0026-895X/89/020195-10$02.00/0

MOLECULAR PHARMACOLOGY, 35:195-204



# Muscarinic Receptor Regulation of Cytoplasmic $Ca^{2+}$ Concentrations in Human SK-N-SH Neuroblastoma Cells: $Ca^{2+}$ Requirements for Phospholipase C Activation

STEPHEN K. FISHER, LISA M. DOMASK, and RICHARD M. ROLAND

*Neuroscience Laboratory (S.K.F., L.M.D., R.M.R.) and Department of Pharmacology (S.K.F.), University of Michigan, Ann Arbor, Michigan 48104-1687*

Received August 3, 1988; Accepted November 16, 1988

## SUMMARY

The relationship between muscarinic receptor-mediated inositol lipid hydrolysis and the generation of $Ca^{2+}$ signals has been examined in human SK-N-SH neuroblastoma cells. The resting cytoplasmic calcium concentration ($[Ca^{2+}]_i$) as determined by fura-2 fluorescence measurements was 59 ± 2 nM. Upon the addition of oxotremorine-M, there was a 4-fold increase in $[Ca^{2+}]_i$ (293 ± 18 nM), with half-maximal stimulation obtained at an agonist concentration of 8 μM, a value similar to that previously observed for the enhancement of phosphoinositide hydrolysis. Addition of partial muscarinic agonists for phosphoinositide turnover (bethanechol, oxo-2, and arecoline) elicited correspondingly smaller increases in $[Ca^{2+}]_i$ than did oxotremorine-M. Inclusion of EGTA lowered the basal $[Ca^{2+}]_i$ within 2 min and markedly reduced (> 60%) the magnitude of the agonist-induced rise in $[Ca^{2+}]_i$. Addition of muscarinic agonists to SK-N-SH cells that had been prelabeled with [$^3$H]inositol led to the rapid (5–15 sec) release of inositol mono-, bis-, and trisphosphates. When assayed under conditions similar to those employed for the fluorescence measurements, EGTA also inhibited both the basal and oxotremorine-M-stimulated release of inositol phosphates by 45–61%. Conversely, ionomycin both elevated $[Ca^{2+}]_i$ and stimulated the release of inositol phosphates. The addition of $Ca^{2+}$ (10 nM–2 μM) to digitonin-permeabilized cells directly stimulated the release of labeled inositol mono-, bis-, and trisphosphates by 3–4-fold with a half-maximal effect ($EC_{50}$) observed at 145 nM free $Ca^{2+}$ ($Ca^{2+}_f$). A further (6-fold) calcium-dependent increase in inositol phosphate release was obtained by inclusion of either guanosine-5-*O*-(3-thio)-trisphosphate (GTPγS) or oxotremorine-M. In the combined presence of agonist and GTPγS, a synergistic release of all three inositol phosphates occurred, with half-maximal stimulation observed at 35–40 nM $Ca^{2+}_f$, a value similar to the $[Ca^{2+}]_i$ in quiescent cells. These results indicate (i) that the magnitude of the initial rise in $[Ca^{2+}]_i$ is directly related to the production of phosphoinositide-derived second messenger molecules and (ii) that the phospholipase C-mediated breakdown of inositol lipids in SK-N-SH cells is particularly sensitive to regulation by physiologically relevant $Ca^{2+}$ concentrations. It is concluded that, in SK-N-SH cells, either an elevation above or reduction below basal $[Ca^{2+}]_i$ can modulate the extent of hydrolysis of inositol lipids and the subsequent generation of calcium signals.

Neurotumor cells provide a valuable alternative to the more complex preparations such as brain slices or synaptosomes for studies related to the coupling of mAChRs to functional effectors in the nervous system (1, 2). One such biochemical consequence of mAChR activation that is frequently observed both in the central nervous system and in tumor cells of neural origin is the phospholipase C-mediated hydrolysis of inositol phospholipids (for review see Ref. 3). Although it is known that one of the products, namely $IP_3$, can mobilize an intracellular pool of $Ca^{2+}$ (4), the precise relationship between the extent of mAChR-stimulated PPI turnover and the magnitude of the rise in cytoplasmic calcium ($Ca^{2+}_i$) has been technically difficult to assess in the neuroblastoma cell lines employed thus far, because the calcium signals generated after receptor activation are often minimal (5–7).

Recently, we have described some of the properties of a human neuroblastoma cell line, SK-N-SH, that possesses a relatively high density of mAChRs (500 fmol/mg of protein)

This work was supported by National Institutes of Health Grant NS 23831 and National Institute of Mental Health Grant MH 42652.

**ABBREVIATIONS:** mAChR, muscarinic acetylcholine receptor; PPI, phosphoinositide; $IP_1$, D-myo-inositol monophosphate; $IP_2$, D-myo-inositol bisphosphate; $IP_3$, D-myo-inositol trisphosphate; $IP_4$, D-myo-inositol tetrakisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[Ca^{2+}]_i$, concentration of cytoplasmic $Ca^{2+}$; $[Ca^{2+}]_f$, concentration of free $Ca^{2+}$; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N*-tetraacetic acid; fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-amino-benzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N*-tetraacetic acid; fura-2/AM, pentaacetoxymethyl ester derivative of fura-2; quin2, 2-{[2-bis(carboxymethyl)-amino-5-methyl phenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline; quin2/AM, tetraacetoxymethyl ester derivative of quin2; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); GDPβS, guanosine-5′-*O*-(2-thiodiphosphate); TCA, trichloroacetic acid; 2,3-DPG, 2,3-bisphosphoglyceric acid.

that are coupled to PPI turnover (8, 9). Based upon pharmacological criteria (10), mAChRs on these cells have been classified as belonging to the $M_3$ subtype (11). In addition, these receptors exhibit a higher molecular weight than those in either the cerebral cortex or heart (11–13) and may represent the $m_3$/$HM_4$ subtype described from molecular cloning studies (14, 15). In the present study, we demonstrate that exposure of SK-N-SH cells to muscarinic agonists results in a pronounced increase in $[Ca^{2+}]_i$ (up to 5-fold), the extent of which is closely linked to the magnitude of PPI hydrolysis elicited by the particular agonist. A further observation is that both calcium signaling and inositol lipid turnover in intact cells are markedly influenced by inclusion of either the calcium chelator EGTA or the calcium ionophore ionomycin. Using digitonin-permeabilized cells, we demonstrate that the increase in phospholipase C activity mediated by addition of either a muscarinic agonist or GTPγS, a nonhydrolyzable analog of GTP, is regulated by nanomolar concentrations of free calcium. Thus, in SK-N-SH cells, the enzymic machinery that underlies the generation of intracellular calcium signals may itself be particularly sensitive to changes in $[Ca^{2+}]_i$ in the physiological range.

## Materials and Methods

myo-[2-$^3$H]Inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). [$^3$H]*N*-Methylscopolamine (80 Ci/mmol) and [$^3$H]inositol-1,4,5-trisphosphate (20 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Atropine, carbamoylcholine, digitonin, ATP, bethanechol, arecoline, pilocarpine, and 2,3-DPG were obtained from Sigma Chemical Co. (St. Louis, MO). Fura-2, quin2/AM, and ionomycin were obtained from Calbiochem (La Jolla, CA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR). GTPγS and GDPβS were obtained from Boehringer-Manheim (Indianapolis, IN). Oxotremorine-M was obtained from Research Biochemicals (Waltham, MA). Tissue culture supplies were obtained from Corning Glass Works (Corning, NY). Powdered Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from GIBCO (Grand Island, NY). Dowex-1 (100–200 mesh; ×8 in the formate form) was obtained from Bio-Rad (Rockville Center, NY). The source of SK-N-SH neuroblastoma cells was as previously described (8).

### Cell Culture Conditions

Human SK-N-SH neuroblastoma cells were cultured under conditions that have previously been described (8). In the majority of experiments, 10- to 14-day-old cells were utilized. Cells were removed from the tissue culture dishes by the addition of Puck's $D_1$ solution (16), collected by centrifugation, and resuspended in buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose, and 30 mM $Na^+$ HEPES buffer, pH 7.4).

### Radioligand Binding Assays

The binding of [$^3$H]NMS to mAChRs on intact SK-N-SH cells was monitored as previously described (9).

### Measurement of Cytoplasmic Calcium Concentrations

Intracellular $Ca^{2+}$ concentrations were determined by the use of the fluorescent $Ca^{2+}$ indicators, quin2 or fura-2. SK-N-SH cells, suspended in 10 ml of buffer A at a concentration of approximately 3 mg of protein/ml, were loaded with either 1 μM fura-2/AM or 25 μM quin2/AM for 15 min at 37°. The loaded cells were then diluted to 40 ml with buffer A, collected by centrifugation at 500 × g for 60 sec and resuspended in an additional 10 ml of buffer A. The centrifugation step was then repeated and cells were resuspended in buffer A at a concentration of approximately 4 mg of protein/ml. Before measurement of fluorescence, fura-2 and quin2-loaded cells were allowed to remain at room temperature. Only minimal amounts of fura-2 and quin2 leaked from dye-loaded cells that had been extensively washed in buffer A and then maintained at this lower temperature. Fluorescence measurements were made on 1-ml volumes of SK-N-SH cells constantly stirred and maintained at 37°. The fluorometer used was an Aminco-Bowman spectrophotofluorometer ($\lambda_{excitation}$ = 340 nm; $\lambda_{emission}$ = 490 nm), interfaced to both a strip chart recorder and an LED readout, from which quantitative fluorescence values were obtained. After measurement of the fluorescence ($F$), cells were lysed by the addition of 100 μM digitonin, Tris base was added (pH > 8), and a minimum fluorescence value ($F_{min}$) was obtained after chelation of $Ca^{2+}$ by the addition of 20 mM EGTA. A maximum fluorescence value ($F_{max}$) was then obtained after the addition of 20 mM $CaCl_2$. Autofluorescence ($F_{auto}$) was monitored by the addition of 20 mM $MnCl_2$. Intracellular cytoplasmic calcium concentrations ($[Ca^{2+}]_i$) were calculated from the equation $[Ca^{2+}]_i = (F - F_{min}/F_{max} - F) \times K_d$ (Ref. 15), where $K_d$ is the affinity of either fura-2 or quin2 for $Ca^{2+}$ (assumed to be 224 nM and 115 nM, respectively, at 37°, see Refs. 17 and 18). The relationship between $F_{min}$, $F_{auto}$, and $F_{max}$ is given by the equation $F_{min} = (F_{max} - F_{auto})\beta$, where $\beta$ = 0.27 and 0.16 for fura-2 and quin2, respectively. In some experiments, we monitored $[Ca^{2+}]_i$ in fura-2-loaded cells, using the dual wavelength method of Grynkiewicz *et al.* (18) by manual switching of the excitation wavelengths. Under these conditions, $[Ca^{2+}]_i = (R - R_{min}/R_{max} - R)B \cdot K_d$, where $R$, $R_{min}$, and $R_{max}$ are the ratios of the fluorescence obtained at 340 nm and 380 nm, correlated for autofluorescence. $B$ is the ratio of fluorescence of $Ca^{2+}$ free/$Ca^{2+}$ saturated fluorescence signals at 380 nm. For the purpose of $[Ca^{2+}]_i$ calculations, it was assumed that a single homogeneous population of SK-N-SH cells was present and that the changes induced in $[Ca^{2+}]_i$ were synchronous.

