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Elevation of Cytoplasmic Calcium Concentration Stimulates Hydrolysis of Phosphatidylinositol Bisphosphate in Chick Heart Cells: Effect of Sodium Channel Activators

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SUMMARY

The sodium channel activators veratridine and batrachotoxin, the sodium ionophore gramicidin, and the calcium ionophore ionomycin stimulated phosphoinositide breakdown, as indicated by the increased accumulation of [^{3}H]inositol monophosphate in embryonic chick heart cells. The levels of [^{3}H]inositol trisphosphate and [^{3}H]inositol bisphosphate were also increased by veratridine, indicating that there was increased hydrolysis of phosphatidylinositol bisphosphate by phospholipase C. The response to veratridine required both extracellular sodium and calcium, suggesting that calcium entry via Na/Ca exchange might activate phospholipase C. Fluorescence measurements with fura-2 confirmed that the sodium agents greatly increased the cytoplasmic calcium concentration. Veratridine (100 μM) increased cytoplasmic calcium from 94 ± 4 nM to 862 ± 103 nM, giving a maximal calcium increase in about 2 min. Batrachotoxin (1 μM) induced an even greater increase in calcium but required a longer time. Gramicidin also induced a large increase in cytoplasmic calcium which was maximal within 0.5 min. To directly test the calcium dependency of phospholipase C, we permeabilized the chick heart cells with saponin and monitored the production of inositol phosphates at different calcium concentrations. Raising the calcium concentration from 3 to 1000 nM increased the accumulation of [^{3}H] inositol phosphates by nearly 4-fold with a half-maximal effect at about 200 nM calcium. The guanine nucleotide guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) also stimulated accumulation of the InsPs and the response to (GTPγS) was potentiated by increasing the calcium concentration. The data suggest that the effect of the sodium agents on phosphoinositide hydrolysis results from an elevation of intracellular calcium which increases GTP-dependent phospholipase C activity. Thus, drugs or other conditions that elevate cytoplasmic calcium in heart cells may increase the hydrolysis of membrane phosphoinositides.

Veratridine and batrachotoxin activate sodium channels in the plasma membrane of excitable cells, promoting the entry of sodium into the cytoplasm from the extracellular fluid (1, 2). Recently, these agents have also been found to stimulate the production of [^{3}H]InsP from [^{3}H]inositol-labeled phospholipids in synaptosomes, brain, and cardiac preparations (3–5). The production of [^{3}H]InsP in these tissues in response to the sodium agents presumably results from activation of phospholipase C to cleave membrane phosphoinositides, but the details of this process have not been elucidated. One proposal is that the sodium agents stimulate calcium entry into the cell, increasing the cytoplasmic calcium concentration to the point where the phospholipase C is activated (5). In previous studies utilizing sodium agents, however, there was no demonstration that cytoplasmic calcium or calcium influx was increased by these agents, nor was it shown that increasing calcium could effect phospholipase C activity. Also, the source of [^{3}H]InsP formed in response to the sodium agents has not been established; potential substrates for phospholipase C which could ultimately increase [^{3}H]InsP production include phosphatidylinositol, phosphatidylinositol 4-phosphate, and PIP_2 (6).

In the present study, we extend the observation that sodium agents cause hydrolysis of membrane phosphoinositides to another preparation, the embryonic chick heart cell. To define the pathway through which this occurs, we have determined which inositol phosphates are produced in response to veratridine. We also show that the sodium agents greatly elevate cytoplasmic calcium in these same cells. Furthermore, inositol polyphosphate production is stimulated in permeabilized cells in which the calcium level is increased and the calcium appears

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ABBREVIATIONS: InsP, inositol monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; TCA, trichloroacetic acid; fura-2, {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid;, fura-2/AM, the cell-permeant pentaacetoxymethyl ester derivative of fura-2; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; quin 2, 2-{[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline.

to augment activation of phospholipase C by a GTP-binding protein.

Materials and Methods

Chick heart cell preparation. The hearts from 13-day chick embryos were removed and minced in a Krebs-Henseleit buffer containing (in mM): 118 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 glucose, 20 HEPES. The tissue pieces were then placed in a buffer without added calcium or magnesium and incubated in 0.25% trypsin for 40 min at 37°. Bovine serum albumin (1%) was added to stop the enzymatic digestion; the cells were triturated and washed several times with the Krebs-Henseleit buffer described above. Typical yields were 7 × 10^6 cells/heart at 83–93% viability (trypan blue exclusion).

