unchanged (Fig. 2, a–c). As the dose of InsP3 used (1 μM) was submaximal (Fig. 1) (22), the response to InsP3 was suppressed strongly by a combination of decreased potency and maximal effect.

In these experiments, responses were transient because of InsP3 metabolism and Ca2+ resequestration (21, 22). The unchanged kinetics in Fig. 2 are consistent, therefore, with the data in Table I which show that neither the rate nor the route of InsP3 metabolism was affected by pretreatment with carbachol for 3–24 h.

Fig. 1. InsP3-induced mobilization of 45Ca2+ from permeabilized SH-SY5Y cells pretreated with carbachol for 3–24 h. SH-SY5Y cells in culture were not pretreated or were exposed to muscarinic agonist for 3–24 h. Cells were then permeabilized and were incubated with 45Ca2+ for 15 min in buffer A, and were added to tubes containing stimuli at the final concentrations indicated. 45Ca2+ released was assessed after incubation for 2 min at 20 °C. a, InsP3-induced 45Ca2+ mobilization (n = 5) from untreated cells (O) or from cells pretreated with 2 mM carbachol for 3 h ( ), 6 h ( ), or 24 h ( ). Error bars have been omitted for clarity. b, 45Ca2+ mobilization (n = 5) from cells pretreated for 2 mM carbachol in response to 45Ca2+ from untreated cells ( ), 10 μM InsP3 ( ), or 2 mM carbachol ( ). c, 45Ca2+ mobilization (n = 3) in response to 10 μM InsP3 from cells pretreated for 24 h with 0.05 mM muscarine ( ) or with 0–2 mM carbachol in the absence ( ) or presence ( ) of 10 μM atropine.

Fig. 2. Kinetics of Ca2+ mobilization in suspensions of permeabilized control or carbachol-pretreated SH-SY5Y cells. SH-SY5Y cells in culture were not pretreated (a) or were exposed to 2 mM carbachol for 3 h (b), 24 h (c), or 0–6 h (d). Cells were then harvested, permeabilized, and assayed for Ca2+ release with a Ca2+-sensitive electrode as described under “Experimental Procedures.” Addition of InsP3 (a–c, final concentration of 1 μM) and InsP3 (d, final concentration of 10 μM) were made at the points indicated by arrowheads. Data shown are representative of ≥3 independent experiments with similar results.
carbachol. In control and carbachol-pretreated permeabilized SH-SYSY cells, as in other cells types (2, 23), metabolism of InsP₃ proceeded initially via both InsP₃ 5-phosphatase to inositol 1,4-bisphosphate and InsP₃ 3-kinase to inositol 1,3,4,5-tetrakisphosphate. These metabolites were then dephosphorylated further to primarily inositol 4-phosphate and inositol 1,3,4-trisphosphate, respectively (Table 1).

Table 1. Radioactivity (% of total) of inositol phosphates in control and carbachol-pretreated cells

<table>
<thead>
<tr>
<th>Inositol phosphate</th>
<th>Control cells</th>
<th>Pretreated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol 1-phosphate</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Inositol 4-phosphate</td>
<td>0.9</td>
<td>6.8</td>
</tr>
<tr>
<td>Inositol 1,3-bisphosphate</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Inositol 1,4-bisphosphate</td>
<td>34.0</td>
<td>59.1</td>
</tr>
<tr>
<td>Inositol 1,3,4-trisphosphate</td>
<td>1.2</td>
<td>9.5</td>
</tr>
<tr>
<td>Inositol 1,4,5-trisphosphate</td>
<td>56.5</td>
<td>8.6</td>
</tr>
<tr>
<td>Inositol 1,3,4,5-tetrakisphosphate</td>
<td>7.3</td>
<td>15.4</td>
</tr>
</tbody>
</table>