### Measurement of (PPI) Turnover

**Intact cells.** SK-N-SH cells were allowed to prelabel for 3 days at 37° in Dulbecco's modified Eagle's medium/10% fetal calf serum containing 7.5 μCi/ml [$^3$H]inositol, in an atmosphere of 90% air/10% $CO_2$. Cells were detached in Puck's $D_1$ solution prewarmed to 37°, centrifuged, and washed twice in an equal volume of buffer A. Labeled cells were then resuspended in buffer A and incubated for 30 min at 37° in the same buffer containing 10 mM LiCl and either oxotremorine-M, EGTA, and/or ionomycin (8). Reactions were terminated by the addition of an equal volume (0.5 ml) of 20% TCA. The TCA extract was washed with five 2-ml aliquots of $H_2O$-saturated diethyl ether and neutralized with $KHCO_3$. A total inositol phosphate fraction was quantitated as previously described (8). In other experiments, the individual inositol phosphates ($IP_4$, $IP_3$, $IP_2$, and $IP_1$) were fractionated by anion-exchange chromatography (19, 20). $IP_3$ and $IP_4$ were separated by the methods of either Batty *et al.* (21) or Takazawa *et al.* (22). Recovery of labeled inositol phosphates after acid extraction was > 80%. In some experiments, incorporation of [$^3$H]inositol into the lipid fraction was monitored as previously described (20).

**Permeabilized cells.** After the prelabeling period, cells were detached from the dishes with Puck's $D_1$ solution, washed twice with $D_1$ solution, and then resuspended in KGEH buffer (139 mM $K^+$ glutamate, 2 mM ATP, 4 mM $MgCl_2$, 10 mM LiCl, 10 mM EGTA, and 30 mM $Na^+$ HEPES buffer, pH 7.4) containing 20 μM digitonin, as described by Eberhard and Holz (23). Cells were allowed to permeabilize for 5 min at 37°, at a protein concentration of approximately 3–4 mg/ml. Permeabilized cells were centrifuged and washed with an equal volume of KGEH buffer (minus digitonin) and then resuspended in the same buffer. Incubations were routinely allowed to continue for 15 min at 37°. The required free $Ca^{2+}$ concentrations ($Ca^{2+}_f$) were achieved by the addition of various amounts of $CaCl_2$ to the KGEH buffer containing 10 mM EGTA, and the *actual* $[Ca^{2+}]_f$ was monitored by the addition of 20 μM fura-2 ($K^+$ salt). Reactions were terminated by rapid cooling to 0°, followed by centrifugation at 4000 × *g* for 5 min. Supernatants were collected and the release of labeled inositol phosphates from the cells was monitored after the addition of an equal volume of 20% TCA. The procedures for extraction, separation, and quantitation of inositol phosphates were the same as those used for intact cells. Protein was determined by the method of Geiger and Bessman (24).

### Data analysis

Values quoted are means ± standard errors for the number of observations stated in parentheses. Where single traces of changes in

$[Ca^{2+}]_i$ are shown, similar results were obtained from at least three separate experiments. Student's one- or two-tailed *t*-tests were used to evaluate the statistical differences of the means of paired and unpaired sets of data.

## Results

**Measurement of cytoplasmic $Ca^{2+}$ concentrations in quiescent and stimulated SK-N-SH cells using quin2 or fura-2 fluorescence.** In initial studies, both basal and stimulated $[Ca^{2+}]_i$ values in SK-N-SH cells were monitored using either quin2 or fura-2 fluorescent probes. The basal $[Ca^{2+}]_i$ value for quin2-loaded cells was 44 ± 5 nM ($n$ = 18), whereas that for cells loaded with fura-2 was 59 ± 2 nM ($n$ = 96). Using dual wavelength measurements ($\lambda_{340}$:$\lambda_{380}$ nm) for fura-2-loaded cells, a resting $[Ca^{2+}]_i$ value of 37 ± 4 nM ($n$ = 8) was obtained. Thus, quiescent SK-N-SH cells appear to maintain a relatively low $[Ca^{2+}]_i$ of approximately 50 nM. When SK-N-SH cells were exposed to 1 mM oxotremorine-M, (a quaternary $N^+$ analog of oxotremorine) (25) both the magnitude and duration of the $Ca^{2+}$ signal were found to be dependent upon the probe used. Agonist addition to fura-2-loaded cells resulted in a rapid (~5 sec) increase $[Ca^{2+}]_i$ to a value of 293 ± 18 nM ($n$ = 20). This increase was transient and was followed by a decline within 2 min to a steady $[Ca^{2+}]_i$ (plateau phase), which was approximately 70% higher than basal $[Ca^{2+}]_i$. This elevated $[Ca^{2+}]_i$ persisted for as long as the agonist occupied the mAChR (Fig. 1A). The peak $[Ca^{2+}]_i$ achieved in quin2-labeled cells was smaller (180 ± 23 nM; $n$ = 9), whereas the increase in $[Ca^{2+}]_i$ was sustained over a longer period (Fig. 1B). The smaller increase in $[Ca^{2+}]_i$ monitored by quin2 fluorescence, in addition to the slower time-course for decline of the $Ca^{2+}$ signal, may reflect the higher intracellular concentration of free quin2 and its greater buffering capacity for $Ca^{2+}$ (17, 18). Accordingly, we selected fura-2 for routine monitoring of changes in $[Ca^{2+}]_i$ in SK-N-SH cells.

**Relationship between agonist concentration and rise in $[Ca^{2+}]_i$.** Concentrations of oxotremorine-M as low as 1 μM were observed to elicit an increase in $[Ca^{2+}]_i$ with a maximum response observed at a 1 mM concentration (Fig. 2). At each agonist concentration, a peak value of $[Ca^{2+}]_i$ was observed, which was followed by a decline to a plateau $[Ca^{2+}]_i$ higher than the resting $[Ca^{2+}]_i$. The magnitude of the initial rise in $[Ca^{2+}]_i$ was dependent upon agonist concentration, whereas the plateau $[Ca^{2+}]_i$ achieved was the same for agonist concentrations of 1 μM–1 mM. The rate of decline of $[Ca^{2+}]_i$ from its peak value appeared to be more rapid in the presence of high agonist concentrations. The collective data for peak and plateau $[Ca^{2+}]_i$ levels obtained from a number of experiments are shown in Fig. 3. The half-maximal increase ($EC_{50}$) in peak $[Ca^{2+}]_i$ elicited by the addition of oxotremorine-M was approximately 8 μM, a value similar to that previously obtained for stimulation of PPI turnover in these cells (2.5 μM) (8). A comparable value for the plateau phase of the calcium signal could not be calculated but was less than 1 μM. The dissociation constant for oxotremorine-M binding to the mAChR on SK-N-SH cells was 75 ± 17 μM ($n$ = 3). Thus, both the rise in $[Ca^{2+}]_i$ and stimulated PPI turnover are observed at agonist concentrations that are somewhat lower than those required to occupy all available mAChR sites.

**Comparison of changes in $[Ca^{2+}]_i$ induced by full and partial muscarinic agonists for PPI turnover.** We have previously shown that muscarinic agonists differ widely in their ability to promote PPI hydrolysis in SK-N-SH cells (8). These differences are also manifested in the magnitude of the initial rise in $[Ca^{2+}]_i$. Addition of agonists such as carbamoylcholine or oxotremorine-M, known to maximally stimulate PPI hydrolysis, resulted in a 4–5-fold increase in $[Ca^{2+}]_i$. In contrast, partial agonists for PPI turnover, such as oxo-2, bethanechol, or arecoline are markedly less effective (Fig. 4). The addition of either pilocarpine or McN-A-343 did not elicit a measureable

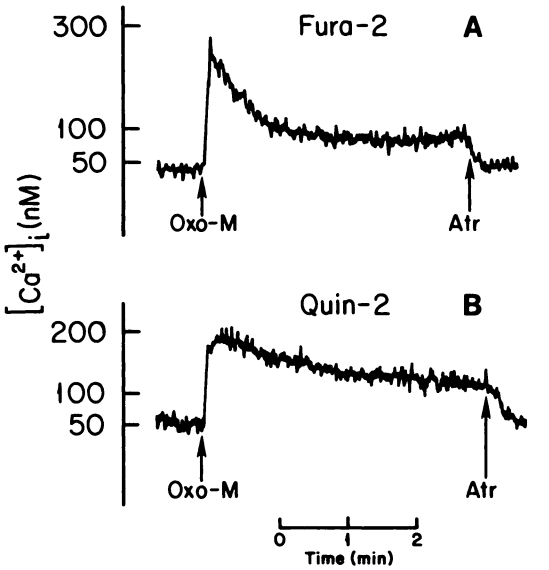


**Fig. 1.** Comparison of the effect of oxotremorine-M on $[Ca^{2+}]_i$ in SK-N-SH cells, as monitored by either fura-2 or quin2 fluorescence. Cells were first loaded with either 1 μM fura-2/AM or 25 μM quin2/AM for 15 min at 37°. At the times indicated, the cells were exposed to 1 mM oxotremorine-M (*Oxo-M*) or 10 μM atropine (*Atr*).

