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Relationships between Phosphoinositide and Calcium Responses to Muscarinic Agonists in Astrocytoma Cells

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SUMMARY

Activation of muscarinic receptors in human astrocytoma (1321N1) cells stimulates phosphoinositide metabolism and calcium mobilization. The muscarinic effect on phosphoinositide turnover is evidenced by increased formation of [^{3}H]inositol 1-phosphate (Ins1P) and by increased [^{3}H]inositol incorporation into PtdIns. The muscarinic effect on calcium mobilization is seen as a large increase in unidirectional $^{45}Ca^{2+}$ efflux from cells equilibrated with $^{45}Ca^{2+}$ and a small increase in unidirectional $^{45}Ca^{2+}$ influx. A series of muscarinic agonists was used to explore the relationship between phosphoinositide metabolism and unidirectional $^{45}Ca^{2+}$ efflux. The maximal increases in [^{3}H]Ins1P formation produced by carbachol and acetylcholine are similar and are much larger than those caused by oxotremorine and pilocarpine. The effects of these agonists on $^{45}Ca^{2+}$ efflux are similar: carbachol and acetylcholine cause equivalent maximal increases in the rate of $^{45}Ca^{2+}$ efflux whereas oxotremorine and pilocarpine cause submaximal $^{45}Ca^{2+}$ efflux reponses. The K_{act} values of carbachol and acetylcholine for stimulation of [^{3}H]Ins1P formation are 40 μM and 1.5 μM, respectively. These values are only 2- to 3-fold higher than the respective K_{act} values for stimulating $^{45}Ca^{2+}$ efflux. The finding that each of the muscarinic agonists tested has nearly identical efficacy and similar potency for stimulating [^{3}H]Ins1P formation and $^{45}Ca^{2+}$ efflux supports the idea that hormonal stimulation of phosphoinositide hydrolysis leads to calcium mobilization.

INTRODUCTION

Muscarinic cholinergic receptor activation leads to several distinct biochemical events. These events include increased turnover of PtdIns[4] and the polyphosphoinositides[5] (1–5), calcium mobilization (1, 2, 6), and changes in cyclic AMP and cyclic GMP metabolism (2, 3, 7, 8). While it is clear that all of these biochemical events occur concurrently, the relationships between them remains a subject of intense investigation. One proposal that has recently attracted much attention is that hydrolysis of PtdIns or the polyphosphoinositides mediates hormonally stimulated calcium mobilization (1–4). Although there are several lines of evidence that support this theory (1–4, 9–11), other data are inconsistent with it (12–15). As a result, the relationship between phosphoinositide hydrolysis and calcium mobilization is still a matter of active debate.

Recent work from our laboratory has demonstrated a potentially useful characteristic of the inositol phospholipid response in chick heart cells (8), which is also seen in synaptosomes (16). In these systems, phosphoinositide turnover is greatly increased by carbachol but is only slightly increased by oxotremorine, a muscarinic agonist that is potent and specific in other regards (8, 17, 18). If phosphoinositide turnover is the signal for calcium mobilization, oxotremorine might also cause little calcium mobilization.

Meeker and Harden (18) have recently reported that activation of muscarinic receptors on 1321N1 astrocytoma cells attenuates cyclic AMP accumulation through a mechanism that appears to be secondary to calcium mobilization. Interestingly, oxotremorine is as effective as carbachol in causing the activation of phosphodiesterase in this homogeneous cell system (18). At face value, this finding suggests that oxotremorine is also a full

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[4] The abbreviations used are: PtdIns, phosphatidylinositol; PSS, physiological salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ins1P, inositol 1-phosphate; PtdIns 4P, phosphatidylinositol 4-phosphate; PtdIns 4,5P_2, phosphatidylinositol 4,5-bisphosphate.

[5] "Inositol phospholipids" and "phosphoinositides" are collective terms for PtdIns, PtdIns 4P, and PtdIns 4,5P_2. "Polyphosphoinositides" refers only to PtdIns 4P and PtdIns 4,5P_2.

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agonist for causing calcium mobilization and that there may be a dissociation between receptor-mediated phosphoinositide and calcium responses in these cells. In the studies presented here, we have established methods for studying inositol phospholipid metabolism and $^{45}Ca^{2+}$ fluxes in 1321N1 cells and have used this system to examine the differential effects of cholinergic agonists on these two cellular responses.

EXPERIMENTAL PROCEDURES

Cell culture. All cell culture and experiments were carried out at the University of California, San Diego. Cell stocks were maintained in 250-ml flasks (Nunc) in growth medium composed of 95% Dulbecco's modified Eagle's medium and 5% fetal calf serum (Irvine Scientific). Cultures were grown in a humidified incubator at 37° in an atmosphere of 10% carbon dioxide/90% air. Cells were subcultured every 7 days; medium was changed every 3 days. For subculturing, cells were released from the monolayer with 0.25% trypsin without calcium or magnesium (Irvine Scientific) and were plated onto 250-ml flasks or 35-mm plates (Nunc) at a density of 8,000 cells/cm^2.

