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Agonist-Induced Desensitization of Muscarinic Receptor-Mediated Calcium Efflux without Concomitant Desensitization of Phosphoinositide Hydrolysis

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SUMMARY

Phosphoinositide hydrolysis does not appear to desensitize in 1321N1 astrocytoma cells. The evidence for this is that 1) the rate of accumulation of [^{3}H]inositol 1-phosphate is linear for up to 90 min in the presence of carbachol, 2) pretreatment of cells with 100 μM carbachol for 75 min does not diminish the subsequent ability of carbachol to increase [^{3}H]inositol 1-phosphate accumulation, and 3) the production of all of the [^{3}H]inositol phosphates including the polyphosphoinositide metabolites [^{3}H]inositol bis- and trisphosphate continues for up to 75 min in the presence of carbachol and declines rapidly when the muscarinic receptor antagonist atropine is added. Only when cells are treated with carbachol for 2.5 hr or longer is there a reduction in carbachol-stimulated phosphoinositide hydrolysis, and this is associated with a decrease in muscarinic receptor number. There does appear to be desensitization of hormone-stimulated Ca^{2+} mobilization in 1321N1 cells, because treatment of these cells with carbachol for 75 min leads to loss of the subsequent ability of carbachol to stimulate unidirectional $^{45}Ca^{2+}$ efflux. Histamine-stimulated $^{45}Ca^{2+}$ efflux also is lost in cells pretreated with carbachol, indicating that the desensitization is heterologous. We conclude that desensitization of hormone-stimulated, unidirectional $^{45}Ca^{2+}$ efflux cannot be accounted for by a loss of receptor-mediated phosphoinositide hydrolysis. If phosphoinositide hydrolysis or inositol trisphosphate formation are signals for calcium mobilization, the site at which the calcium response desensitizes must be distal to the initial receptor-mediated activation of phospholipase C.

INTRODUCTION

Cells have acquired a number of adaptive processes to "tune out" the stimulatory effects of hormones. One such adaptive process is the phenomenon of desensitization, in which responsiveness to agonist is lost subsequent to agonist treatment (1–5). Desensitization can occur rapidly (seconds to minutes) and is not necessarily associated with a decrease in the total number of cellular hormone receptors. Another type of adaptive process that occurs more slowly (hours) is that of receptor down-regulation, in which the number of receptors is decreased by continued agonist treatment (4–7).

Desensitization and receptor down-regulation are general terms that refer to phenomena shared by a variety of receptor-effector systems. It is unlikely that the same molecular events are responsible for agonist-induced loss of responsiveness in every system. In some, desensitization appears to result from changes in the hormone receptor such that agonist can no longer elicit the first in a series of molecular events. An example is the well characterized loss in the ability of the β-adrenergic receptor to activate adenylate cyclase (1, 3, 5). In other systems, desensitization appears to occur distal to the receptor. In such cases, the loss of responsiveness is not specific for the agonist to which the cells were initially exposed and is termed "heterologous desensitization" (2, 5, 8).

The breakdown of phosphoinositides and the resultant production of $InsP_3$[4] have been suggested to be the initial events in the series of molecular responses by which

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[4] The abbreviations used are: InsP, inositol phosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; PSS, physiological salt solution; NMS, *N*-methylscopolamine; QNB, quinuclidinylbenzilate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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certain neurohormones mobilize calcium and elicit calcium-dependent cellular responses (9–11). In 1321N1 cells, muscarinic agonists stimulate phosphoinositide hydrolysis and Ca^{2+} mobilization (12). These events appear to cause the calcium-dependent activation of a phosphodiesterase (13). The activation of phosphodiesterase has been shown to desensitize during 75 min of agonist exposure (14). There is evidence that calcium-dependent responses in other cells also desensitize with continued exposure to agonist (2, 8, 10). It is not known, however, which steps in the series of molecular events leading to these responses are the sites at which desensitization occurs.

One question that has not been adequately addressed is whether receptor-mediated phosphoinositide hydrolysis desensitizes. Cohen *et al.* (15) suggested that there is desensitization of muscarinic receptor-mediated PtdIns turnover, based on studies examining hormonal effects on the synthesis of PtdIns. Kirk and Creba (16) found that agonists cause a smaller decrease in the cellular content of the polyphosphoinositide PtdIns 4,5P in hepatocytes that are pretreated with agonist. Neither study specifically assessed hormonal effects on the receptor-stimulated process of phosphoinositide hydrolysis or on the accumulation of inositol phosphates. In fact, results of several papers (17, 18), while not directly addressing the question of desensitization, demonstrate that inositol phosphate formation is sustained in the continued presence of agonist.

The studies presented here establish that muscarinic receptor-mediated phosphoinositide hydrolysis in 1321N1 cells is not lost during short-term agonist exposure. Under the same conditions of agonist exposure, hormone-stimulated unidirectional $^{45}Ca^{2+}$ efflux is abolished.

MATERIALS AND METHODS

Measurements of inositol phosphates. 1321N1 cells were cultured on 35-mm plates as described previously (12). In most experiments, phosphoinositide hydrolysis was monitored by measuring [^{3}H]InsP accumulation with a modification (19) of the procedure described by Berridge *et al.* (20). Briefly, phosphoinositides were labeled by incubating cells for 18–20 hr with 1 μCi/ml [^{3}H]inositol in Dulbecco's modified Eagle's medium plus 5% fetal calf serum (growth medium). At the start of the assay, cells were washed with PSS comprised of 118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, and 20 mM HEPES, pH 7.4. Cells were then incubated with carbachol and LiCl (10 mM) or LiCl alone for specified time intervals. Incubations were terminated with methanol, phospholipids were extracted, and [^{3}H]InsP was separated from [^{3}H]inositol by anion-exchange chromatography with Dowex AG 1X-8. In some experiments, the organic phase containing [^{3}H]PtdIns was also counted.