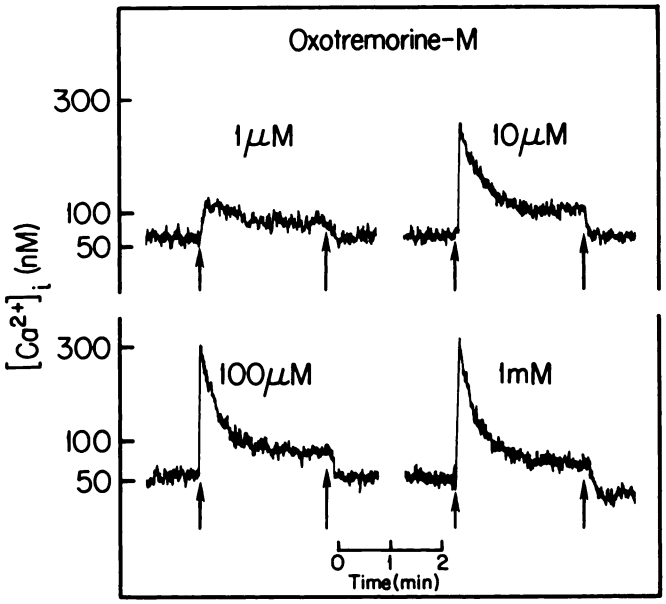


**Fig. 2.** Dependence of the rise in $[Ca^{2+}]_i$ on oxotremorine-M concentration. For each *trace*, the *initial arrow* indicates the point of agonist addition, whereas the *second arrow* indicates the addition of atropine (10 μM). The peak $[Ca^{2+}]_i$ values achieved with 1 μM, 10 μM, 100 μM, and 1 mM oxotremorine-M were 107, 276, 290, and 316 nM, respectively. The corresponding plateau $[Ca^{2+}]_i$ values were 86, 100, 91, and 79 nM, respectively.

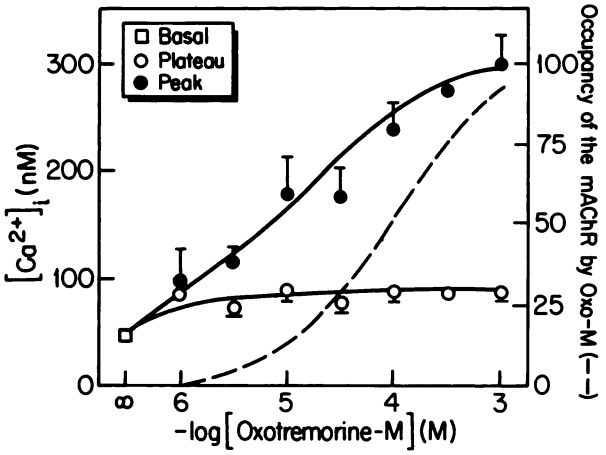


**Fig. 3.** Relationship between peak $[Ca^{2+}]_i$ and plateau $[Ca^{2+}]_i$ values elicited by the addition of oxotremorine-M, and occupancy of the mAChR by oxotremorine-M. Results shown are means ± standard errors for four or five separate experiments, except for data obtained at an agonist concentration of 300 μM, which was derived from a single experiment. The receptor occupancy curve was calculated from a $K_d$ value of 75 ± 17 μM ($n$ = 3) for oxotremorine-M displacement of [$^3$H]NMS from the mAChR on intact SK-N-SH cells.

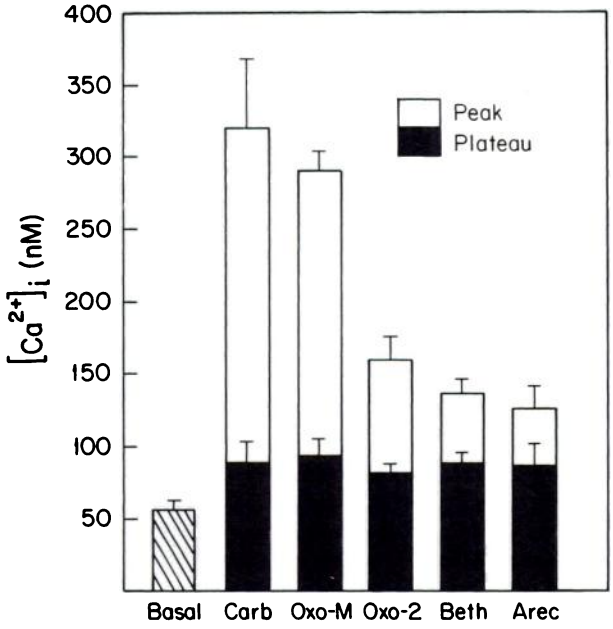


**Fig. 4.** Differential ability of muscarinic agonists to elicit increases in $[Ca^{2+}]_i$. To fura-2-loaded SK-N-SH cells was added either 1 mM oxotremorine-M (*Oxo-M*), 10 mM carbamoylcholine (*Carb*), 3 mM oxo-2 (*Oxo-2*), 10 mM bethanechol (*Beth*), or 1 mM arecoline (*Arec*). Both peak and plateau $[Ca^{2+}]_i$ values were monitored. Results shown are means ± standard errors for four separate experiments. The peak and plateau $[Ca^{2+}]_i$ values were different from basal values of $[Ca^{2+}]_i$ ($p < 0.01$).

increase in $[Ca^{2+}]_i$. The plateau $[Ca^{2+}]_i$ levels achieved for all agonists capable of increasing the $[Ca^{2+}]_i$ were essentially identical (82–93 nM). We also measured the latency between agonist addition and peak $[Ca^{2+}]_i$ levels for both full and partial agonists. The values obtained for carbamoylcholine, oxotremorine-M, oxo-2, bethanechol, and arecoline were 6 ± 1, 5 ± 1, 9 ± 1, 8 ± 3, and 7 ± 2 sec, ($n$ = 4 or 5). Thus, both full and partial agonists appear to maximally increase $[Ca^{2+}]_i$ within the same time-frame and, furthermore, no obvious differences in latency are observed for a lipophilic agonist (e.g., oxo-2, a dimethylamino analog of oxotremorine-M) (25) and a hydrophilic agonist (e.g., bethanechol). Once an increase in $[Ca^{2+}]_i$ had been elicited by the addition of either a full or partial agonist, the subsequent addition of the same agonist did not result in a further rise in $[Ca^{2+}]_i$. However, if SK-N-SH cells were first exposed to bethanechol, a partial agonist, the subsequent addition of oxotremorine-M resulted in a further rise in $[Ca^{2+}]_i$ (Fig. 5A). If oxotremorine-M was added first, the subsequent addition of bethanechol failed to increase $[Ca^{2+}]_i$ further (Fig. 5B). These results indicate that both full and partial agonists mobilize the same finite pool of cellular $Ca^{2+}$, but to different extents.

**Effect of EGTA on calcium signaling.** Reduction in the concentration of extracellular $Ca^{2+}$ to approximately 300 nM $Ca^{2+}$, by the addition of 4 mM EGTA resulted in three distinct consequences (Fig. 6). First, the resting $[Ca^{2+}]_i$ was reduced to 38 ± 5 nM ($n$ = 17) within 2 min for fura-2-loaded cells and a similar reduction was observed for quin2-loaded cells (17 ± 3 nM; $n$ = 4). This suggests that extracellular EGTA may cause the depletion of cytoplasmic $Ca^{2+}$, as previously noted for aortic smooth muscle cells (26). Second, the magnitude of the initial rise in $[Ca^{2+}]_i$ following the addition of oxotremorine-M to SK-N-SH cells was reduced to 128 ± 15 nM ($n$ = 9) from a normal value of approximately 250–300 nM. However, the ability of oxotremorine-M to elevate $[Ca^{2+}]_i$ was maintained even when the extracellular $[Ca^{2+}]_i$ was lowered to 70 nM by the addition of 10 mM EGTA. When SK-N-SH cells were incubated in $Ca^{2+}$-free buffer A in the absence of added EGTA, little or no reduction in the initial rise in $[Ca^{2+}]_i$ induced by the addition of oxotremorine-M was observed (data not shown). A third consequence of EGTA inclusion was the abolition of the plateau

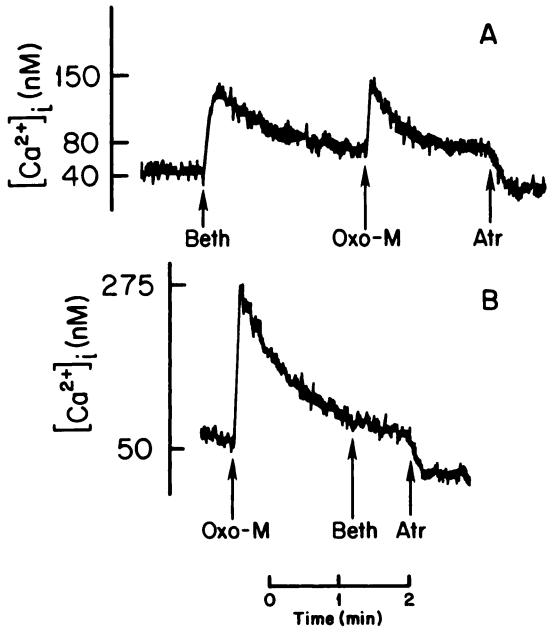


**Fig. 5.** Full and partial muscarinic agonists mobilize the same metabolic pool of $Ca^{2+}$. A, To fura-2-loaded cells was added 10 mM bethanechol (*Beth*), followed by 1 mM oxotremorine-M (*Oxo-M*). Note that the addition of oxotremorine-M after an initial exposure of the cells to bethanechol elicited a further rise in $[Ca^{2+}]_i$. The peak $[Ca^{2+}]_i$ values achieved with bethanechol and oxotremorine-M were 132 and 141 nM, respectively. The corresponding plateau $[Ca^{2+}]_i$ values were 80 and 85 nM. B, If cells were first challenged with oxotremorine-M, the subsequent addition of bethanechol had no effect. The *traces* are from one of three experiments that gave similar results. *Atr*, atropine.