Assay incubation conditions. All assays were performed at 37° with confluent 7-day-old cultures grown on 35-mm plates. The incubation medium was PSS, pH 7.4, composed 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. Prior to most assays, growth medium was removed by aspiration and cells were washed three times with 1.5 ml of PSS. Monolayers were incubated with an additional 1.5 ml of PSS for at least 10 min before the assay was initiated. When antagonists were used, they were added at least 5 min before assays were initiated.

Phosphoinositide assays. Phosphoinositide hydrolysis was monitored by measuring ^{3}H-labeled Ins1P accumulation in the presence of LiCl, an inhibitor of the enzyme that converts Ins1P to inositol (19, 20). Phosphoinositides were radiolabeled by incubating 1321N1 cell monolayers with 1.0 ml of growth medium containing [*myo*-2-^{3}H]inositol (1.0 μCi/ml) (New England Nuclear Corporation) for 18–20 hr. Just prior to the assay, monolayers were washed and incubated as described above. Assays were initiated by the addition of warmed PSS containing LiCl (10 mM) and drugs. To terminate the incubation, the drug solution was rapidly removed by aspiration, the monolayer was washed three times with 1.5 ml of PSS, and 0.5 ml of cold methanol was added. Cells were removed from the dishes by scraping, and each dish was rinsed with an additional 0.5 ml of cold methanol. Chloroform (500 μl) and water (0.4 ml) were added to the pooled methanol washes to achieve a final solution of chloroform/methanol/water (5:10:4), and the samples were sonicated for 15 sec with a Kontes cell disrupter set at the maximal power setting. An additional 0.5 ml each of chloroform and water were added and the resulting two-phase solution was clarified by centrifugation at 1500 × *g* for 15 min at 4°.

[^{3}H]Ins1P was quantitated as described previously (5). Briefly stated, 1.6 ml of the upper (aqueous) phase was transferred to a column containing approximately 175 mg of anion exchange resin (Bio-Rad AG 1X8, 100–200 mesh, formate form). Columns were washed with 10 ml of water to remove [^{3}H]inositol. Labeled Ins1P was eluted with 8 ml of 200 mM ammonium formate/100 mM formic acid and was counted in an equal volume of Liquiscint (National Diagnostics). In some experiments, the lower phases of the cell extract were analyzed for [^{3}H]PtdIns as described previously (5).

In experiments where the synthesis of PtdIns was studied, unlabeled cell monolayers were washed and transferred to PSS as described above. Cellular inositol pools were labeled by incubating 1321N1 cells for 60 min with PSS containing [^{3}H]inositol (1 μCi/ml). Vehicle or drugs were then added for various times. Incubations were terminated, and [^{3}H]PtdIns was quantitated as described above.

Unidirectional $^{45}Ca^{2+}$ efflux assays. Unidirectional $^{45}Ca^{2+}$ flux assays were performed essentially as described by Brown *et al.* (21). For the efflux assays, cells were labeled with $^{45}Ca^{2+}$ by incubation with 1.0 ml of growth medium containing $^{45}CaCl_2$ (5 μCi/ml) (Amersham) for 18–20 hr. This period of labeling allowed the cellular $^{45}Ca^{2+}$ to reach isotopic equilibrium with exchangable calcium (achieved by 16 hr). To initiate the efflux assay, the growth medium containing extracellular $^{45}Ca^{2+}$ was removed by aspiration, monolayers were quickly (<8 sec) washed three times with 3 ml of PSS (37°) without $^{45}Ca^{2+}$, and PSS (37°) containing drugs was added. Incubations were terminated by rapidly washing the monolayers four times with 3 ml of a buffer containing lanthanum (118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 0.5 mM EDTA, 20 mM Hepes, and 5 mM $LaCl_3$, pH 7.4). Lanthanum was used in the termination buffer because it displaces extracellular $^{45}Ca^{2+}$ while it traps intracellular $^{45}Ca^{2+}$ (22, 23). Cells were solubilized in 0.5 ml of a solution containing 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 1 mM Hepes, and 3% (w/v) Triton X-100 and were removed from the plate by scraping. The solubilized cells were transferred into minivials, and dishes were rinsed with 0.5 ml of the solubilizing solution.

In each experiment, the data were normalized to the $^{45}Ca^{2+}$ present in cells that received the wash protocol without any intervening efflux interval. The time course data from some experiments were fit to a first-order exponential function with two kinetic components of efflux (21, 24):

$$\frac{A_t}{A_0} = \lambda_1 e^{(-k_1 t)} + \lambda_2 e^{(-k_2 t)}$$

where A_t is the cellular $^{45}Ca^{2+}$ present at time t; A_0 is the zero time cellular $^{45}Ca^{2+}$; and λ_1, λ_2, k_1, and k_2 are the first-order amplitudes and rate constants, respectively. Curve-fitting was performed with the noniterative least-squares algorithm of Marquardt (25) on a Tektronix 4052 minicomputer.

At short times (0–0.8 min), $^{45}Ca^{2+}$ efflux in the presence of a maximal concentration of carbachol approximated a first-order exponential with only a single kinetic component (See Fig. 9). To compare efflux responses to various concentrations of cholinergic agonists, we measured the cellular $^{45}Ca^{2+}$ after 0.5 min of efflux ($A_{0.5}$) and used this value to calculate a rate constant of efflux (k_1) according to the equation

$$k_1 = \frac{-2.303 \log(A_{0.5}/A_0)}{0.5 \text{ min}}$$

where A_0 is the zero time cellular $^{45}Ca^{2+}$.