The procedure for measuring [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ differed somewhat from that described above. Cells were labeled with 10 μCi/ml [^{3}H]inositol, rather than 1 μCi/ml. Incubations were terminated with 0.5 ml of ice-cold 10% trichloroacetic acid, rather than methanol. To prepare these samples for anion-exchange chromatography, cells were removed by scraping, and dishes were rinsed with an additional 0.5 ml of trichloroacetic acid. Samples were sonicated for 10 sec with a Kontes cell disrupter set at the maximal power setting and centrifuged at 12,000 × *g* for 30 sec in a Beckman Microfuge. The supernatants were transferred to glass tubes, and the trichloroacetic acid was removed by five washes with 4 volumes of diethyl ether. The samples were then applied to anion-exchange columns. [^{3}H]Inositol was eluted with 10 ml of water. [^{3}H]InsP was eluted with 8 ml of 200 mM ammonium formate/100 mM formic acid, and the columns were then washed with an additional 32 ml of this buffer; [^{3}H]$InsP_2$ was eluted with 16 ml of 450 mM ammonium formate/100 mM formic acid, and columns washed with an additional 24 ml of this buffer; [^{3}H]$InsP_3$ was then eluted with 8 ml of 1 M ammonium formate/100 mM formic acid.

$^{45}Ca^{2+}$ assays. Unidirectional $^{45}Ca^{2+}$ efflux assays were performed as previously described (12). Briefly, cell monolayers in 35-mm plates were labeled to isotopic equilibrium by incubation in growth medium containing 5 μCi/ml $^{45}Ca^{2+}$ for 18–20 hr. The efflux assay was initiated by aspiration of the growth medium and addition of carbachol or vehicle in PSS without $^{45}Ca^{2+}$. Incubations were terminated by washing the monolayers in a buffer containing $LaCl_3$ (5 mM), and cellular $^{45}Ca^{2+}$ was determined. Data were expressed relative to intracellular $^{45}Ca^{2+}$ content in cultures that received no efflux interval (time zero cellular $^{45}Ca^{2+}$). In some experiments, the effects of carbachol on net cellular $^{45}Ca^{2+}$ ($^{45}Ca^{2+}$ at isotopic equilibrium) rather than on unidirectional $^{45}Ca^{2+}$ efflux were measured. For these experiments, 1321N1 cells equilibrated for 18–20 hr with $^{45}Ca^{2+}$ were exposed to carbachol in the continued presence of $^{45}Ca^{2+}$ at the same specific activity used during equilibration. At various time after the addition of carbachol, cultures were rapidly washed, and cellular $^{45}Ca^{2+}$ was determined as in the unidirectional efflux assays.

Receptor-binding assays. Muscarinic receptors were quantitated by measuring the binding of [^{3}H]NMS to intact 1321N1 cells in monolayer culture. Cells were washed with PSS and incubated in 2.5 ml of PSS containing [^{3}H]NMS for 75 min at 37°. [^{3}H]NMS binding was at equilibrium at this time (data not shown). Specific binding was defined as that inhibited by 10 μM atropine. Assays were terminated by rapidly washing the monolayers four times with 3 ml of PSS. Cells were solubilized in 0.5 ml of a solution containing 5 mM EGTA, 1 mM HEPES, and 3% (w/v) Triton X-100 and were removed from the plate by scraping. Solubilized cells were transferred into minivials, dishes were rinsed with 0.5 ml of the solubilizing solution, and samples were counted in Liquiscint. Data were analyzed by Scatchard plots. Receptor-binding assays were also performed on membranes from 1321N1 cells prepared essentially as described (13). These assays were carried out at 37° for 60 min in 5 ml of PSS containing saturating concentrations of [^{3}H]NMS (0.8 nM) or [^{3}H]QNB (0.1 nM) and approximately 300 μg of membrane protein/assay tube.

Materials. Dulbecco's medium and fetal calf serum were from Irvine Biological. Dowex was from Bio-Rad. [^{3}H]Inositol, [^{3}H]QNB, and [^{3}H]NMS were from New England Nuclear; $^{45}Ca^{2+}$ was from Amersham. Other drugs and chemicals were from Sigma.

RESULTS

Carbachol-stimulated phosphoinositide hydrolysis was monitored by measuring the accumulation of [^{3}H]InsP in the presence of lithium and carbachol. Under these conditions, in which the breakdown of [^{3}H]InsP is blocked by lithium (20), the accumulation of [^{3}H]InsP is a linear function of time for at least 90 min (Fig. 1). This finding indicates that the process by which carbachol stimulates phosphoinositide hydrolysis does not attenuate in the continued presence of agonist.

The effect of agonist pretreatment on muscarinic receptor stimulation of phosphoinositide hydrolysis also was examined. Cells were treated with 100 μM carbachol for various times during overnight labeling with [^{3}H]inositol. The cells were then washed, and phosphoinositide hydrolysis was examined by measuring [^{3}H]InsP accumulation during a 10-min re-exposure to various concentrations of carbachol in the presence of lithium (Fig. 2). Pretreatment with carbachol for 75 min does not decrease the maximal [^{3}H]InsP formation in response to carbachol and does not significantly alter the agonist concentration-response curve. When cells are treated with carbachol for longer periods of time, there is a progressive reduction in the maximal response to carbachol. The ability of carbachol to stimulate phos-