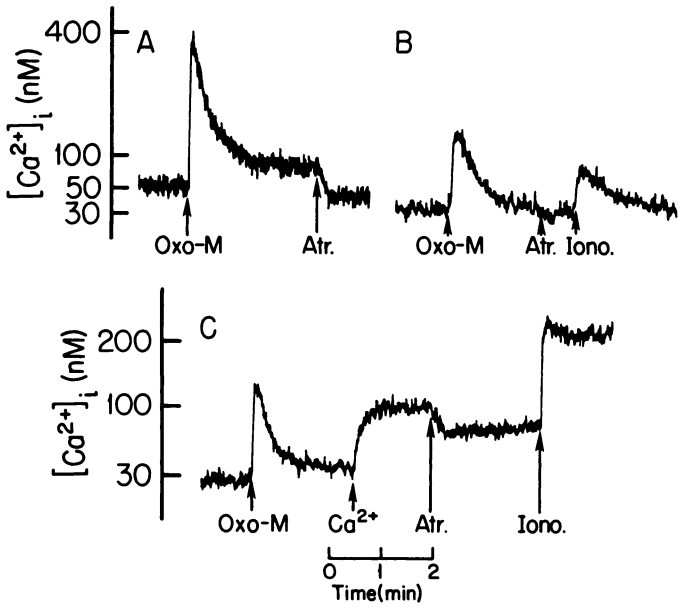


**Fig. 6.** Effect of EGTA on calcium signals generated by muscarinic agonist addition. A, Fura-2-loaded SK-N-SH cells, resuspended in buffer A (2.2 mM $Ca^{2+}$), were exposed to 1 mM oxotremorine-M (*Oxo-M*). The peak and plateau $[Ca^{2+}]_i$ values were 406 and 107 nM, respectively, whereas basal $[Ca^{2+}]_i$ was 60 nM. B, To cells from the same preparation was added 4 mM EGTA (extracellular $[Ca^{2+}]_f$ ~300 nM) before the addition of agonist. Note that the peak $[Ca^{2+}]_i$ achieved (129 nM) is less than in A and that $[Ca^{2+}]_i$ returns to basal values (34 nM) within 60–90 sec. The addition of 10 μM ionomycin (*Iono*) elicits a further increase in $[Ca^{2+}]_i$. In C, cells were treated as in B but, after the return of $[Ca^{2+}]_i$ to basal values, 4 mM $CaCl_2$ (*$Ca^{2+}$*) was added. The subsequent addition of 10 μM atropine (*Atr*) reduced the $[Ca^{2+}]_i$ from 114 to 84 nM. This 30 nM calcium signal was similar to the value obtained for the plateau phase of the calcium signal in A.

phase of the calcium signal. Thus, in a $Ca^{2+}$-deficient medium, the rise in $[Ca^{2+}]_i$ declined to basal values within 60–90 sec after agonist addition. A subsequent addition of ionomycin (10 μM) to these cells could, however, evoke a further rise in $[Ca^{2+}]_i$, presumably due to the discharge of other intracellular stores of $Ca^{2+}$ (Fig. 6B). Upon the readdition of $Ca^{2+}$ to cells previously exposed to EGTA, the plateau phase of the $Ca^{2+}$ signal could be quantitatively restored (Fig. 6C). When SK-N-SH cells were incubated in $Ca^{2+}$-containing buffer A and exposed to 10 μM ionomycin, there was a rapid rise in $[Ca^{2+}]_i$ (346 ± 41 nM, $n = 10$), which was sustained for several minutes (Fig. 7A). The subsequent addition of oxotremorine-M did not result in a further rise in $[Ca^{2+}]_i$. In the presence of EGTA, the addition of ionomycin induced a rapid but transient increase in $[Ca^{2+}]_i$, (210 ± 13 nM; $n = 8$) and no further rise in $[Ca^{2+}]_i$ could be detected after the addition of oxotremorine-M (Fig. 7B). The data presented in Figs. 6 and 7 indicate that, whereas ionomycin and oxotremorine-M can both discharge intracellular pools of $Ca^{2+}$ within SK-N-SH cells, the ionophore is able to mobilize approximately twice the amount of intracellular $Ca^{2+}$ as that released by oxotremorine-M.

**Inositol lipid hydrolysis in intact and permeabilized SK-N-SH cells: dependence on calcium availability.** When SK-N-SH cells were prelabeled with [$^3$H]inositol for 3 days and then exposed to either carbamoylcholine or oxotremorine-M, a rapid release (5–15 sec) of $IP_3$, $IP_2$, and $IP_1$ occurred (Fig. 8). Although $IP_3$ was often the most rapidly released inositol phosphate following the addition of agonist, in other experiments the appearances of $IP_3$ and $IP_2$ were kinetically indistinguishable. Agonist-induced increases in $IP_4$ formation were not consistently observed. Because total inositol phosphate accumulation was linear with time in the presence of $Li^+$ for at least 60 min, longer incubation periods (15–30 min) were routinely employed in subsequent experiments. In the absence of an agonist, only a limited release of inositol phosphates from the prelabeled cells occurred during a 30-min incubation (0.20 ± 0.03% of radioactivity present initially in inositol lipid fraction; $n = 4$). Addition of oxotremorine-M increased this release to 7.4 ± 2.0%, whereas, in the presence of 10 μM ionomycin, inositol phosphate release increased to 1.5 ± 0.4%. Oxotremorine-M and ionomycin induced 33- and 5-fold increases in inositol phosphate release over control, respectively (Table 1). However, when 4 mM EGTA was included in the assays, reductions in both the basal and stimulated PPI turnover occurred. Thus, in the presence of EGTA, the addition of oxotremorine-M resulted in only a 13-fold increase in inositol phosphate release when compared with buffer A control incubations, whereas that induced by the addition of ionomycin was essentially abolished (Table 1). The inhibitory effect of EGTA on oxotremorine-M-stimulated PPI turnover could be detected after 2 min of incubation (shortest time assayed) and thereafter remained constant (on a percentage basis) for up to 60 min of incubation (data not shown). Because the *accumulation* of inositol phosphates is being measured in these experiments, it is likely that the inhibitory effect of EGTA on stimulated PPI turnover is immediate, rather than delayed. To assess more directly the role played by physiologically relevant $Ca^{2+}$ concentrations in the regulation of the phospholipase C enzyme, prelabeled SK-N-SH cells were first permeabilized in digitonin-containing KGEH buffer and then exposed to $Ca^{2+}$-EGTA buffer solutions, in which the $[Ca^{2+}]_f$ had been directly measured by the addition of fura-2 (free acid). The release of labeled inositol phosphates into the medium surrounding the cells (rather than the cell pellet) was then quantitated after a 15-min incubation. Elevation of $[Ca^{2+}]_f$ from 10 nM to 2 μM resulted in a 3–4-fold increase in inositol phosphate release, with an $EC_{50}$ of 145 ± 29 nM ($n = 3$) (Fig. 9). An increase in $[Ca^{2+}]_f$ from 10 to 60 nM resulted in an increased release of $IP_3$, $IP_2$, and $IP_1$ (170 ± 30, 172 ± 32, and 193 ± 33% of control, respectively; $n = 4$). The addition of 50 μM GTPγS further potentiated this $Ca^{2+}$-dependent release of inositol phosphates with an $EC_{50}$ for $Ca^{2+}$ of approximately 60 nM (Fig. 9). The permeabilized cells also responded to the addition of oxotremorine-M in the presence of increasing $[Ca^{2+}]_f$ (Fig. 10). This ability of the agonist to enhance PPI hydrolysis presumably reflects the presence of endogenous guanine nucleotides, because inclusion of GDPβS (3 mM) substantially reduced this stimulation. The effects of oxotremorine-M were potentiated by the inclusion of 50 μM GTPγS, such that, under these conditions, the $[Ca^{2+}]_f$ required to elicit a 50% increase in PPI hydrolysis was reduced to 35–40 nM. When SK-N-SH cells were incubated in the presence of 50–60 nM free $Ca^{2+}$ (which approximates that in the cytoplasm of quiescent cells), the addition of GTPγS or oxotremorine-M increased the release of total inositol phosphates to 830 ± 76% and 725 ± 146% of control, respectively. When the two agents were present simultaneously, the stimulated release of inositol phosphates was 2658 ± 466% of control ($n = 4$), a value that compares favorably with the degree of stimulation obtained in intact cells (see

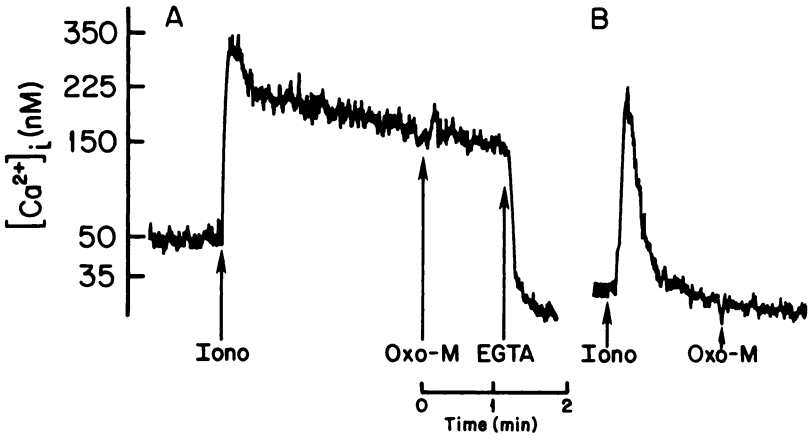


**Fig. 7.** Increase in $[Ca^{2+}]_i$ elicited by the addition of ionomycin (*Iono*) in the presence or absence of extracellular $Ca^{2+}$. A, Addition of 10 μM ionomycin to cells incubated in buffer A containing 2.2 mM $Ca^{2+}$ resulted in a rise in $[Ca^{2+}]_i$, sustained for several minutes. Addition of oxotremorine-M (*Oxo-M*) did not result in any further increase in $[Ca^{2+}]_i$. In B, cells were incubated with buffer A containing 4 mM EGTA for 2 min before the addition of ionomycin. Note the transient rise in $[Ca^{2+}]_i$ and the inability of oxotremorine-M to further increase $[Ca^{2+}]_i$.