Unidirectional $^{45}Ca^{2+}$ influx assays. Monolayers of unlabeled 1321N1 cells were washed and incubated as described under Assay Incubation Conditions. To initiate influx assays, $^{45}Ca^{2+}$ was added to the cells to a final concentration of 5 μCi/ml. Incubations were terminated, and cellular $^{45}Ca^{2+}$ was quantitated exactly as described for unidirectional $^{45}Ca^{2+}$ efflux studies. The data were analyzed as a first-order approach to equilibrium (21, 24):

$$\frac{A_\infty - A_t}{A_\infty} = \lambda_1 e^{(-k_1 t)} + \lambda_2 e^{(-k_2 t)}$$

where A_t is the cellular $^{45}Ca^{2+}$ at time t; A_∞ is the cellular $^{45}Ca^{2+}$ at isotopic equilibrium; and λ_1, λ_2 k_1, and k_2 are the first-order amplitudes and rate constants, respectively. A_∞ was determined from the cellular $^{45}Ca^{2+}$ in sister cultures incubated overnight with growth medium containing $^{45}Ca^{2+}$. The data were plotted as the natural log of $\frac{A_\infty - A_t}{A_\infty}$.

RESULTS

Inositol phospholipid hydrolysis was monitored by first labeling cellular phosphoinositides with [^{3}H[inositol and then measuring hormone-induced accumulation of [^{3}H]Ins1P in the presence of lithium. Hormonal stimulation of inositol phospholipid hydrolysis by a phospholipase C results in the formation of diacylglycerol and inositol phosphates. The inositol phosphates, which are normally

rapidly dephosphorylated, are trapped as Ins1P in the presence of lithium, an inhibitor of the enzyme that converts Ins1P to inositol (19, 20). In 1321N1 cells, the cholinergic agonist carbachol causes a large increase in the rate of [^{3}H]Ins1P accumulation (Fig. 1A). This finding indicates that carbachol stimulates phospholipase C hydrolysis of phosphoinositides. The observation that there is no change in the amount of [^{3}H]PtdIns in these cells during the 30-min exposure to carbachol (Fig. 1B) suggests that the increase in the metabolism of labeled phosphoinositides is balanced by a compensatory increase in [^{3}H]PtdIns synthesis.

Cholinergic stimulation of [^{3}H]Ins1P accumulation is antagonized by the muscarinic receptor antagonist atropine but not by the nicotinic receptor antagonist *d*-tubocurarine (Table 1). The apparent K_I for atropine inhibition of the phosphoinositide response is 3 nM (data not shown). The divalent cation ionophore A23187 does not mimic the effects of carbachol on [^{3}H]Ins1P accumulation (Table 1), although this concentration of ionophore (10 μM) does cause calcium mobilization (data not shown) and phosphodiesterase activation in these cells (18). These data indicate that the effect of carbachol on [^{3}H]Ins1P accumulation in 1321N1 cells is mediated through muscarinic receptors and that it is not secondary to receptor-mediated calcium mobilization.

A comparison of several cholinergic agonists demonstrates that acetylcholine and carbachol cause similar maximal increases in [^{3}H]Ins1P accumulation (Fig. 2). The K_{act} for acetylcholine is 1.5 μM and that for carbachol is 40 μM. Thus, acetylcholine is about 25 times more potent than carbachol regarding stimulation of inositol phospholipid hydrolysis. In contrast, the muscarinic agonists oxotremorine and pilocarpine have much smaller effects on [^{3}H]Ins1P accumulation (Fig. 2). The difference in the responses to carbachol and oxotremorine is not due to differences in their ability to access receptors, since 100 μM oxotremorine completely blocks the increase in [^{3}H]Ins1P accumulation caused by 100 μM carbachol (data not shown), and oxotremorine competes with high affinity for [^{3}H]quinuclidinyl benzilate binding sites in membranes from these cells (18).

To test the possibility that the [^{3}H]Ins1P response to oxotremorine is lost as a result of rapid desensitization or rapid agonist breakdown, the effect of oxotremorine on [^{3}H]Ins1P accumulation was examined at short times. Although carbachol (100 μM) significantly increases [^{3}H]Ins1P accumulation within 30 sec, oxotremorine (100

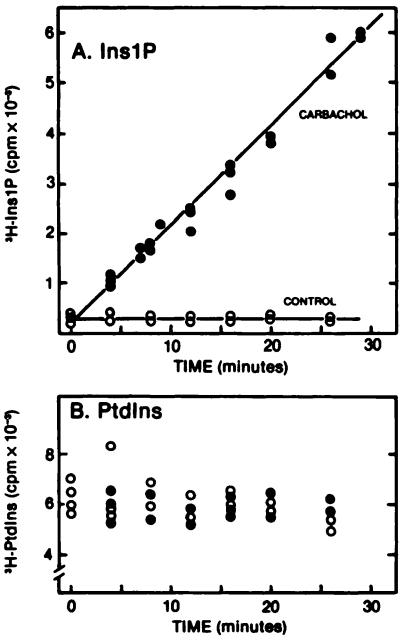

FIG. 1. *Time course of stimulation of phosphoinositide hydrolysis by carbachol*

Cultures of 1321N1 cells labeled overnight with [^{3}H]inositol were incubated with LiCl (control, ○) or with LiCl and 1 mM carbachol (carbachol, ●) for various times. Cellular [^{3}H]Ins1P and [^{3}H]PtdIns were quantitated as described under Experimental Procedures.