Similarly, carbachol pretreatment had no overt effect on the kinetics of release when ⁴⁵Ca⁺ mobilization was examined at low temperature. Under these conditions, ATP-dependent ⁴⁵Ca⁺ reuptake is blocked and thus unidirectional ⁴⁵Ca⁺ efflux can be measured. Incubation of ²⁵⁴Ca⁺-labeled control or pretreated permeabilized cells at 1-2 °C resulted in a gradual fall in ⁴⁵Ca⁺ content (20 ± 3% in 5 min), reflecting gradual leakage from ⁴⁵Ca⁺ stores (30) in a manner described by a single exponential function (Fig. 3). In control cells, a maximal dose of InsP₃ (10 μM) caused a rapid burst of ⁴⁵Ca⁺ efflux, accounting for 71 ± 1% of that sequestered, that was complete by 0.5 min and which was followed by a return to gradual leakage (Fig. 3). In carbachol-pretreated cells the rate of leakage was unchanged but the extent to which InsP₃ lowered ⁴⁵Ca⁺ content in the initial burst was reduced to 24 ± 6% (Fig. 3).

Effects of Carbachol Pretreatment on Ca²⁺ Pools—It is clear from Fig. 1 that SH-SYSY cells contain both InsP₃-sensitive and InsP₃-insensitive Ca²⁺ pools, as InsP₃ releases 65% of sequestered Ca²⁺ whereas ionomycin, which mobilizes all intravesicular Ca²⁺, releases 84% (Fig. 1). Thus, approximately three-quarters of the Ca²⁺ sequestered during the 15-min incubations with ⁴⁵Ca⁺ is InsP₃-sensitive. The effect of carbachol pretreatment was to cause a diminution in size of this pool such that only approximately one-third of sequestered Ca²⁺ was InsP₃-sensitive (Fig. 1). Importantly, this occurred without alteration of the basic metabolic character-
and InsP3 in combination released no more "Ca2+" than did InsP3 alone. Thus, in contrast to previous studies with liver and brain (31, 32), our findings indicate that thapsigargin releases only part of the InsP3-sensitive Ca2+ store.

Finally, the data in Fig. 2 show that responses to repeated additions of InsP3 do not diminish in size even after carbachol pretreatment (Fig. 2, a-c) and that Ca2+ released by InsP3s is not resequestered more rapidly or completely after exposure to carbachol (Fig. 2d). This indicates that the InsP3-insensitive store has retained the same Ca2+ uptake characteristics and Ca2+ content after its expansion following exposure to carbachol.

Analysis of Binding to the InsP3 Receptor—As a change in the properties of the InsP3 receptor might account for the inhibition of InsP3-induced "Ca2+" mobilization, the effect of pretreatment with carbachol on InsP3 binding was examined.

[32P]InsP3 binding to membranes from untreated cells was displaced by coincubation with non-radioactive InsP3 and a range of concentrations of non-radioactive InsP, (Fig. 5). This site exhibits similar binding affinity to the well-characterized receptor in cerebellum that binds InsP3 with strict stereo and positional specificity (4-6, 24). Fig. 5 also shows that pretreatment with 1 mM carbachol for 24 h did not alter the affinity of InsP3 (Kd = 57 ± 3 nM) but reduced significantly (p < 0.01) the abundance of binding sites (Bmax = 1.0 ± 0.1 pmol/mg protein). Heterogeneity of InsP3 binding was not apparent, as the slopes of binding curves in both control and carbachol-pretreated cells are close to unity (Fig. 5).

The decrease in the number of InsP3-binding sites was half-maximal and maximal at 3 h and 6 h, respectively, was mimicked by muscarine and blocked by atropine (Fig. 5). Thus, muscarinic receptors mediate the inhibitory effects of carbachol on both the extent and the time course of the decrease in InsP3 binding correlated well with the decrease in InsP3-induced "Ca2+" mobilization seen in Fig. 1.