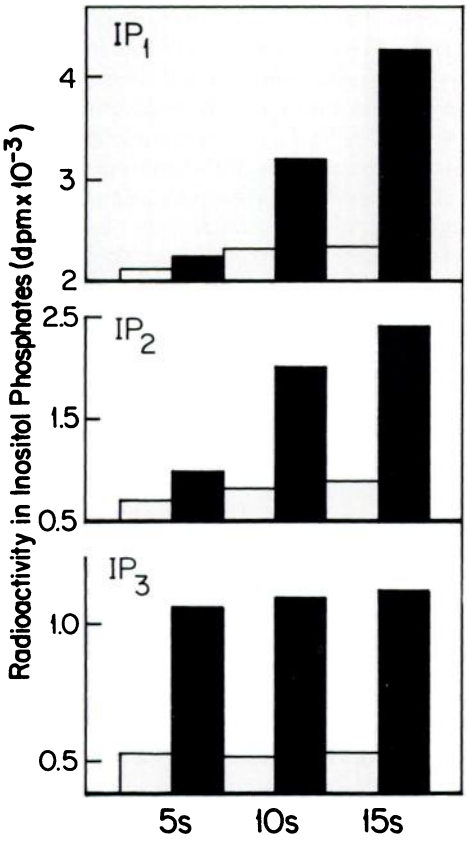


**Fig. 8.** Rapid release of inositol phosphates from SK-N-SH cells in the presence of muscarinic agonists. Cells that had been prelabeled for 3 days with [$^3$H]inositol were detached, washed in buffer A, and then incubated in the presence of either buffer A (□) or 10 mM carbamoylcholine (■). Reactions were terminated by the addition of TCA and inositol phosphates were extracted and separated. Results shown are the means of duplicate determinations, which differed by less than 20%. In the experiment shown, label in $IP_3$ appeared before that in $IP_2$. In other experiments, the appearance of labeled $IP_3$ and $IP_2$ was kinetically indistinguishable but was always detected within 5–10 sec of agonist addition.

Table 1). Thus, GTPγS and oxotremorine-M together elicit a synergistic (rather than additive) increase in PPI hydrolysis. If SK-N-SH cells were exposed to KGEH buffer in the absence of digitonin, only a small increase in the release of labeled inositol phosphates was observed in the presence of oxotremorine-M, GTPγS, or a combination of both (< 15% of that detected in the presence of digitonin).

TABLE 1

**Calcium dependency of PPI turnover in intact SK-N-SH cells. Cells were allowed to prelabel with [$^3$H]inositol for 3 days. Labeled cells (approximately 1 mg of protein) were allowed to incubate in buffer A containing either oxotremorine-M (1 mM), EGTA (4 mM), or ionomycin (10 μM). Reactions were terminated after 30 min and inositol phosphates were isolated and quantitated. Results are expressed as per cent increase in the release of inositol phosphates relative to control buffer A incubations from three or four separate experiments (means ± standard errors). In some experiments, the individual inositol phosphates were separated by anion-exchange chromatography. Both oxotremorine-M and ionomycin elicited increases in $IP_1$, $IP_2$, and $IP_3$. The amount of radioactivity recovered in the lipid fraction at the beginning of the incubations was 348,000 ± 36,000 dpm.**

| | Radioactivity in inositol phosphates | |
|---|---|---|
| | Buffer A | Buffer A + EGTA |
| | *% of control* | |
| Control | 100 | 55 ± 12 |
| Oxotremorine-M | 3469 ± 600 | 1360 ± 170 |
| Ionomycin | 644 ± 138 | 115 ± 23 |

Both the effect of oxotremorine-M alone and the synergistic increase in inositol phosphate release from permeabilized cells could be prevented by inclusion of atropine (Fig. 11). When the individual inositol phosphates were separated on anion-exchange columns, statistically significant increases in $IP_1$, $IP_2$, and $IP_3$ were detected in response to oxotremorine-M, GTPγS, or a combination of these agents (Fig. 12). However, larger increases were observed for $IP_1$ and $IP_2$ than for $IP_3$. For example, inclusion of oxotremorine-M increased the release of $IP_3$, $IP_2$, and $IP_1$ to 216 ± 36, 460 ± 142, and 900 ± 325% of control, respectively ($n = 4$). Corresponding values for GTPγS stimulation were 296 ± 48, 647 ± 140, and 666 ± 208%. In the presence of both oxotremorine-M and GTPγS, the release of $IP_3$, $IP_2$, and $IP_1$ increased to 543 ± 146, 2084 ± 539, and 2872 ± 1200% of control, respectively. Inclusion of 10 mM 2,3-DPG, an inhibitor of $IP_3$ phosphatase (27), resulted in a further accumulation of $IP_3$ (42–109% above incubations in which 2,3-DPG was omitted; $p < 0.05$). When shorter incubation times were used (2 or 5 min), stimulated $IP_3$ release was again observed. However, the label recovered in the individual $IP_1$ and $IP_2$ fractions still exceeded that in the $IP_3$ fraction by 3–8-fold. To determine whether a rapid $IP_3$ phosphatase activity was responsible for the preponderance of label in $IP_1$ and $IP_2$, [$^3$H]inositol 1,4,5-trisphosphate was added to unlabeled permeabilized cells and its rate of degradation was determined. Little or no loss (≤ 10%) of [$^3$H]$IP_3$ could be detected within

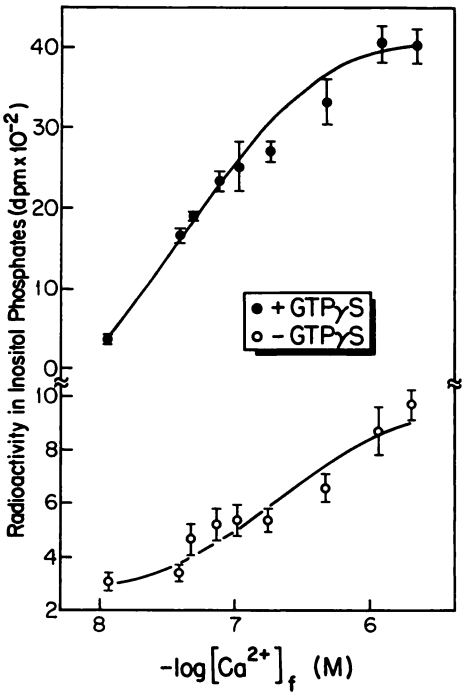


**Fig. 9.** Increases in $[Ca^{2+}]_f$ cause the release of inositol phosphates from permeabilized SK-N-SH cells. [$^3$H]Inositol-prelabeled cells (1.01 mg of protein) were permeabilized and incubated in KGEH buffer with added $Ca^{2+}$ to achieve the required $[Ca^{2+}]_f$ (as monitored by the addition of fura-2). Cells were incubated for 15 min either in the absence (○) or presence (●) of 50 μM GTPγS. Results shown are means ± standard errors for triplicate replicates. The $EC_{50}$ values for basal and GTPγS-stimulated inositol phosphate release were 136 and 63 nM $[Ca^{2+}]_f$, respectively. In a second experiment performed under identical conditions, $EC_{50}$ values of 100 and 58 nM $[Ca^{2+}]_f$ were obtained. Release of inositol phosphates at zero time was 321 dpm. $[Ca^{2+}]_f$ values of 11, 39, 46, 71, 102, 187, 477, 1166, and 1926 nM were obtained at $Ca^{2+}$/EGTA molar ratios of 0, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40, 0.50, and 0.55, respectively, with EGTA maintained at a concentration of 10 mM.

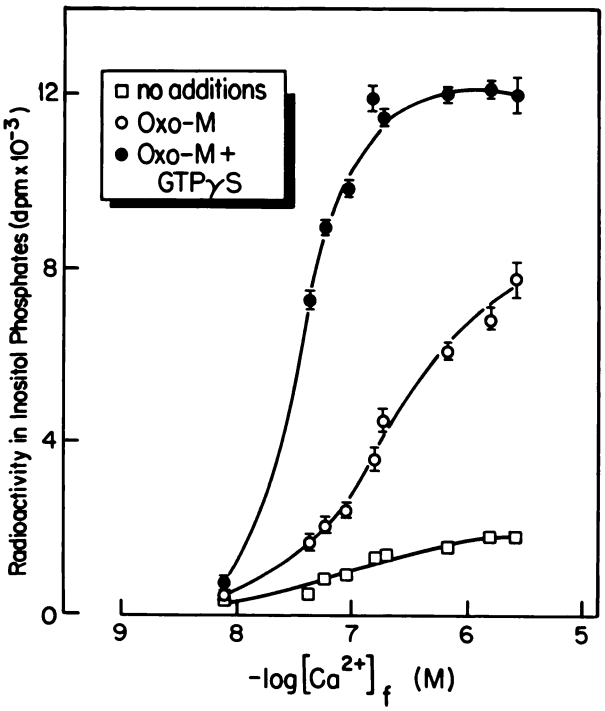


**Fig. 10.** Increases in $[Ca^{2+}]_f$ facilitate the release of inositol phosphates from permeabilized cells (0.79 mg of protein) induced by the addition of either 1 mM oxotremorine-M (*Oxo-M*) or 1 mM oxotremorine-M plus 50 μM GTPγS. The $EC_{50}$ for oxotremorine-M/GTPγS-stimulated assays was 40 nM $[Ca^{2+}]_f$. Results shown are means ± standard errors for triplicate replicates. Release of inositol phosphates at zero time was 581 dpm. In a second experiment performed under identical conditions, an $EC_{50}$ value of 35 nM $[Ca^{2+}]_f$ was obtained.

the first 2 min of incubation. Thereafter, [$^3$H]$IP_3$ was degraded such that, at 5 and 15 min, 38% and 55% of initial label was lost. Inclusion of 10 mM 2,3-DPG blocked this breakdown of $IP_3$ by > 85%. Collectively, these results indicate that at least part of the inositol phosphate release that occurs in SK-N-SH cells is derived from the direct phosphodiesteratic breakdown of phosphatidylinositol 4,5-bisphosphate and that the phospholipase C-mediated hydrolysis of inositol lipids in these cells can be regulated by changes in $[Ca^{2+}]_f$ that are physiologically relevant.