TABLE 1

Effects of antagonists and A23187 on phosphoinositide hydrolysis

Cultures of 1321N1 cells labeled overnight with [^{3}H]inositol were incubated as described under Experimental Procedures for 10 min with LiCl in the presence or absence of drug. The [^{3}H]Ins1P in each culture dish was determined as described under Experimental Procedures. The data are the means ± standard error of number of samples shown in parentheses. The average control value was 177 ± 27 cpm/dish. Atropine and *d*-tubocurarine alone had no efect on [^{3}H]Ins1P accumulation.

Treatment	[^{3}H]Ins1P
	% of control
Control	100 ± 3 (11)
Carbachol (100 μM)	618 ± 20 (6)
Carbachol (100 μM) + atropine (10 μM)	78 ± 4 (3)
Carbachol (100 μM) + *d*-tubocurarine (10 μM)	657 ± 29 (3)
A23187 (10 μM)	115 ± 10 (7)

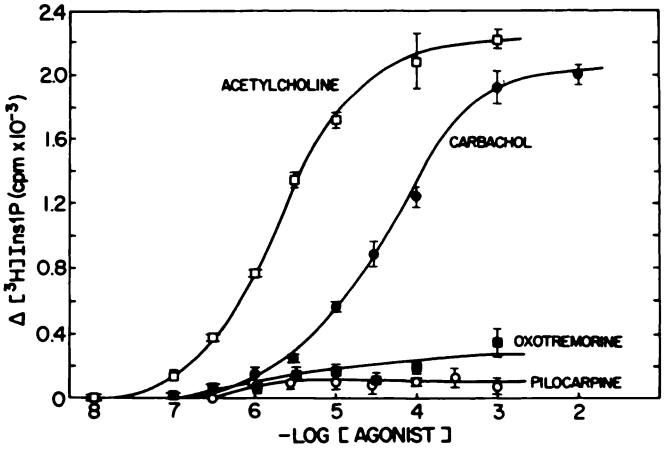

FIG. 2. *Differential agonist effects on phosphoinositide hydrolysis*

Cultures of 1321N1 cells labeled overnight with [^{3}H]inositol were incubated for 10 min with LiCl and various concentrations of acetylcholine (□), carbachol (●), oxotremorine (■), or pilocarpine (○). Cellular [^{3}H]Ins1P was determined as described under Experimental Procedures. The increase above control in counts per minute of [^{3}H]Ins1P per dish is plotted as a function of agonist concentration. Values represent means ± standard error for three to five samples. Oxotremorine, acetylcholine, and carbachol caused increases in [^{3}H]Ins1P accumulation that were significantly different from control ($p < 0.05$) when their effects were tested by one-way analysis of variance and the Dunnett test. Pilocarpine did not significantly increase [^{3}H]Ins1P accumulation.

μM) causes little stimulation of the rate of [^{3}H]Ins1P accumulation even at these short times (Fig. 3).

In most tissues an increase in the rate of PtdIns synthesis accompanies hormonal stimulation of inositol phospholipid breakdown. To monitor PtdIns synthesis, we added [^{3}H]inositol to previously untreated cells for a short period of time (60 min) and then examined the effects of agonists on [^{3}H]inositol incorporation into PtdIns (Fig. 4). Under these conditions, carbachol markedly increases [^{3}H]inositol incorporation into PtdIns whereas oxotremorine is virtually ineffective. These findings indicate that the disparate effects of oxotremorine and carbachol on the phosphoinositide response are not artifacts of the particular assay used to measured hormonal stimulation of phosphoinositide metabolism. There is a lag preceding the carbachol-induced increase in [^{3}H]inositol incorporation into PtdIns (Fig. 4). This is consistent with the idea that hormonal stimulation of PtdIns synthesis is secondary to stimulation of inositol phospholipid hydrolysis.

To examine the relationship between phosphoinositide hydrolysis and calcium mobilization, we studied $^{45}Ca^{2+}$ fluxes in 1321N1 cells. Carbachol stimulates unidirectional $^{45}Ca^{2+}$ efflux from cells equilibrated overnight with $^{45}Ca^{2+}$ (Fig. 5). Nonlinear least-squares analysis was used to fit the time courses of $^{45}Ca^{2+}$ efflux displayed in Fig. 5 to the biphasic exponential function described under Experimental Procedures. In the control situation, the fast component ($k_1 = 0.33$ min^{-1}) corresponds to 48% of the cellular $^{45}Ca^{2+}$ and the slow component represents 52%. In the presence of carbachol, the rate constant of the fast component of efflux (k_1) was increased to 1.31 min^{-1} and its amplitude was increased to 80% of the cellular $^{45}Ca^{2+}$. It thus appears from this simplified kinetic treatment that carbachol markedly increases both the efflux rate and the size of the rapidly exchanging component of $^{45}Ca^{2+}$.