Effects of Pretreatment with Forskolin, PDbu, and Retanoic Acid—Other agents were examined for effects on InsP3-induced "Ca2+" mobilization (Table II). Forskolin, which acti-

---

**Table II**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>InsP3</th>
<th>Carbachol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum</td>
<td>EC50</td>
<td>Maximum</td>
</tr>
<tr>
<td>% release</td>
<td>μM</td>
<td>% release</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>InsP3</th>
<th>Carbachol</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment</td>
<td>60 ± 4</td>
<td>0.35 ± 0.05</td>
</tr>
<tr>
<td>1 mM carbachol for 24 h</td>
<td>32 ± 1*</td>
<td>0.94 ± 0.09*</td>
</tr>
<tr>
<td>10 μM forskolin for 24 h</td>
<td>62 ± 3</td>
<td>0.42 ± 0.08</td>
</tr>
<tr>
<td>1 μM PDbu for 24 h</td>
<td>63 ± 2</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td>10 μM PDbu for 20 min</td>
<td>62 ± 2</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>10 μM retanoic acid for 72 h</td>
<td>60 ± 3</td>
<td>0.46 ± 0.05</td>
</tr>
</tbody>
</table>

* p < 0.01.
* p < 0.02.
TABLE III

Time course of carbachol-induced stimulation of InsP₃ concentration in intact SH-SY5Y cells

SH-SY5Y cells were cultured in multiwell dishes and were assessed for InsP₃ content after exposure to 1 mM carbachol for 5 min–24 h as described under “Experimental Procedures” (n = 3). Protein concentration/well (0.64 ± 0.05 mg; n = 3) was unaltered by exposure to carbachol.

<table>
<thead>
<tr>
<th>Time of incubation (with 1 mM carbachol)</th>
<th>InsP₃ (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (basal)</td>
<td>6.7 ± 1.9</td>
</tr>
<tr>
<td>5 min</td>
<td>53.4 ± 8.9*</td>
</tr>
<tr>
<td>5 h</td>
<td>26.3 ± 2.0*</td>
</tr>
<tr>
<td>24 h</td>
<td>13.7 ± 0.8*</td>
</tr>
</tbody>
</table>

* p ≤ 0.05 comparing stimulated with basal values.

It is well known that persistent agonist stimulation of GTP-binding protein-linked cell-surface receptors can, via diverse mechanisms, lead to desensitization of receptor function (18). Such is the case for muscarinic receptors that stimulate phosphoinositide metabolism (35, 38, 39) and indeed, in SH-SY5Y cells, exposure to 1 mM carbachol for >1 h reduces by approximately 50% the density of cell-surface M3 receptors. In the present study, however, we have focused on other possible sites of adaptation to agonists that activate phosphoinositide hydrolysis and reveal that pretreatment of SH-SY5Y cells with muscarinic agonists can suppress the ability of InsP₃ to mobilize intracellular Ca²⁺. This effect was mediated by muscarinic receptors, was half-maximal and maximal after pretreatment of cells for 3 and 6 h, respectively, and was reversible. Although our data represent the first demonstration of this phenomenon in a neuron-like cell, an analogous study with rabbit pancreatic acinar cells (40) also showed that carbachol inhibited the action of InsP₃, albeit following relatively brief (30 min) pretreatment of cells. Thus, inhibition of InsP₃-induced Ca²⁺ mobilization following receptor-mediated activation of phosphoinositidase C may be a widespread phenomenon.

In addition to characterizing the suppression of InsP₃ action, we have investigated possible mechanisms by which it might occur. The mechanisms considered most likely were (a) that binding of InsP₃ to its receptor was modified, (b) that the rate of InsP₃ metabolism was increased, and (c) that the Ca²⁺ content and the metabolic characteristics of Ca²⁺ stores were altered.