## Discussion

Although SK-N-SH neuroblastoma cells maintain a relatively low resting $[Ca^{2+}]_i$ (approximately 50 nM), the results obtained with the fluorescent probe fura-2 indicate that the addition of muscarinic agonists results in a rapid and pronounced increase in the concentration of cytoplasmic $Ca^{2+}$ in these cells. Temporally, the calcium signal can be divided into three phases, i.e., a rapid increase in $[Ca^{2+}]_i$ to a peak value, followed by a slower decline and a maintained (plateau) $[Ca^{2+}]_i$, which remains above that observed for quiescent cells for as long as the mAChR is occupied by the agonist. Similar results have previously been obtained following mAChR activation in 1321N1 astrocytoma, parotid, pancreatic acinar, and gastric parietal cells (28–31). However, the pattern of calcium

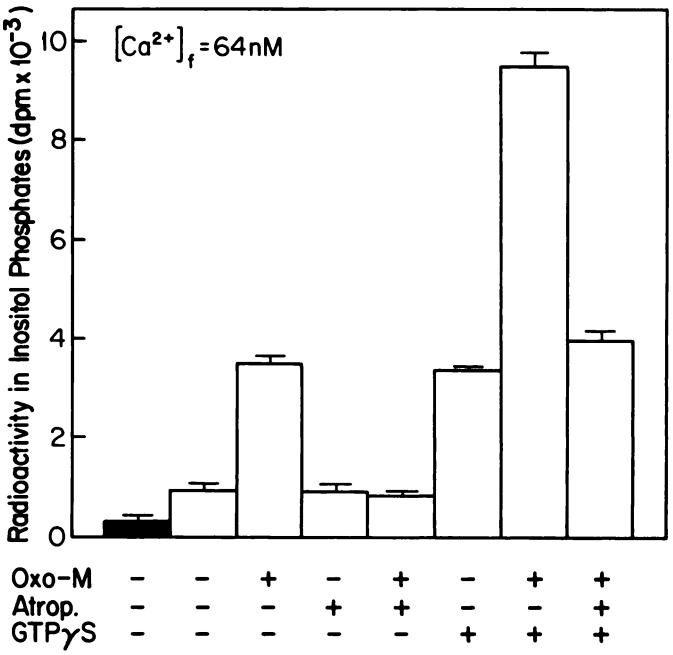


**Fig. 11.** Effect of atropine on oxotremorine-M- and GTPγS-induced inositol phosphate release. Permeabilized cells (1.02 mg of protein) were incubated for 15 min in KGEH buffer and sufficient $Ca^{2+}$ added to provide a $[Ca^{2+}]_f$ of 64 nM, in the presence of either atropine (*Atrop.*) (25 μM), oxotremorine-M (*Oxo-M*) (1 mM), or GTPγS (50 μM). The *solid bar* indicates the release of inositol phosphates obtained at zero time. Results shown are means ± standard errors for quadruplicate replicates.

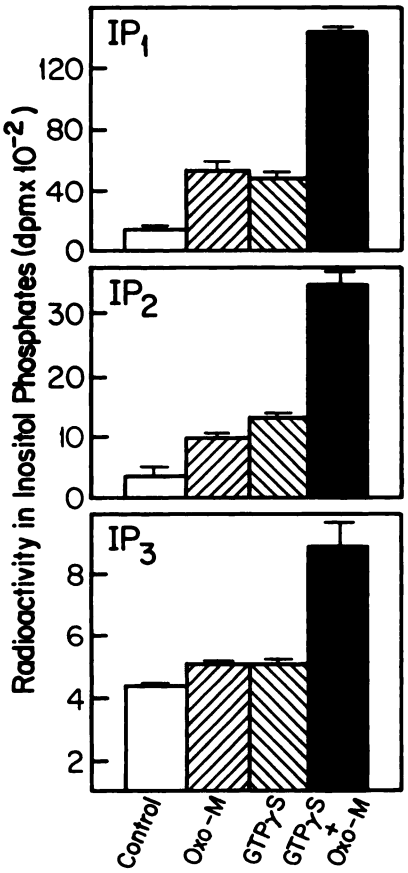


**Fig. 12.** Separation of individual inositol phosphates released from permeabilized SK-N-SH cells (1.20 mg of protein) in the absence or presence of 1 mM oxotremorine-M (*Oxo-M*), 50 μM GTPγS, or a combination of both. The $[Ca^{2+}]_f$ was maintained at 60 nM. For all three inositol phosphates, the release observed in the combined presence of oxotremorine-M plus GTPγS was greater than the sum of radiolabel released for each agent.

signaling in SK-N-SH cells differs considerably from that observed in avian salt gland, in which the initial rise in $[Ca^{2+}]_i$ after mAChR activation is sustained for several minutes and declines only slowly (32). The rapid initial rise in $[Ca^{2+}]_i$ that accompanies the activation of many PPI-linked receptors, including the mAChR, is thought to result from the release of inositol 1,4,5-trisphosphate ($IP_3$) and subsequent mobilization of intracellular calcium stores from the endoplasmic reticulum (33, 34). The results obtained for the SK-N-SH cell indicate that the time course for release of $IP_3$ is compatible with such a role and, furthermore, that the magnitude of the rise in $[Ca^{2+}]_i$ is proportional to the extent of PPI hydrolysis. Evidence in support of the latter suggestion comes from the observation that the $EC_{50}$ values for both oxotremorine-M-stimulated PPI hydrolysis and rise in $[Ca^{2+}]_i$ are very similar (2.5–8.0 μM; Fig. 3 and Ref. 8). Moreover, whereas partial and full agonists for PPI turnover mobilize $Ca^{2+}$ from the same metabolic pool, the former group of agonists elicits correspondingly smaller increases in $[Ca^{2+}]_i$ than does either oxotremorine-M or carbamoylcholine. These results suggest that the increase in $[Ca^{2+}]_i$ is closely coupled to the production of $IP_3$ and that few if any "spare" $IP_3$ receptors exist for calcium mobilization. The relationship between the plateau phase of the calcium signal and PPI hydrolysis remains obscure. As noted previously for the parotid gland (29), lower agonist concentrations are required to elicit this phase of the calcium signal than are required for the initial rise in $[Ca^{2+}]_i$. Furthermore, in SK-N-SH cells, the same plateau $[Ca^{2+}]_i$ values are obtained for both full and partial muscarinic agonists. Previously, a role for inositol, 1,3,4,5-tetrakisphosphate ($IP_4$) in the maintenance of calcium influx across the plasma membrane has been suggested (35). However, stimulated $IP_4$ formation was difficult to reliably detect in SK-N-SH cells after mAChR activation, even though $IP_3$ kinase activity could be demonstrated.[1] Because $IP_4$ is derived metabolically from $IP_3$, if the appearance of $IP_4$ underlies the continued influx of extracellular $Ca^{2+}$, it follows that only small increases in this inositol phosphate would be required to fulfill such a role in SK-N-SH cells.

Both the initial rise in $[Ca^{2+}]_i$ and the plateau $[Ca^{2+}]_i$ were dependent upon the availability of extracellular calcium. As observed previously for other cells (28, 29, 31), incubation of SK-N-SH cells in a calcium-deficient medium abolished the plateau phase of the $Ca^{2+}$ signal, a result consistent with a requirement for influx of extracellular calcium. However, whereas the rapid rise in $[Ca^{2+}]_i$ following mAChR activation in other cells is largely unaffected by a reduction of extracellular calcium (Refs. 28, 29, and 31, but see also Ref. 5), that induced in SK-N-SH cells was markedly reduced by inclusion of EGTA. Although this result could conceivably reflect a role for the influx of extracellular $Ca^{2+}$ in the generation of the initial rise in $[Ca^{2+}]_i$, our results offer an alternative possibility, i.e., that phospholipase C activity (and thereby the production of $IP_3$ and mobilization of intracellular calcium) may itself be very sensitive to changes in $[Ca^{2+}]_i$ induced by the addition of the chelator. The evidence in favor of this suggestion is 2-fold. First, under conditions in which inclusion of EGTA lowered both the basal and stimulated $[Ca^{2+}]_i$ levels, a similar reduction in both the basal and oxotremorine-M-stimulated release of inositol phosphates was also observed. In addition, the effects of EGTA on both PPI hydrolysis and $Ca^{2+}$ signaling were detectable within the same time-frame of 2–4 min. Second, results obtained with permeabilized SK-N-SH cells clearly demonstrated that the basal and oxotremorine-M- and GTPγS-mediated release of $IP_1$, $IP_2$, and $IP_3$ can be regulated by $[Ca^{2+}]_f$ levels that are physiologically relevant (i.e., 20–400 nM). In the presence of oxotremorine-M and GTPγS, a $[Ca^{2+}]_f$ of less than 50 nM was required for half-maximal activation of PPI hydrolysis. Because this also approximates the $[Ca^{2+}]_i$ in quiescent cells, it is evident that small reductions in $[Ca^{2+}]_i$ could significantly reduce phospholipase C activity. A further indication of the role that calcium availability may play in inositol lipid hydrolysis was obtained from experiments with ionomycin. Addition of the ionophore to intact cells resulted in both a 5-fold increase in inositol phosphate release and a comparable increase in $[Ca^{2+}]_i$. Although the extent of ionomycin-stimulated PPI hydrolysis was only 15% of that induced by oxotremorine-M, it remains possible that the rise in $[Ca^{2+}]_i$ that follows mAChR activation may make some further contribution to inositol lipid hydrolysis. Although the stimulatory effects of calcium ionophores on platelet phospholipase C activity have been attributed to an indirect effect of arachidonate metabolites (36), neither arachidonate itself nor inhibitors of its metabolism have any effect on enzyme activity in the pancreas (37). Although we cannot presently exclude the pos-

[1] A. M. Heacock and S. K. Fisher, unpublished results.

sibility that such metabolites may also contribute (in part at least) to the ability of the ionophore to stimulate phospholipase C activity in SK-N-SH neuroblastoma cells, the observation that arachidonate is present in relatively low concentrations in phospholipids extracted from these cells,[2] renders this possibility less likely. Taken collectively, the results obtained for both intact and permeabilized SK-N-SH neuroblastoma cells suggest that either a reduction below, or an increase above, the basal $[Ca^{2+}]_i$ may directly regulate the extent of PPI hydrolysis in these cells.