We also examined the effects of carbachol on unidirectional $^{45}Ca^{2+}$ influx. Carbachol causes a small increase in $^{45}Ca^{2+}$ influx into 1321N1 cells (Fig. 6). Since cholinergic stimulation of unidirectional $^{45}Ca^{2+}$ efflux is much greater than its effect on $^{45}Ca^{2+}$ influx, we used the efflux response as an index of calcium mobilization in further studies.

Cholinergic stimulation of unidirectional $^{45}Ca^{2+}$ efflux is blocked by atropine and is not affected by *d*-tubocurarine (Fig. 7). The observation that this response is mediated through muscarinic, rather than nicotinic, receptors is important since activation of nicotinic receptors opens an ion channel that is permeable to calcium (21). However, it should be noted that activation of

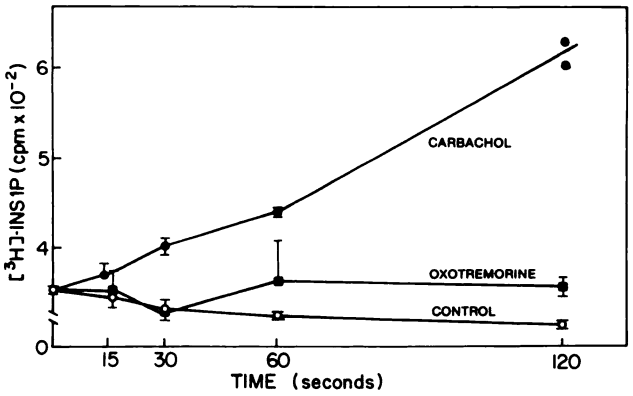

FIG. 3. *A comparison of oxotremorine and carbachol effects on phosphoinositide hydrolysis at short times*

Cultures of 1321N1 cells labeled overnight with [^{3}H]inositol were incubated with LiCl and drugs for short periods of time. Cellular [^{3}H]Ins1P was determined as described under Experimental Procedures. Values represent mean counts per minute of [^{3}H]Ins1P per dish ± standard error for three or four samples, or individual values for duplicate samples. Control, ○; 1 mM oxotremorine, □; 1 mM carbachol, ●).

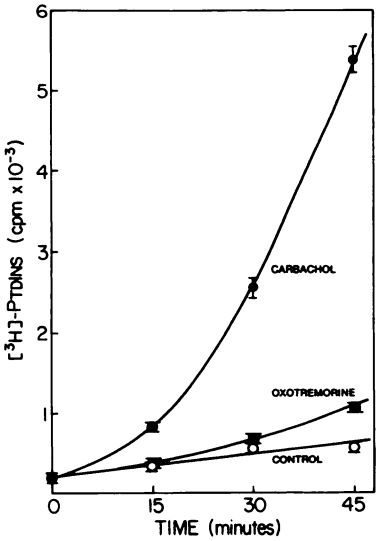

FIG. 4. *Carbachol and oxotremorine effects on [^{3}H]inositol incorporation into PtdIns*

Cultures of 1321N1 cells labeled for 60 min with [^{3}H]inositol were incubated with PSS and drugs. [^{3}H]PtdIns was quantitated as described under Experimental Procedures. Values represent the mean counts per minute of [^{3}H]PtdIns per dish ± standard error for three or four samples. Control, ○; 1 mM oxotremorine, ■; 1 mM carbachol, ●.

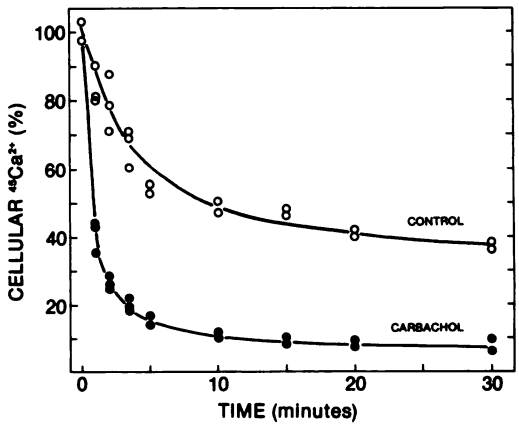

FIG. 5. *Effects of carbachol on unidirectional $^{45}Ca^{2+}$ efflux*

Cultures of 1321N1 cells equilibrated overnight with $^{45}Ca^{2+}$ were washed free of extracellular $^{45}Ca^{2+}$ and were incubated for various times in PSS with (●) or without (○) carbachol (100 μM). Incubations were terminated and cellular $^{45}Ca^{2+}$ was determined as described under Experimental Procedures. Values represent the percentages of time zero cellular $^{45}Ca^{2+}$ (15,500 cpm/dish).