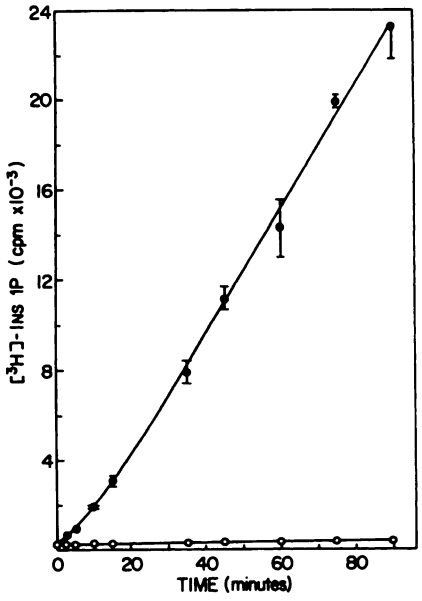

FIG. 1. *The rate of [^{3}H]InsP accumulation in the presence of lithium and carbachol is sustained for up to 90 min*

Cells were labeled overnight with [^{3}H]inositol, washed, and then incubated for various times with lithium (O) or lithium and 1 mM carbachol (●). Data are mean ± standard error (n = 3) and are expressed as counts/min of [^{3}H]InsP per dish.

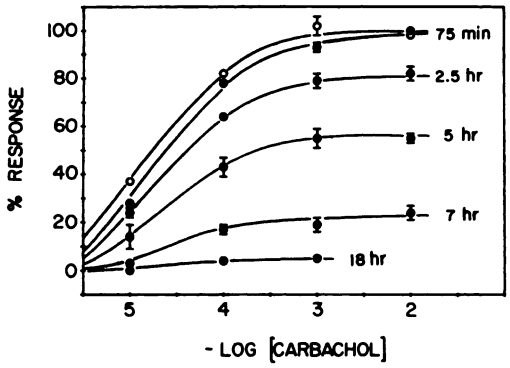

FIG. 2. *Concentration-response relationships for stimulation of [^{3}H] InsP accumulation by carbachol after various periods of agonist pretreatment*

Cells were labeled overnight with [^{3}H]inositol and treated with vehicle (O) or 100 μM carbachol (●) for the indicated period of time during the labeling with [^{3}H]inositol. Cells were then washed and exposed to lithium and different concentrations of carbachol for 10 min. The [^{3}H]InsP formed in cultures treated with lithium alone was subtracted. Values were normalized to the maximal increases in [^{3}H] InsP caused by carbachol in vehicle-treated cells. Values are means ± standard error of 3–4 samples. The presence of carbachol during [^{3}H] inositol labeling resulted in a slightly greater (20–50%) amount of [^{3}H] in PtdIns.

phoinositide hydrolysis is practically abolished after 18 hr of treatment with carbachol (Fig. 2).

The finding that the maximal [^{3}H]InsP response to carbachol decreases only after relatively long periods of agonist pretreatment suggests that this loss results from down-regulation of muscarinic receptors. The number of muscarinic receptors on intact 1321N1 cells was determined with the radioligand [^{3}H]NMS. Since binding of this ligand to intact 1321N1 cells had not been examined

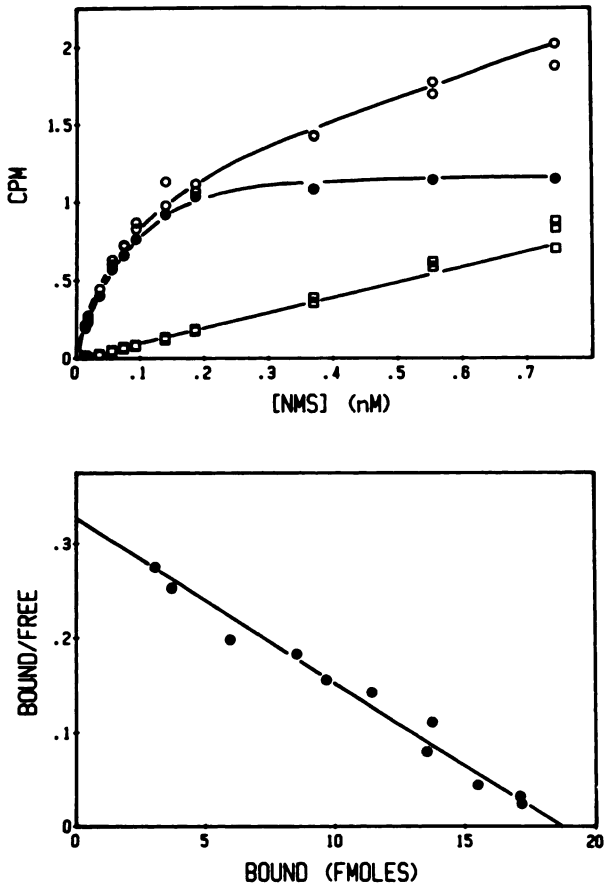

FIG. 3. *Saturation binding isotherm and Scatchard analysis of [^{3}H] NMS binding to intact 1321N1 cells*

The total (O), specific (●), and nonspecific (□) binding of various concentrations of [^{3}H]NMS to intact 1321N1 cells is shown in the upper panel. Scatchard analysis of the specific binding (lower panel) indicates that [^{3}H]NMS binds to a single class of receptors with a K_D of 56 pM and that there are approximately 20 fmol of receptors/35-mm culture dish. This corresponds to approximately 8,500 receptors/cell.

previously, we first demonstrated that the specific binding of [^{3}H]NMS is saturable, and this radioligand binds to a single population of binding sites with a K_D of 56 pM (Fig. 3). Cells pretreated with carbachol show a time-dependent loss in their number of [^{3}H]NMS-binding sites (Table 1). There is no change in the affinity of the muscarinic receptor for [^{3}H]NMS in cells that are pretreated with carbachol (data not shown). The total number of [^{3}H]QNB-binding sites in membranes from 1321N1 cells also has been shown to decrease during agonist treatment (14). We used both [^{3}H]NMS and [^{3}H] QNB to quantitate muscarinic receptors in membrane preparations from cells that had been exposed to carbachol for various times (Table 1). Using either radioligand, one sees the same per cent loss of receptors following carbachol pretreatment (Table 1). These values agree well with the per cent loss measured by equilibrium binding of [^{3}H]NMS to intact cells (Table 1). It is notable that the total numbers of binding sites for [^{3}H]QNB and [^{3}H]NMS differ markedly (Table 1). This phenomenon has been reported recently for 1321N1 cells (21) and is currently under investigation.