Analysis of the interaction of InsP₃ with SH-SY5Y cell membranes revealed that pretreatment with muscarinic agonists did indeed modify InsP₃ binding. An apparent reduction in the density of receptors with no change in the affinity of the remaining binding sites was observed. It should be noted that although to the best of our knowledge this is the first demonstration of a reduction in number of InsP₃-binding sites following hormone/neurotransmitter stimulation, InsP₃ receptor plasticity in response to hormones acting at plasma membrane receptors has been recently demonstrated (16) and inferred (40). In contrast to our work, however, Mauger et al. (16) observed heterogeneity in InsP₃ binding and that acute (5 min) pretreatment of rat liver with vasopressin caused interconversion of high and low affinity binding sites. Thus, this study (16) and that in pancreatic acinar cells (40) differs significantly from ours in SH-SY5Y cells, in which inhibition of InsP₃ binding and action required pretreatment with carbachol for long periods (>1 h) and heterogeneity of InsP₃-binding sites was not observed.

Since the carbachol-induced reduction in density of InsP₃-binding sites correlated well in terms of extent and time course with reduction in the ability of a maximal concentration of InsP₃ to mobilize intracellular Ca²⁺, the possibility exists that there is a causal relationship between the apparent change in receptor number and the decrease in responsiveness to InsP₃. This view is supported by observations that appear to eliminate the other proposed mechanisms by which the activity of InsP₃ might be suppressed. Neither the destruction of InsP₃ by 5-phosphatase or 5-kinase enzymes nor the overall size or metabolic characteristics of the pools that rapidly sequester Ca²⁺ were altered by carbachol pretreatment (as judged by the extent and kinetics of Ca²⁺ uptake, the amount of ionomycin and thapsigargin-induced Ca²⁺ release and the reuptake characteristics of Ca²⁺ released by InsP₃ or InsP₃S₃). Finally, it has been suggested that GTP facilitates movement of Ca²⁺ to InsP₃-sensitive stores (2, 9, 41). However, inclusion of 100 μM GTP in incubations did not reverse the inhibitory effects of carbachol on responsiveness to InsP₃. Clearly then, the reduction in maximal responsiveness to InsP₃, which reflects an apparent shift of Ca²⁺ from the InsP₃-sensitive pool to the InsP₃-insensitive pool, can be ascribed to a partial loss of functional InsP₃ receptors from the former.

However, current evidence suggesting that InsP₃ acts by opening Ca²⁺ channels composed of tetromers of InsP₃ receptors (11, 14) indicates that decreasing the density of InsP₃ receptors and therefore the number of channel-forming units (11–14) should merely slow the rate of InsP₃-induced Ca²⁺ efflux without affecting the amount of Ca²⁺ released max-

mally. This prediction is clearly not borne out by our data and is incompatible with our measurements of the kinetics of Ca\textsuperscript{2+} mobilization which indicate that the rate of Ca\textsuperscript{2+} release is unchanged by carbachol pretreatment and that it is the amount of Ca\textsuperscript{2+} released in the initial rapid burst of efflux that is reduced. It is significant that this latter finding is remarkably similar to recent data showing that both submaximal and maximal doses of InsP\textsubscript{3} release Ca\textsuperscript{2+} only during an initial phase (28–30, 42). This “quantal” (42) Ca\textsuperscript{2+} release has been explained in terms of an intraluminal Ca\textsuperscript{2+} recognition site that, when occupied by Ca\textsuperscript{2+}, increases the affinity of InsP\textsubscript{3} for its receptor (43). Thus, when InsP\textsubscript{3} releases stored Ca\textsuperscript{2+} and intraluminal Ca\textsuperscript{2+} concentration falls, the affinity of InsP\textsubscript{3} for its receptor decreases and the Ca\textsuperscript{2+} channels that mediate Ca\textsuperscript{2+} efflux close (43). However, if, as it appears, InsP\textsubscript{3} receptor number is the only parameter within the Ca\textsuperscript{2+} release system that changes after carbachol pretreatment, this model does not accommodate our data, since after carbachol pretreatment, Ca\textsuperscript{2+} efflux in response to a maximal concentration of InsP\textsubscript{3} was terminated after only a minor (24%) fall in Ca\textsuperscript{2+} content. Our data would fit the model, however, if it is envisaged that carbachol pretreatment causes additional alteration of the events leading to Ca\textsuperscript{2+} release (e.g. the release mechanism becomes more sensitive to falling intraluminal Ca\textsuperscript{2+}). An alternative explanation of our data and those of others (28–30, 42) is that InsP\textsubscript{3} receptors, when activated, release a finite quantum of Ca\textsuperscript{2+} and then no more. If this is indeed the case, then a simple reduction in the density of InsP\textsubscript{3} receptors would reduce the maximal extent of InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release and occupation of a fraction of InsP\textsubscript{3} receptors with a submaximal dose of InsP\textsubscript{3} would release only part of the InsP\textsubscript{3}-sensitive Ca\textsuperscript{2+} store. This proposal is particularly attractive as it can explain simply the apparent carbachol-induced reduction in the size of the InsP\textsubscript{3}-sensitive pool, while taking into account the unchanged uptake characteristics and content of Ca\textsuperscript{2+}.