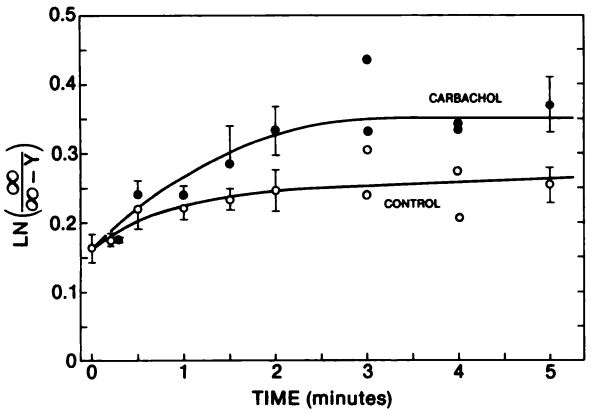

FIG. 6. *Effects of carbachol on unidirectional $^{45}Ca^{2+}$ influx*

Cultures of 1321N1 cells were incubated for various times with $^{45}Ca^{2+}$ in the absence (○) or the presence (●) of 1 mM carbachol. Incubations were initiated and terminated, and cellular $^{45}Ca^{2+}$ was quantitated as described under Experimental Procedures. Values represent means ± standard error for three to six determinations or individual values for duplicate samples. The effect of carbachol was significantly different from control ($p < 0.01$) when the counts per minute of cellular $^{45}Ca^{2+}$ per dish were analyzed by two-way analysis of variance.

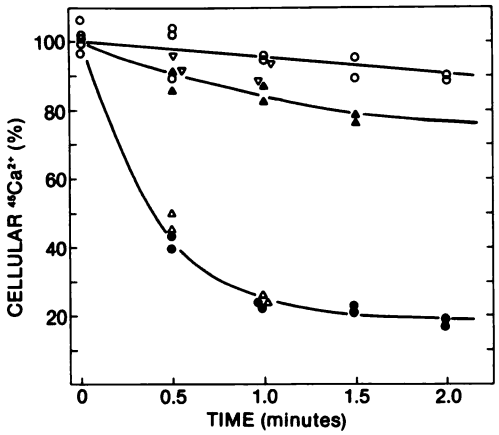

FIG. 7. *Effects of cholinergic antagonists on unidirectional $^{45}Ca^{2+}$ efflux*

Cultures of 1321N1 cells were treated as described in Fig. 5. *d*-Tubocurarine alone did not increase the rate of $^{45}Ca^{2+}$ efflux. Control, ○; 10 μM atropine, ▽; 10 μM atropine and 100 μM carbachol, ▲; 10 μM *d*-tubocurarine and 100 μM carbachol, △; 100 μM carbachol, ●.

nicotinic receptors should allow calcium to flow down its concentration gradient, and this would be reflected as a large increase in unidirectional $^{45}Ca^{2+}$ influx and a relatively small increase in $^{45}Ca^{2+}$ efflux.

A comparison of the responses to carbachol and oxotremorine shows that the effect of oxotremorine on unidirectional $^{45}Ca^{2+}$ efflux is smaller than that caused by an equivalent concentration of carbachol (Fig. 8). In order to compare the concentration-effect relationships for several agonists, it was necessary to define a time when the $^{45}Ca^{2+}$ efflux response is under initial rate conditions. The log of the cellular $^{45}Ca^{2+}$ content in the presence of a maximal concentration of carbachol, expressed as a percentage of time zero cellular $^{45}Ca^{2+}$, is a linear function of time for at least 0.85 min (Fig. 9). When the time course data for carbachol shown in Fig.

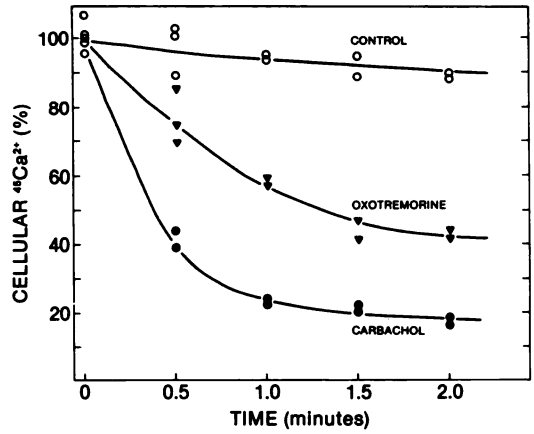

FIG. 8. *Comparison of oxotremorine and carbachol effects on unidirectional $^{45}Ca^{2+}$ efflux*

Cultures of 1321N1 cells were treated as described in Fig. 5. Control, ○; 100 μM oxotremorine, ▼; 100 μM carbachol ●.

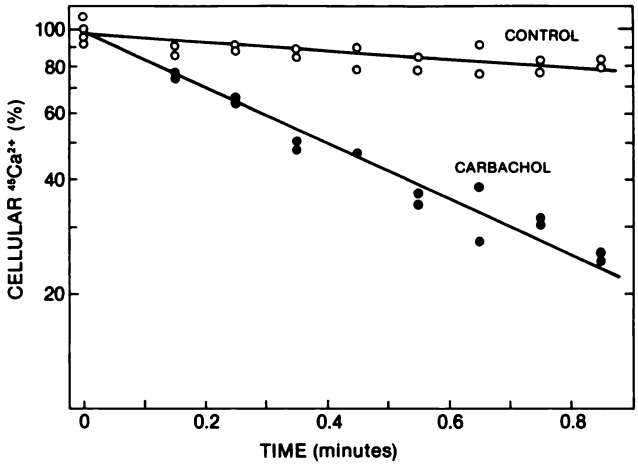

FIG. 9. *Kinetics of muscarinic stimulation of $^{45}Ca^{2+}$ efflux*

Cultures of 1321N1 cells were treated as described in Fig. 5. Values represent the percentages of time zero cellular $^{45}Ca^{2+}$ for cultures incubated in the absence (○) or the presence (●) of 1 mM carbachol.