In 1321N1 cells, carbachol increases the accumulation

TABLE 1

Carbachol-induced decrease in muscarinic receptor number in intact cells and cell membranes

Intact 1321N1 cells were exposed to 100 μM carbachol for the indicated times. The cells were washed and radioligand-binding assays were carried out on the intact cells or on membranes prepared from them. Receptor number in intact cell studies was determined from Scatchard analysis of saturation binding isotherms. Receptor number in membranes was estimated by using a saturating concentration of radioligand. Values represent means ± standard error for 3–4 determinations. The number in parentheses is the percentage of receptors remaining relative to untreated cells.

Treatment time	Intact cells [^{3}H]NMS		Cell membranes			
	fmol/10^6 cells	%	[^{3}H]NMS		[^{3}H]QNB	
			fmol/mg protein	%	fmol/mg protein	%
0	10.2 ± 0.2	100	33 ± 1	100	57 ± 1	100
75 min	7.9 ± 0.3	78	26 ± 1	79	46 ± 1	81
2.5 hr	5.7 ± 0.1	56	20 ± 4	61	35 ± 1	61
3.5 hr	4.5 ± 0.2	44				
5 hr	3.5 ± 0.1	35				
15.5 hr	1.5 ± 0.1	14				

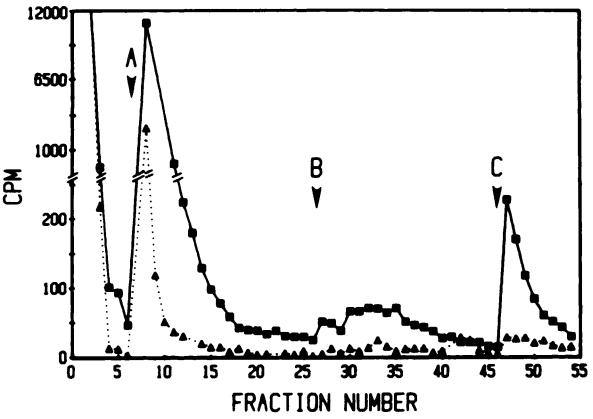

FIG. 4. *Anion-exchange chromatography of aqueous extracts of 1321N1 cells*

Cells labeled overnight with [^{3}H]inositol were washed and then treated for 30 min with vehicle (△) or 1 mM carbachol (■) (lithium was not present). Aqueous extracts were applied to anion-exchange columns and 2-ml fractions were collected. The eluting solutions were water and 100 mM formic acid with 200 mM (*A*), 450 mM (*B*), and 1 M (*C*) ammonium formate as described in Materials and Methods.

of radioactivity eluting as [^{3}H]InsP_2 and [^{3}H]InsP_3, as well as [^{3}H]InsP (Fig. 4). This observation indicates that the polyphosphoinositides are hydrolyzed in response to activation of muscarinic receptors in these cells. The time course of carbachol-stimulated [^{3}H]InsP, [^{3}H]InsP_2, and [^{3}H]InsP_3 formation was examined (Fig. 5). These experiments were carried out in the absence of lithium so that the normal interconversion of the three inositol phosphates was not perturbed. Since cellular [^{3}H]inositol and [^{3}H]PtdIns are at steady state during the time course of these incubations (data not shown), changes in the amount of the radiolabeled [^{3}H]inositol phosphates should parallel changes in the concentrations of these inositol phosphates. Carbachol causes a rapid rise in the concentrations of [^{3}H]InsP, [^{3}H]InsP_2, and [^{3}H]InsP_3 (Fig. 5). After several minutes, the [^{3}H]inositol phosphate levels reach a maximum and remain elevated (compared to control) over the next 75 min of agonist exposure. The addition of 10 μM atropine for an additional 10 min (arrow, Fig. 5) decreases the concentrations of all three inositol phosphates, indicating that muscarinic receptor-stimulated inositol phosphate formation (i.e., phosphoinositide hydrolysis) is ongoing at this time.

Activation of muscarinic receptors also stimulates unidirectional $^{45}Ca^{2+}$ efflux from 1321N1 cells equilibrated with $^{45}Ca^{2+}$ (12). To assess the effects of agonist pretreatment on unidirectional $^{45}Ca^{2+}$ efflux, cells labeled overnight with $^{45}Ca^{2+}$ were exposed to carbachol or vehicle for 75 min, in the continued presence of $^{45}Ca^{2+}$. After this period of treatment, the carbachol-pretreated cells have 70–80% of the cellular $^{45}Ca^{2+}$ of cells treated with vehicle. The cells were then washed, and the basal and carbachol-stimulated rates of unidirectional $^{45}Ca^{2+}$ efflux were measured. The basal rate of $^{45}Ca^{2+}$ efflux is not different in cells pretreated with carbachol and in normal cells (Fig. 6). However, carbachol fails to increase the rate of unidirectional $^{45}Ca^{2+}$ efflux in cells that have been pretreated with carbachol (Fig. 6).