Although there is general agreement that PPI hydrolysis is less sensitive to $Ca^{2+}$ depletion than is the attendant physiological response (e.g., contraction, secretion, cell-cell communication), the possibility that phospholipase C itself might be subject to regulation by $[Ca^{2+}]_f$ levels that occur in the cell cytoplasm has been a controversial issue, with evidence presented both in favor of (23, 37–40) and against (41–44) this possibility. Four considerations are pertinent in this regard. First, in some studies measurements of $[Ca^{2+}]_i$ and PPI turnover were not obtained under the same assay conditions, thus rendering direct comparisons difficult. Second, there has been a reliance on $[Ca^{2+}]_f$ values obtained from $Ca^{2+}$-EGTA-calculating computer programs, rather than by direct measurement. In our studies, we found it essential to monitor the *actual* $[Ca^{2+}]_f$ using fura-2, because computed $[Ca^{2+}]_f$ values were invariably lower than those actually obtained. A similar discrepancy between computed and actual $[Ca^{2+}]_f$ values has previously been noted (42). A third consideration is that tissues may differ considerably in the sensitivity of their phospholipase C activity to $[Ca^{2+}]_f$. For example, elevation of $[Ca^{2+}]_i$ in brain slices by means of $K^+$ depolarization or addition of A23187 or calcium agonists is able to promote the hydrolysis of inositol lipids (45–47). Similarly, ionomycin enhances PPI hydrolysis in SK-N-SH cells, embryonic chick heart cells (38), and, to a lesser extent, in pancreatic acini (37). Conversely, in neutrophils, elevation of $[Ca^{2+}]_f$ above 100 nM (resting level) does not result in any further inositol lipid hydrolysis (48). A further consideration is the fact that SK-N-SH cells possess a relatively low resting $[Ca^{2+}]_i$ (50 nM), which may render them more susceptible to the effects of calcium chelators than cells in which the resting $[Ca^{2+}]_i$ is higher.

One of the most notable features of stimulated PPI turnover in SK-N-SH cells is that mAChR-effector coupling in permeabilized cells is as effective as that observed in intact cells. In contrast, permeabilization causes a marked reduction in the coupling efficiency of mAChRs in pancreatic cells (40). Thus, in the presence of GTPγS and oxotremorine-M, PPI hydrolysis in permeabilized SK-N-SH cells was increased by 26-fold, a value similar to that obtained for intact cells stimulated by the addition of agonist (Table 1) (8, 9). That GTPγS alone was able to enhance inositol lipid hydrolysis suggests the involvement of a guanine nucleotide-binding protein in the transduction process, as noted for mAChRs in other tissues (49–51). Although oxotremorine-M could promote the release of inositol phosphates in the absence of GTPγS, this ability presumably reflects the presence of endogenous guanine nucleotides still retained within the permeabilized cells. However, in the combined presence of GTPγS and agonist, a synergistic increase in PPI hydrolysis was detected. A key feature of the role that guanine nucleotides may play in PPI hydrolysis is a reduction in the $[Ca^{2+}]_f$ required for phospholipase C activity (52, 53). In SK-N-SH cells, stimulated PPI hydrolysis displayed an absolute dependence on the availability of a $[Ca^{2+}]_f$ of > 10 nM (Fig. 10), whereas a half-maximal ($EC_{50}$) increase in inositol phosphate release observed in the presence of oxotremorine-M occurred at a $[Ca^{2+}]_f$ of > 150 nM. However, in the presence of GTPγS, only 35–40 nM $[Ca^{2+}]_f$ was necessary for the agonist to half-maximally stimulate inositol lipid turnover. The ability of GTPγS to enhance the agonist-mediated PPI hydrolysis was most marked at $[Ca^{2+}]_f$ levels of 40–80 nM. Thus, guanine nucleotides appear to sensitize the transduction process such that a muscarinic agonist most effectively initiates PPI hydrolysis at a $[Ca^{2+}]_i$ that is encountered in quiescent cells.

In studies with both permeabilized and intact SK-N-SH cells, it was observed that most of the label recovered in inositol phosphates was present in $IP_1$ and $IP_2$, rather than $IP_3$. This pattern of inositol phosphate release could reflect rapid $IP_3$ and $IP_2$ phosphatase actions or, alternatively, phospholipase C activation may result in the direct hydrolysis of all three inositol lipids. Although the current amount of information precludes a definite conclusion regarding these two alternatives, the evidence presently available favors the possibility of the breakdown of more than phosphatidylinositol-4,5-bisphosphate alone. Thus, (i) the amount of radiolabel recovered in $IP_2$ and $IP_1$ exceeded that present in $IP_3$ even under conditions of acute stimulation (10–15 sec), (ii) in permeabilized cells incubated with 2,3-DPG, the amount of $IP_3$ was increased but was still considerably less than that present in either $IP_2$ or $IP_1$, and (iii) inclusion of 2,3-DPG reduced the breakdown of $[^3H]IP_3$ added exogenously to permeabilized SK-N-SH cells by > 85%. A simultaneous release of $IP_3$ and $IP_2$ emanating from phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol 4-phosphate breakdown has also been proposed to occur in $GH_3$ pituitary cells when stimulated by thyrotropin-releasing hormone (54). The fact that the variables used in the present study (i.e., calcium availability and presence of agonist and guanine nucleotides) appeared to influence the release of labeled $IP_3$, $IP_2$, and $IP_1$ in a similar manner but to a different extent suggests that the release of the individual inositol phosphates may reflect the different amounts of radiolabel associated with the parent inositol lipids present in the plasma membrane.

In summary, the results demonstrate that mAChR activation in SK-N-SH cells results in a rapid rise in $[Ca^{2+}]_i$ and that the magnitude of this increase is related to the extent of PPI hydrolysis. Phospholipase C activity in these cells appears to be sensitive to small changes in $[Ca^{2+}]_i$, such that PPI hydrolysis, and by inference calcium signaling, can be modulated by either a decrease or an increase in $[Ca^{2+}]_i$. From experiments with permeabilized cells, we conclude that guanine nucleotides sensitize the transduction process, thereby facilitating agonist-induced inositol lipid hydrolysis at a $[Ca^{2+}]_f$ found in the cytoplasm of quiescent cells.

**Acknowledgments**

The authors wish to thank Ms. Jo Ann Kelsch for preparation of the manuscript and Dr. R. W. Holz and D. A. Eberhard for their helpful suggestions.

***Note added in Proof***

Although nicotinic cholinergic receptors may also be present on SK-N-SH cells (*Pharmacologist 30:* A78, 1988), their contribution to the $Ca^{2+}$ signals reported in the present study appears to be minimal for the following reasons: (1) inclusion of optimal concentrations of nocotinic agonists (1,1-dimethyl-4-phenylpiperazinium iodide or nicotine) increased $[Ca^{2+}]_i$ by <15% that elicited by either oxotre-

[2] C.-H. Lee, S. K. Fisher, B. W. Agranoff, and A. K. Hajra, unpublished results.

morine- M or muscarine; (2) inclusion of 10 μM atropine prior to the addition of $10^{-3}$ M oxotremorine-M blocked the increase in $[Ca^{2+}]_i$ by 90%.