9 are fitted to the biphasic exponential function described under Experimental Procedures, the amplitude of the fast component (λ_1) is 100% of the cellular $^{45}Ca^{2+}$. Thus, the unidirectional $^{45}Ca^{2+}$ efflux stimulated by carbachol approximates a single component of efflux for up to 0.8 min. To generate concentration-response data for muscarinic agonists, cellular $^{45}Ca^{2+}$ was measured 0.5 min after drug addition, and a rate constant of $^{45}Ca^{2+}$ efflux was calculated as described under Experimental Procedures.

The relative effects of different cholinergic agonists on unidirectional $^{45}Ca^{2+}$ efflux (Fig. 10) are strikingly similar to their relative effects on phosphoinositide hydrolysis (Fig. 2). Acetylcholine and carbachol cause comparable maximal increases in the rate of $^{45}Ca^{2+}$ efflux, whereas oxotremorine and pilocarpine cause much smaller maximal rate increases. The K_{act} for acetylcholine and carbachol are 0.7 μM and 13 μM, respectively. Thus, acetylcholine is about 20-fold more potent than carbachol for stimulation of unidirectional $^{45}Ca^{2+}$ efflux, just as it is about 25-fold more potent than carbachol for stimulation of phosphoinositide hydrolysis. The K_{act} val-

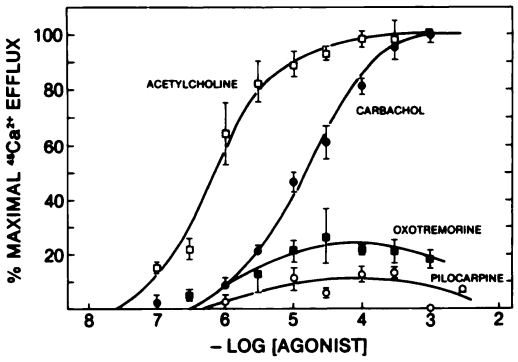

FIG. 10. *Differentiation agonist effects on unidirectional $^{45}Ca^{2+}$ efflux* Cultures of 1321N1 cells equilibrated overnight with $^{45}Ca^{2+}$ were incubated for 0.5 min with different concentrations of acetylcholine (□), carbachol (●), oxotremorine (■), or pilocarpine (○). Rate constants of efflux (k_1) were calculated as described under Experimental Procedures. The control $^{45}Ca^{2+}$ efflux rate (0.21 ± 0.06 min^{-1}) was substracted, and the percentage maximal response was calculated by normalization to the $^{45}Ca^{2+}$ efflux rate caused by 10 mM carbachol (1.45 ± 0.15 min^{-1}). Values are means ± standard error of 3–11 samples. All four agonists caused decreases in $A_{0.5}$ (cellular $^{45}Ca^{2+}$ at t = 0.5 min) that were significantly different from control ($p < 0.05$) when their effects were tested by one-way analysis of variance and the Dunnett test.

ues of these two agonists for the $^{45}Ca^{2+}$ efflux response are 2- to 3-fold lower than their respective K_{act} values for stimulation of phosphoinositide hydrolysis.

DISCUSSION

A variety of hormone receptors, including the muscarinic receptor, are linked to the metabolism of PtdIns and the polyphosphoinositides, PtdIns 4P and PtdIns 4,5P_2 (1–5). Interest has recently focused on the possible second messenger role of this linkage in hormonally mediated calcium mobilization. We have used the differential effects of several cholinergic agonists to explore the relationship between phosphoinositide metabolism and calcium mobilization in a homogeneous cell culture system.

Activation of muscarinic receptors on 1321N1 cells labeled with [^{3}H]inositol induces a large increase in Ins1P formation. This effect can be most readily observed when lithium is used to inhibit the enzyme that normally converts Ins1P to inositol, although the hormonal increase in [^{3}H]Ins1P also can be measured in the absence of lithium.[6] [^{3}H]Ins1P formation is an indicator of phospholipase C hydrolysis of phosphoinositides, since this labeled metabolite can only arise from phospholipase C hydrolysis of PtdIns or the polyphosphoinositides. In some systems, there is evidence that the polyphosphoinositides are the primary phospholipase C substrates in hormonal responses (15, 26–28). This may also be the case in 1321N1 cells, since preliminary data from our laboratory show that carbachol stimulates the formation of inositol-bisphosphate and inositol-trisphosphate, phospholipase C hydrolysis products of the polyphosphoinositides.[6]

PtdIns 4P and PtdIns 4,5P_2 are present in only small quantities in cells (usually 1–5% of the cellular PtdIns) (1). When these small pools of phospholipids are depleted, they must be replenished by phosphorylation of their precursor, PtdIns. It is therefore likely that the large quantity of [^{3}H]Ins1P formed in 1321N1 cells in the presence of carbachol is ultimately derived from PtdIns. Since the amount of [^{3}H]PtdIns in 1321N1 cells is not decreased by carbachol, metabolized PtdIns must be resynthesized rapidly from intracellular pools of [^{3}H] inositol. This possibility is further supported by our observation that carbachol markedly stimulates PtdIns synthesis. Thus, it appears that in 1321N1 cells carbachol stimulates a cycle of inositol phospholipid metabolism that is initiated by increased inositol phospholipid hydrolysis and sustained by increased inositol phospholipid synthesis.