The loss of the unidirectional $^{45}Ca^{2+}$ efflux response occurs at a time when inositol phosphate concentrations are at steady state and are elevated above control (Fig. 5). It is possible that a change in the rate of InsP_3 production, rather than the instantaneous concentration, is the signal necessary for calcium mobilization. To test this possibility, cells treated with carbachol for 75 min were treated for an additional 10 min with 100 nM atropine. The reason for adding this receptor antagonist was to stop inositol phosphate production and thus reduce cellular InsP_3 levels (as shown in Fig. 5). Both drugs were washed away and the cells were rechallenged with carbachol. Under this protocol, carbachol stimulates [^{3}H]InsP_3 reaccumulation in normal and pretreated cells (Fig. 7). Nonetheless, carbachol does not increase unidirectional $^{45}Ca^{2+}$ efflux in cells that are pretreated with carbachol (Fig. 8). These findings provide further evidence that agonist-stimulated unidirectional $^{45}Ca^{2+}$ efflux is lost under conditions in which agonist-stimulated phosphoinositide hydrolysis appears normal.

We also considered the possibility that hormone treatment depletes the total cellular pools of calcium available for exchange. Carbachol does cause a large and rapid decrease in net cellular $^{45}Ca^{2+}$ in 1321N1 cells at isotopic equilibrium (Fig. 9). However, during the 75-min period of carbachol treatment, the cells take up and largely

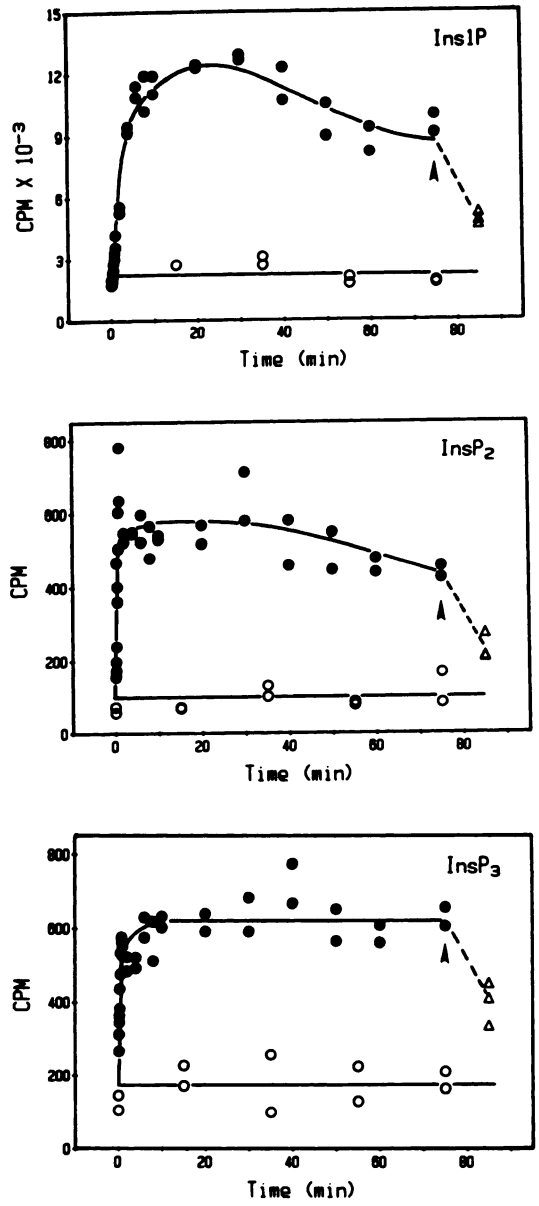

FIG. 5. *Accumulation of [³H]InsP, [³H]InsP₂, and [³H]InsP₃ in 1321N1 cells*

Cells labeled overnight with [³H]inositol were washed and then treated for various times with vehicle (○) or 1 mM carbachol (●) (lithium was not present). Several cultures that were treated for 75 min with carbachol received 10 μM atropine (arrow) for an additional 10 min (△).

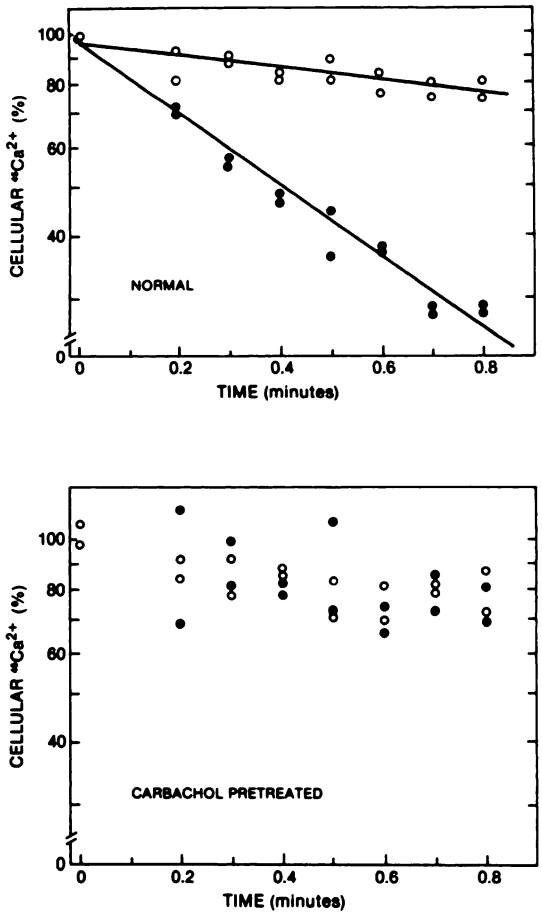

FIG. 6. *Carbachol does not stimulate unidirectional $^{45}Ca^{2+}$ efflux from cells that are pretreated with carbachol for 75 min*

Cells were labeled overnight with $^{45}Ca^{2+}$ and were treated with vehicle (NORMAL) or 100 μM carbachol (CARBACHOL-PRETREATED) during the last 75 min of labeling. At time zero, cells were washed free of extracellular $^{45}Ca^{2+}$ and incubated for various times with vehicle (○) or 1 mM carbachol (●). The time zero cellular $^{45}Ca^{2+}$ was 11,000 cpm/dish for normal cultures and 7,800 cpm/dish for cultures pretreated with carbachol. The difference in the time zero cellular $^{45}Ca^{2+}$ values was due to the effects of carbachol on net cellular $^{45}Ca^{2+}$ (see Fig. 9).

replenish their stores of $^{45}Ca^{2+}$ (Fig. 9; also see Ref. 12). Thus, the loss of carbachol-stimulated unidirectional $^{45}Ca^{2+}$ efflux does not appear to be due to the depletion of *total* exchangeable calcium.