However, these postulates do not provide a simple explanation of why the EC\textsubscript{50} of InsP\textsubscript{3} for Ca\textsuperscript{2+} release was increased by pretreatment with carbachol, since in the absence of a “reservoir” effect a simple reduction in InsP\textsubscript{3} receptor number should not alter agonist potency. Evidence for a reservoir effect on InsP\textsubscript{3} in SH-SY5Y cells was not apparent as the K\textsubscript{D} for InsP\textsubscript{3} binding was less than the EC\textsubscript{50} for Ca\textsuperscript{2+} release and was also not seen in other tissues when binding and Ca\textsuperscript{2+} mobilization were measured under identical conditions (6, 16). However, the relationship between EC\textsubscript{50} and InsP\textsubscript{3} binding is clearly complex, as Ca\textsuperscript{2+} channel opening is cooperative in nature (27) and InsP\textsubscript{3} exhibits different EC\textsubscript{50} values when Ca\textsuperscript{2+} release is assessed during the initial phase of efflux and at steady-state (28). Thus, it cannot be ruled out that, in an unknown way, the reduction in InsP\textsubscript{3} receptor density also accounts for the increase in the EC\textsubscript{50} of InsP\textsubscript{3}. It is, however, possible that another mechanism accounts for the increase in EC\textsubscript{50} as pretreatment of SH-SY5Y cells with phorbol ester, which did not alter InsP\textsubscript{3} binding, also increased the EC\textsubscript{50} of InsP\textsubscript{3}. These data and similar results in pancreatic acinar cells (40) suggest that protein kinase C, which in agonist-stimulated cells would be activated by diacylglycerol (1), can reduce the potency of InsP\textsubscript{3} as a Ca\textsuperscript{2+} releasing agent without altering its maximal effect. Whether this reflects phosphorylation of the InsP\textsubscript{3} receptor at a site separate from that which binds InsP\textsubscript{3}, or of a protein associated with the Ca\textsuperscript{2+} release mechanism remains to be established.

In addition to the actions of phorbol ester, other evidence points toward the products of PIP\textsubscript{2} hydrolysis as being mediators of the inhibitory effects of carbachol on InsP\textsubscript{3} action.

First, SH-SY5Y cell muscarinic receptors are of the M3 subtype (19) and are linked primarily to stimulation of PIP\textsubscript{2} hydrolysis (37, 44), although it should be noted that carbachol can also increase cAMP levels in related SK-N-SH cells (45) and that cAMP-dependent phosphorylation can increase the EC\textsubscript{50} of InsP\textsubscript{3} on Ca\textsuperscript{2+} mobilization (46). However, in the present study, raising cAMP concentration with forskolin had no effect on the response to InsP\textsubscript{3}. Second, differentiation of cells with retinoic acid, which enhances adenylyl cyclase activation but does not alter phosphoinositide hydrolysis (33), did not alter responsiveness to InsP\textsubscript{3}. Finally, the potency of carbachol as an inhibitor of InsP\textsubscript{3}-induced Ca\textsuperscript{2+} mobilization (half-maximal effect at ~5 μM) was close to the EC\textsubscript{50} of carbachol as a stimulus of phosphoinositide hydrolysis (17–42 μM) and Ca\textsuperscript{2+} mobilization (4–12 μM) in intact cells (20, 36, 37, 47, 48).