Muscarinic receptor activation in 1321N1 cells causes a striking increase in unidirectional $^{45}Ca^{2+}$ efflux and a much smaller increase in unidirectional $^{45}Ca^{2+}$ influx. These observations suggest that the net effect of muscarinic receptor stimulation is calcium efflux from the cell. This idea is further supported by our preliminary finding that carbachol causes a net decrease in cellular $^{45}Ca^{2+}$ content under isotopic equilibrium labeling conditions.[6] The increase in calcium efflux could be secondary to an elevation in cytosolic free calcium caused by the release of internal calcium pools. Alternatively, the efflux could be due to direct stimulation of active extrusion of calcium. We favor the former explanation because muscarinic agonists appear to activate a phosphodiesterase by increasing the concentration of intracellular calcium (18).

The relationship between hormonally stimulated phosphoinositide hydrolysis and mobilization of calcium by the same hormone is poorly defined. It is unlikely that the inositol phospholipid response in 1321N1 cells is secondary to calcium mobilization, because the phosphoinositide response is not mimicked by the divalent cation ionophore A23187. In situations where the major site of hormonally stimulated calcium release is from intracellular sites, there must be a messenger that carries the signal of cell-surface receptor activation to the intracellular site of calcium release. The nature of this intracellular messenger is unknown. One theory is that one of the metabolites in the phosphoinositide cycle serves as the intracellular signal for calcium mobilization (1–4). Although the putative messenger has not been unequivocally identified, there is recent evidence that micromolar concentrations of inositol-trisphosphate can release calcium from within permeabilized cells (10, 11).

Our results from experiments with various cholinergic agonists support the idea that the inositol phospholipid response participates in calcium mobilization. The magnitude of the calcium mobilization response corresponds to that of the phosphoinositide response for a variety of cholinergic agonists. Acetylcholine and carbachol cause comparable maximal phosphoinositide and calcium mobilization responses, whereas oxotremorine and pilocarpine are partial agonists that cause submaximal inositol phospholipid and calcium mobilization responses. It is noteworthy that, in contrast to the situation in hepato-

[6] S. B. Masters, and J. H. Brown, manuscript submitted for publication.

cytes (3, 15) or in the blowfly salivary gland (29), there is little difference between agonist K_{act} values for eliciting phosphoinositide and calcium responses in 1321N1 cells. The similarity of the K_{act} values and the relative efficacies of agonists for causing phosphoinositide and $^{45}Ca^{2+}$ efflux responses suggest that in these cells maximal increases in calcium efflux would occur only when phosphoinositide hydrolysis is maximally stimulated.

A response that appears to be more distal to muscarinic receptor activation in 1321N1 cells is calcium-dependent activation of a cyclic nucleotide phosphodiesterase (18). This muscarinic effect results in attenuation of hormonally stimulated cyclic AMP accumulation. In contrast to the differential effects of carbachol and oxotremorine on both [^{3}H]Ins1P accumulation and unidirectional $^{45}Ca^{2+}$ efflux in 1321N1 cells, oxotremorine is as efficacious as carbachol for inhibiting isoproterenol-stimulated cyclic AMP accumulation (18). Preliminary data have demonstrated that changes in cyclic AMP metabolism in these cells are triggered at a concentration of carbachol (30) that causes relatively little phosphoinositide hydrolysis and calcium mobilization. These findings suggest that the phosphodiesterase is sensitive to relatively small increases in the concentration of intracellular calcium. Another possibility is that there is a synergistic effect of the phosphoinositide and the calcium mobilization responses on phosphodiesterase activation. In platelets, for example, it has been shown that submaximal increases in intracellular calcium and activation of the phospholipid-dependent protein kinase have synergistic effects on platelet activation responses (31, 32). Analogously, the modest phosphoinositide response elicited by oxotremorine in 1321N1 cells may provide sufficient diacylglycerol for activation of a phospholipid-dependent protein kinase. The combination of this and a small calcium mobilization response may cause maximal changes in phosphodiesterase activation.

The data presented here demonstrate that stimulation of $^{45}Ca^{2+}$ efflux by different muscarinic agonists closely parallels their effects on phosphoinositide hydrolysis. The most parsimonious explanation for these results is that stimulation of phosphoinositide hydrolysis leads to calcium mobilization. The possibility that these are parallel and independent cellular responses cannot be dismissed, however, until methods for selectively inhibiting hormone-stimulated phosphoinositide hydrolysis are identified.

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