It has been shown in several systems that treatment with one hormone can produce heterologous desensitization to the calcium-mobilizing effects of other hormones (8, 22–25). This finding has led to the suggestion that these hormones may utilize a common pool of calcium. In 1321N1 cells, histamine can also stimulate $^{45}Ca^{2+}$ efflux. When cells are pretreated with carbachol, the ability of histamine to stimulate $^{45}Ca^{2+}$ efflux is lost (Fig. 10). This finding indicates that carbachol causes loss of hormonally stimulated $^{45}Ca^{2+}$ efflux at a site that is distal to the muscarinic receptor and common to the actions of both carbachol and histamine.

DISCUSSION

There is abundant evidence that physiological responses to calcium-mobilizing hormones are not maintained in the continued presence of agonist (2, 4, 8, 10, 14). There is also considerable evidence that calcium mobilization is mediated through phosphoinositide hydrolysis (9–11, 26–30). The possibility that Ca^{2+} mobilization desensitizes due to loss of receptor-mediated phosphoinositide hydrolysis has been raised (8, 30), and there is evidence that under some circumstances agonist exposure can diminish subsequent agonist effects on phosphoinositide turnover (15, 16, 30). These previous studies suggest that there are adaptive changes in PtdIns resynthesis or in the extent to which phosphoinositides are depleted, but they do not directly examine agonist-in-

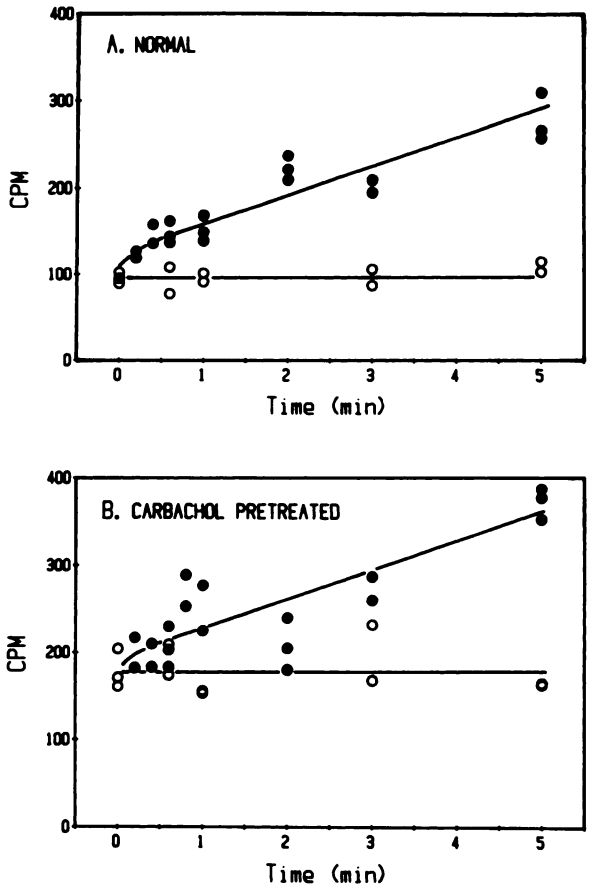

FIG. 7. *Stimulation of [^{3}H]InsP$_3$ accumulation in normal cells and cells pretreated with carbachol*

Cells were labeled overnight with [^{3}H]inositol and were treated for 75 min with vehicle (A) or 100 μM carbachol (B) in the continued presence of [^{3}H]inositol. Atropine (100 nM) was then added for an additional 10 min in the continued presence of $^{45}Ca^{2+}$. At time zero, cells were washed and exposed to vehicle (O) or 1 mM carbachol (●) for various times. Data represent cpm of [^{3}H]InsP$_3$/dish. The effect of carbachol in normal as well as in carbachol-pretreated cells was highly significant ($p < 0.001$) by analysis of variance.

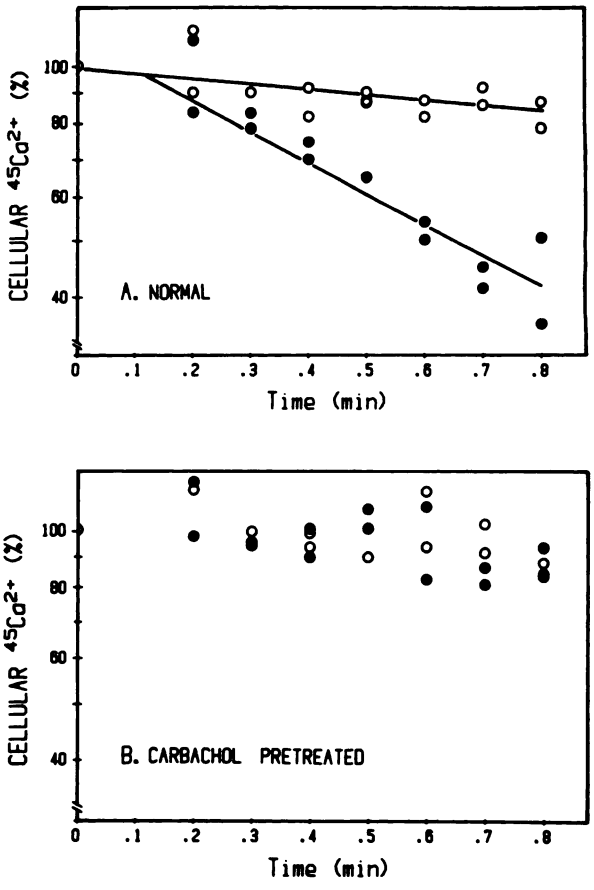

FIG. 8. *Unidirectional $^{45}Ca^{2+}$ efflux from cells treated with atropine alone or with carbachol and atropine*