## References


1. Richelson, E. The use of cultured cells in neurobiological studies. *Int. Rev. Biochem. (Physiol. and Pharmacol. Biochem.)* **26**:81–120 (1979).
2. Nathanson, N. M. Molecular properties of the muscarinic acetylcholine receptor. *Annu. Rev. Neurosci.* **10**:195–236 (1987).
3. Fisher, S. K., and B. W. Agranoff. Receptor activation and inositol lipid hydrolysis in neural tissues. *J. Neurochem.* **48**:999–1017 (1987).
4. Gil, D. L., T. Ueda, S.-H. Chueh, and M. W. Noel. $Ca^{2+}$ release from endoplasmic reticulum is mediated by a guanine nucleotide regulatory mechanism. *Nature (Lond.)* **320**:461–464 (1986).
5. Sher, E., C. Gotti, A. Pandiella, L. Madeddu, and F. Clementi. Intracellular calcium homeostasis in a human neuroblastoma cell line: modulation by depolarization, cholinergic receptors, and α-latrotoxin. *J. Neurochem.* **50**:1708–1713 (1988).
6. Heikkila, J. E., I. G. Scott, L. A. Suominen, and K. E. O. Akerman. Differentiation-associated decrease in muscarinic receptor sensitivity in human neuroblastoma cells. *J. Cell. Physiol.* **130**:157–162 (1987).
7. Snider, R. M., M. McKinney, C. Forray, and E. Richelson. Neurotransmitter receptors mediate cyclic GMP formation by involvement of arachidonic acid and lipoxygenase. *Proc. Natl. Acad. Sci. USA* **81**:3905–3909 (1984).
8. Fisher, S. K., and R. M. Snider. Differential receptor occupancy requirements for muscarinic cholinergic stimulation of inositol lipid hydrolysis in brain and in neuroblastomas. *Mol. Pharmacol.* **32**:81–90 (1987).
9. Fisher, S. K. Recognition of muscarinic cholinergic receptors in human SK-N-SH neuroblastoma cells by quaternary and tertiary ligands is dependent upon temperature, cell integrity, and the presence of agonists. *Mol. Pharmacol.* **33**:414–422 (1988).
10. Dooda, H. N., M.-J. Mathy, D. Davidesko, K. J. van Charldorp, A. de Jonge, and P. A. van Zwieten. Selectivity of muscarinic antagonists in radioligand and *in vivo* experiments for the putative $M_1$, $M_2$, and $M_3$ receptors. *J. Pharmacol. Exp. Ther.* **242**:257–262 (1987).
11. Fisher, S. K., and A. M. Heacock. A putative $M_3$ muscarinic cholinergic receptor of high molecular weight couples to phosphoinositide hydrolysis in human SK-N-SH neuroblastoma cells. *J. Neurochem.* **50**:984–987 (1988).
12. Venter, J. C. Muscarinic cholinergic receptor structure: receptor size, membrane orientation and absence of major phylogenetic structural diversity. *J. Biol. Chem.* **258**:4842–4848 (1983).
13. Hunter, D. D., and N. M. Nathanson. Biochemical and physical analyses of newly synthesized muscarinic acetylcholine receptors in cultured embryonic chicken cardiac cells. *J. Neurosci.* **6**:3739–3748 (1986).
14. Bonner, T. I., N. J. Buckley, A. C. Young, and M. R. Brann. Identification of a family of muscarinic acetycholine receptor genes. *Science (Wash. D. C.)* **237**:527–532 (1987).
15. Peralta, E. G., A. Ashkenazi, J. W. Winslow, D. H. Smith, J. Ramachandran, and D. J. Capon. Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO J.* **6**:3923–3929 (1987).
16. Honegger, P., and E. Richelson. Biochemical differentiation of mechanically dissociated mammalian brain in aggregating cell culture. *Brain Res.* **109**:335–354 (1976).
17. Tsien, R. Y., T. Pozzan, and T. J. Rink. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new intracellularly trapped fluorescent indicator. *J. Cell Biol.* **94**:324–334 (1982).
18. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
19. Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212**:374–382 (1983).
20. Fisher, S. K., and R. T. Bartus. Regional differences in the coupling of muscarinic receptors to inositol phospholipid hydrolysis in guinea pig brain. *J. Neurochem.* **45**:1085–1095 (1985).
21. Batty, I. R., S. R. Nahorski, and R. F. Irvine. Rapid formation of inositol 1,3,4,5-tetrakisphosphate following muscarinic receptor stimulation of rat cerebral cortical slices. *Biochem J.* **232**:211–215 (1985).
22. Takazawa, K., H. Passareiro, J. E. Dumont, and C. Erneaux. $Ca^{2+}$/calmodulin-sensitive inositol 1,4,5-trisphosphate 3-kinase in rat and bovine brain tissues. *Biochem. Biophys. Res. Commun.* **153**:632–641 (1988).
23. Eberhard, D. A., and R. W. Holz. Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. *J. Neurochem.* **49**:1634–1643 (1987).
24. Geiger, P. J., and S. P. Bessman. Protein determination by Lowry's method in the presence of sulfhydryl reagents. *Anal. Biochem.* **49**:467–473 (1972).
25. Fisher, S. K., J. C. Figueiredo, and R. T. Bartus. Differential stimulation of inositol phospholipid turnover in brain by analogs of oxotremorine. *J. Neurochem.* **43**:1171–1179 (1984).
26. Capponi, A. M., P. D. Lew, and M. B. Vallotton. Cytosolic free calcium levels in monolayers of cultured rat aortic smooth muscle cells. *J. Biol. Chem.* **260**:7836–7842 (1985).
27. Downes, C. P., M. C. Mussat, and R. H. Michell. The inositol trisphosphate phosphomonoesterase of the human erythrocyte membrane. *Biochem. J.* **203**:169–177 (1982).
28. McDonough, P. M., J. H. Eubanks, and J. H. Brown. Desensitization and recovery of muscarinic and histaminergic $Ca^{2+}$ mobilization in 1321N1 astrocytoma cells. *Biochem. J.* **249**:135–141 (1988).
29. Merritt, J. E., and T. J. Rink. Regulation of cytosolic free calcium in fura-2-loaded rat parotid acinar cells. *J. Biol. Chem.* **262**:17362–17369 (1987).
30. Sung, C. K., S. R. Hootman, E. L. Stuenkel, C. Kuroiwa, and J. A. Williams. Downregulation of protein kinase C in guinea pig pancreatic acini: effects on secretion. *Am. J. Physiol.* **254**:G242–G248 (1988).
31. Chew, C. S., and M. R. Brown. Release of intracellular $Ca^{2+}$ and elevation of inositol trisphosphate by secretagogues in parietal and chief cells isolated from rabbit gastric mucosa. *Biochim. Biophys. Acta.* **888**:116–125 (1986).
32. Snider, R. M., R. M. Roland, R. J. Lowy, B. W. Agranoff, and S. A. Ernst. Muscarinic receptor-stimulated $Ca^{2+}$ signaling and inositol lipid metabolism in avian salt gland cells. *Biochim. Biophys. Acta.* **889**:216–224 (1986).
33. Rasmussen, H., and P. Q. Barrett. Calcium messenger system: an integrated view. *Physiol. Rev.* **64**:938–984 (1984).
34. Berridge, M. J., and R. F. Irvine. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond.)* **312**:315–321 (1984).
35. Irvine, R. F., and R. M. Moor. Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external $Ca^{2+}$. *Biochem. J.* **240**:917–920 (1986).
36. Rittenhouse, S. E. The mobilization of arachidonate and metabolism of phosphoinositides in stimulated human platelets, in *Inositol and Phosphoinositides, Metabolism and Regulation* (J. E. Bleasdale, J. Eichberg, and G. Hauser, eds.). Humana Press, Clifton, N.J., 459–473 (1985).
37. Taylor, C. W., J. E. Merritt, J. W. Putney, Jr., and R. P. Rubin. Effects of $Ca^{2+}$ on phosphoinositide breakdown in exocrine pancreas. *Biochem. J.* **238**:765–772 (1986).
38. McDonough, P. M. D. Goldstein, and J. H. Brown. Elevation of cytoplasmic calcium concentration stimulates hydrolysis of phosphatidylinositol bisphosphate in chick heart cells: effect of sodium channel activators. *Mol. Pharmacol.* **33**:310–315 (1988).
39. Eberhard, D. A., and R. W. Holz. Intracellular $Ca^{2+}$ activates phospholipase C. *Trends. Neurosci.*, **11**: 517–520 (1988).
40. Best. L. A role for calcium in the breakdown of inositol phospholipids in intact and digitonin-permeabilized pancreatic islets. *Biochem. J.* **238**:773–779 (1986).
41. Renard, D., J. Poggioli, B. Berthon, and M. Claret. How far does phospholipase C activity depend on the cell calcium concentration? *Biochem. J.* **243**:391–398 (1987).
42. Wolf, B. A., J. Florholmen, J. Turk, and M. L. McDaniel. Studies of the $Ca^{2+}$ requirements for glucose- and carbachol-induced augmentation of inositol trisphosphate and inositol tetrakisphosphate accumulation in digitonin-permeabilized islets. *J. Biol. Chem.* **263**:3565–3575 (1988).
43. Sasaguri, T., M. Hirata, and H. Kuriyama. Dependence on $Ca^{2+}$ of the activities of phosphatidylinositol 4,5-bisphosphate phosphodiesterase and inositol 1,4,5-trisphosphate phosphatase in smooth muscles of the porcine coronary artery. *Biochem. J.* **231**:497–503 (1985).
44. Vicentini, L. M., A. Ambrosini, F. Di Virgilio, T. Pozzan, and J. Meldolesi. Muscarinic receptor-induced phosphoinositide hydrolysis at resting cytosolic $Ca^{2+}$ concentration in PC12 cells. *J. Cell Biol.* **100**:1330–1333 (1985).
45. Kendall, D. A., and S. R. Nahorski. Dihydropyridine calcium channel activators and antagonists influence depolarization-evoked inositol phospholipid hydrolysis in brain. *Eur. J. Pharmacol.* **115**:31–36 (1985).
46. Gonzales, R. A., C. Theiss, and F. T. Crews. Effects of ethanol on stimulated inositol phospholipid hydrolysis in rat brain. *J. Pharmacol. Exp. Ther.* **237**:92–98.
47. Zernig, G., T. Moshammer, and H. Glossmann. Stereospecific regulation of [$^3$H]inositol monophosphate accumulation by calcium channel drugs from all three main chemical classes. *Eur. J. Pharmacol.* **128**:221–229 (1986).
48. Cockcroft, S. The dependence on $Ca^{2+}$ of the guanine-nucleotide-activated polyphosphoinositide phosphodiesterase in neutrophil plasma membranes. *Biochem. J.* **240**:503–507 (1986).
49. Jones, L. G., D. Goldstein, and J. H. Brown. Guanine nucleotide-dependent inositol trisphosphate formation in chick heart cells. *Circ. Res.* **62**:299–305 (1988).
50. Merritt, J. E., C. W. Taylor, R. P. Rubin, and J. W. Putney, Jr. Evidence suggesting that a novel guanine nucleotide regulatory protein couples receptors to phospholipase C in exocrine pancreas. *Biochem. J.* **236**:337–343 (1986).
51. Evans, T., J. R. Hepler, S. B. Masters, J. H. Brown, and T. K. Harden. Guanine nucleotide regulation of agonist binding to muscarinic cholinergic receptors. *Biochem. J.* **232**:751–757 (1985).
52. Bradford, P. G., and R. P. Rubin. Guanine nucleotide regulation of phospholipase C activity in permeabilized rabbit neutrophils. *Biochem. J.* **239**:97–102 (1986).
53. Smith, C. D., C. C. Cox, and R. Snyderman. Receptor-coupled activation of phosphoinositide-specific phospholipase C by an N protein. *Science (Wash. D. C.)* **232**:97–100 (1986).
54. Martin, T. J. F., S. M. Bajjalieh, D. O. Lucas, and J. A. Kowalchyk. Thyrotropin-releasing hormone stimulation of polyphosphoinositide hydrolysis in $GH_3$ cell membranes is GTP dependent but insensitive to cholera or pertussis toxin. *J. Biol. Chem.* **261**:10041–10049 (1986).


**Send reprint requests to:** Dr. S. K. Fisher, Neuroscience Laboratory Building, University of Michigan, 1103 E. Huron, Ann Arbor, MI 48104-1687.