These data imply that either diacylglycerol or InsP\textsubscript{3} initiate a mechanism that decreases the number of InsP\textsubscript{3} receptors, although a role for the former is unlikely as PDBu did not alter InsP\textsubscript{3} binding. Rather, it is possible that InsP\textsubscript{3} provides the signal for the decrease, either directly by occupying the receptor, or indirectly by depleting Ca\textsuperscript{2+} from the InsP\textsubscript{3}-sensitive store and/or by raising cytosolic Ca\textsuperscript{2+} concentration. This is quite plausible because, remarkably, InsP\textsubscript{3} levels remain significantly elevated above basal for at least 24 h after addition of carbachol, despite the fact that InsP\textsubscript{3} mass peaks at 5–10 s and then, within 2 min, declines to a new steady-state that is dependent upon Ca\textsuperscript{2+} influx (36, 37).

Although thus far we have assumed that SH-SY5Y cell populations are homogeneous, it could be argued that refractoriness of a substantial proportion of cells to muscarinic agonists accounts for the limited extent to which carbachol inhibits InsP\textsubscript{3} action. However, analysis of Ca\textsuperscript{2+} concentration in individual cells with Fura-2 revealed that all cells respond to carbachol and that >80% respond with a biphasic signal that is a product of InsP\textsubscript{3} generation, mobilization of intracellular Ca\textsuperscript{2+} stores, and flux of Ca\textsuperscript{2+} into the cell (36, 37). This does not exclude the possibility, though, that there are differences between cells in the extent to which responsiveness to InsP\textsubscript{3} is reduced after carbachol treatment. To establish this will require development of novel techniques to study InsP\textsubscript{3} reduced Ca\textsuperscript{2+} release in nonmobilized single cells.

The data presented here demonstrate, in a neurone-like cell, that the InsP\textsubscript{3} receptor is a site at which the response to an agonist that simulates phosphoinositide hydrolysis can be modified. This process can, therefore, be included with cell-surface receptor desensitization and down-regulation (18) as part of a cell’s adaptive response to plasma membrane receptor activation. How common this adaptive mechanism is in other cell types is not yet known, but it may be particularly important to those cells, like SH-SY5Y cells, in which phosphoinositide breakdown is only partially desensitized during chronic agonist stimulation. Such cells maintain high InsP\textsubscript{3} concentrations over extremely long periods and thus decreasing responsiveness to InsP\textsubscript{3} represents a “fail-safe” mechanism that enables the cell to limit Ca\textsuperscript{2+} release from internal stores.

While we have no data concerning the mechanism by which the number of InsP\textsubscript{3}-binding sites is reduced, it is likely, in view of the fact that chronic muscarinic receptor activation alters gene and protein expression in neuronal cells (49, 50), that either a decrease in receptor synthesis or an increase in rate of receptor degradation accounts for the change. This clearly represents an important topic for further study, as is the possibility that similar suppression of InsP\textsubscript{3} action occurs.

\footnote{D. G. Lambert and S. R. Nahorski, unpublished data.}
in disease states, when hormones or neurotransmitters are secreted persistently, or during chronic administration of receptor agonists. Indeed, it is intriguing that InsP$_3$-induced Ca$^{2+}$ mobilization is reduced in the brains of aged rats (51), perhaps reflecting an age-related fall in the density of InsP$_3$ receptors, and during transformation of NIH 3T3 cells with Ha-ras (52).

In summary, we have shown that chronic activation of muscarinic receptors in SH-SY5Y cells suppresses the ability of InsP$_3$ to mobilize Ca$^{2+}$. This suppression appears to be causally related to a reduction in the density of InsP$_3$ receptors and a protein kinase C-mediated event. The ability to manipulate InsP$_3$ receptor number in this manner should facilitate greatly studies aimed at defining the mechanism and control of InsP$_3$-induced Ca$^{2+}$ mobilization.

REFERENCES