Cells were labeled overnight with $^{45}Ca^{2+}$ and treated for 75 min with vehicle (A) or with 100 μM carbachol (B) in the continued presence of $^{45}Ca^{2+}$. Atropine (100 nM) was then added for an additional 10 min. At time zero, cells were washed and exposed to vehicle (O) or 1 mM carbachol (●) for various times. The time zero cellular $^{45}Ca^{2+}$ was 7,300 cpm/dish in normal cells and 7,000 cpm/dish in cells pretreated with carbachol.

duced changes in the rate of phosphoinositide hydrolysis or $InsP_3$ formation. The data presented here demonstrate that 1) the accumulation of [^{3}H]InsP is linear for up to 90 min when carbachol is added to cells in the presence of lithium, 2) pretreatment of 1321N1 cells with carbachol for 75 min does not decrease the subsequent magnitude or sensitivity of this response to carbachol, and 3) the polyphosphoinositide metabolites [^{3}H]InsP$_2$ and [^{3}H]InsP$_3$ are continuously formed in the presence of carbachol. We therefore conclude that muscarinic receptor-stimulated, phospholipase C-mediated hydrolysis of phosphoinositides does not desensitize.

Our studies, like those of Meeker and Harden (14), demonstrate that short-term (75-min) treatment of 1321N1 cells with carbachol causes only a small decrease in the total number of muscarinic receptors as quantitated by [^{3}H]NMS or [^{3}H]QNB binding to membranes (Table 1). Our intact cell-binding studies indicate that there is also little decrease in the number of [^{3}H]NMS-binding sites available at the cell surface after 75 min of agonist treatment (Table 1). Several laboratories have

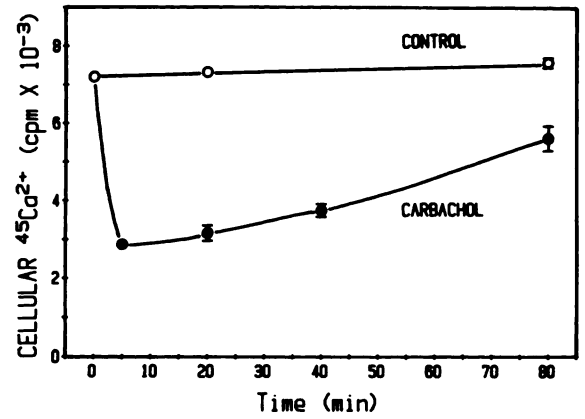

FIG. 9. *The effects of carbachol on cellular $^{45}Ca^{2+}$ in cells equilibrated with $^{45}Ca^{2+}$*

1321N1 cells were labeled overnight with $^{45}Ca^{2+}$. The cells were then treated for various times with vehicle (O) or with 100 μM carbachol (●) in the continued presence of $^{45}Ca^{2+}$ and cellular $^{45}Ca^{2+}$ was determined. Data are mean ± standard error of 3–5 determinations.

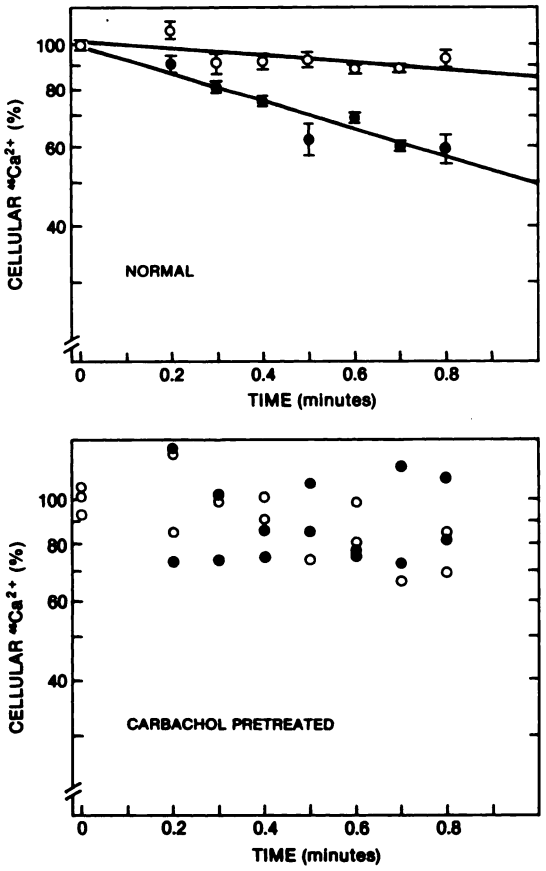

FIG. 10. *Histamine does not stimulate unidirectional $^{45}Ca^{2+}$ efflux from cells that are pretreated with carbachol*

Cells were labeled overnight with $^{45}Ca^{2+}$ and treated for 75 min with vehicle (NORMAL) or 100 μM carbachol (CARBACHOL PRETREATED) in the continued presence of $^{45}Ca^{2+}$. At time zero, cells were washed free of extracellular $^{45}Ca^{2+}$ and incubated with vehicle (○) or 1 mM histamine (●). The data in the upper panel are from normal cultures and are mean ± standard error of three determinations. Those in the lower panel are from cultures pretreated with carbachol. The time zero cellular $^{45}Ca^{2+}$ was 8,700 cpm/dish for normal cultures and 4,400 cpm/dish for cultures pretreated with carbachol (this difference in the time zero values was unusually large).

shown that short-term agonist treatment can lead to the reversible disappearance of [^{3}H]NMS-binding sites on intact cells (31–33), a process suggested to result from alterations in receptor localization (e.g., internalization). Because our intact cell-binding studies were carried out under equilibrium binding conditions and at 37°, any such transient alterations in receptor localization induced by agonist exposure are likely to have been reversed. It will be interesting to determine if carbachol causes changes in the localization of muscarinic receptors on 1321N1 cells, because the finding that muscarinic receptor-stimulated phosphoinositide hydrolysis does not attenuate in the presence of carbachol would then suggest that an internalized or otherwise altered state of the receptor retains the ability to regulate phosphoinositide hydrolysis.

The loss of the phosphoinositide response seen when 1321N1 cells are treated with agonist for periods longer than 75 min seems to be related to receptor down-regulation. The decline in the maximal carbachol-induced [^{3}H]InsP formation coincides with a progressive loss in the number of muscarinic receptors. We have not quantitated precisely the relationship between receptor loss and decrease in phosphoinositide hydrolysis because agonist pretreatment causes some change in the amount of [^{3}H]inositol in total cellular phosphoinositides (see legend to Fig. 2). It is clear, nonetheless, that there is coordinate loss of binding sites and maximal phosphoinositide hydrolysis, consistent with the hypothesis that there is little receptor reserve in the coupling of muscarinic receptors to phosphoinositide hydrolysis (10, 34–36).

Cells exposed to carbachol for 75 min lose their ability to respond to carbachol with an increase in unidirectional $^{45}Ca^{2+}$ efflux. This desensitization does not appear to occur at the level of the muscarinic receptor, because receptors retain their ability to stimulate phosphoinositide hydrolysis at a time when calcium efflux is lost. It thus appears that the loss of the calcium response occurs distal to receptor activation. This idea is further supported by the finding that histamine cannot stimulate $^{45}Ca^{2+}$ efflux following carbachol pretreatment. The conclusion that desensitization of the calcium mobilization response occurs beyond the level of the hormone receptor is consistent with those of earlier studies demonstrating heterologous desensitization to the effects of hormones that share common hormone-sensitive pools of calcium in liver and gland cells (22–25).

A recent series of articles supports the possibility that inositol 1,4,5-trisphosphate is the phosphoinositide metabolite that releases calcium from intracellular stores (26–29). Our data suggest that this putative signal for calcium mobilization is formed in response to muscarinic receptor activation in 1321N1 cells. We examined [^{3}H]$InsP_3$ accumulation after various periods of treatment with carbachol. $InsP_3$ is continually formed during 75 min of carbachol treatment. Furthermore, when the cellular $InsP_3$ levels are lowered by the application of atropine to carbachol-pretreated cells, the subsequent addition of carbachol again increases $InsP_3$ formation but does not restore $^{45}Ca^{2+}$ efflux. These data suggest that loss of the calcium efflux response cannot be accounted for by a reduction in the formation of $InsP_3$. The observation that agonist-stimulated phosphorylase activation is lost in the face of agonist-stimulated PtdIns4,5P2 breakdown led Kirk and Creba (16) to a similar conclusion.

An added complexity regarding agonist-stimulated phosphoinositide hydrolysis has been described recently (37). In the parotid, at least two $InsP_3$ isomers are formed in response to hormones. One is inositol 1,4,5-trisphosphate, the isomer that releases calcium from permeabilized cells. The second is inositol 1,3,4-trisphosphate, an isomer whose effect on cellular calcium stores is unknown. Although our data suggest that loss of the calcium efflux response cannot be accounted for by a decrease in total $InsP_3$ production, it is possible that there is a change in the relative production of the two $InsP_3$ isomers and that the isomer that predominates at the time when the calcium response is desensitized cannot release calcium. Detailed analysis of the existence, relative production, and calcium-mobilizing activity of $InsP_3$

isomers in 1321N1 cells is required before we can determine unequivocally whether desensitization of the calcium mobilization response is due to lack of production of an active isomer of $InsP_3$.

We consider it more likely that desensitization occurs at a site interposed between the production of the active isomer of $InsP_3$ and the release of calcium. In support of this, Prentki *et al.* (29) reported that the ability of exogenous $InsP_3$ to cause calcium release from microsomes desensitizes after a preliminary exposure to this putative second messenger. The loss of calcium mobilization that we observe in agonist-treated 1321N1 cells could result from insensitivity of the mechanism that transduces changes in $InsP_3$ concentration into calcium release (i.e., an $InsP_3$ receptor). Alternatively, there may be depletion of a hormone-sensitive trigger pool of calcium which cannot be replenished in the continued presence of carbachol or $InsP_3$. Further experimentation and more detailed information about the mechanism of action of $InsP_3$ will be needed before we can distinguish between these possibilities.

Our finding that the unidirectional $^{45}Ca^{2+}$ efflux response desensitizes and the previous observation that the calcium-dependent activation of phosphodiesterase in 1321N1 cells also desensitizes (14) indicate that agonist treatment leads to a loss in the ability of muscarinic agonists to increase intracellular calcium. In contrast, phosphoinositide hydrolysis is sustained and inositol phosphate production continues during short-term agonist treatment. It therefore appears that desensitization of muscarinic receptor-mediated calcium mobilization does not occur at the level of the receptor but at a more distal site involving inositol phosphate effects on calcium release or the depletion of a small hormone-sensitive trigger pool of calcium.

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