Analysis of phosphoinositide metabolism. The heart cells were placed in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 1% Fungibact and were plated in 35-mm tissue culture plates. Labeling of the membrane phosphoinositides was achieved by incubating the cells with 1 μCi/ml [^{3}H]inositol overnight at 37° in a 95% air/5% CO_2 atmosphere. For some experiments, measurements of [^{3}H]InsP_2 and [^{3}H]InsP_3 production were facilitated by increasing the concentration of [^{3}H]inositol to 20–40 μCi/ml. Following the overnight incubations with [^{3}H]inositol, the cells were washed with Dulbecco's modified Eagle's medium; 0.75 ml of buffer containing the drug to be tested and 10 mM $LiCl_2$ were then added for an exposure period of either 2 or 30 min. Following the drug exposures, the medium was removed, 10% TCA was added, and the cells were scraped from the plates in 1 ml total volume. The TCA cell extract or the TCA-treated media from the permeabilized cells (see below) were extracted with ether (five times) and the [^{3}H]inositol phosphates were separated by anion exchange column chromatography (7, 8). Results were quantified via liquid scintillation counting of the [^{3}H]InsP, [^{3}H]InsP_2, and [^{3}H]InsP_3 fractions.

Measurement of cytoplasmic calcium concentration. Freshly dissociated cells were separated from the red blood cells via centrifugation in a Percoll density gradient (26,000 × *g* at bottom of gradient) for 30 min. The cells were washed twice via centrifugation to remove the Percoll and resuspended in the Krebs-Henseleit buffer described above. All measurements of cytoplasmic calcium were performed on the same day as the cell preparation. Cells were loaded with the fluorescent calcium indicator fura-2 by incubating the cells in 1 μM fura-2/AM for 30 min. Following this loading, the cells were gently centrifugated and resuspended in buffer that was free of fura-2/AM to begin the fluorescent analysis. Fluorescence measurements were made on 2-ml samples continuously stirred in polystyrene cuvettes (from Fisher, rated for use at 340 nm). Fluorescence was monitored with a SPEX Fluorolog spectrofluorometer equipped with a "chopper" to allow excitation at dual wavelengths. Excitation was at 340 and 380 nm (4.6-nm bandpass). Emission was monitored at 510 nm (10-nm bandpass), and a cutoff filter (Oriel no. 51490) was used to further exclude fluorescence below 480 nm. Calcium concentrations were calculated from the ratio of the 340- and 380-nm signals via the equation:

$$[Ca_i] = K_d B(R-R_{min})/(R_{max}-R)$$

where K_d is the dissociation constant for the fura-2/Ca^{2+} complex (224 nM at 37°) (9), R_{max} is the ratio between the 340- and 380-nm signals when the fura-2 is calcium saturated, R_{min} is the ratio for calcium-free fura-2, and B is the ratio between the calcium-free and calcium-saturated 380-nm signals. To determine R_{max}, R_{min}, and B for the fura-2 within the chick heart cells, 10 μM ionomycin was added to the fura-2-loaded cells in the standard Krebs-Henseleit buffer, allowing sufficient calcium to enter the cells and fully saturate the fura-2, yielding the R_{max}; next, 20 mM EGTA was added (pH > 8), which removed calcium from the cells to yield R_{min} (10). Values of 25.4, 1.04, and 8.33 were obtained for the R_{max}, R_{min}, and B ratios, respectively.

Cell permeabilization. The chick heart cells were permeabilized according to the method of Jones *et al.* (11). Briefly, following the overnight culture and labeling with [^{3}H]inositol, the cells were resuspended in a buffer resembling intracellular fluid, containing (in mM): 110 KCl, 10 NaCl, 1 KH_2PO_4, 20 HEPES, 4 $MgCl_2$, 1 EGTA, 3 Na_2ATP, 8 creatine phosphate, 6 units/ml creatine kinase (pH 7.0), and saponin (100 μg/ml) for 5 min, then washed and resuspended in saponin-free intracellular buffer. To determine the effect of calcium on phosphoinositide metabolism in permeabilized cells, free calcium concentrations from 3 to 5000 nM were prepared by adding different amounts of $CaCl_2$ to the intracellular buffer according to the MS-DOS-compatible computer programs SKIN1A and "FREE1" (obtained from Dr. Chi-Ming Hai, University of Virginia, Charlottesville, VA). These programs calculate free calcium concentration utilizing association constants from Fabiato (12). To start the experimental calcium exposure, the normal intracellular buffer was removed and replaced with the test calcium concentration buffer which also contained 10 mM lithium. Following the exposure period (either 2 or 30 min), the medium from the permeabilized cell samples was removed and acidified with TCA at a final concentration of 10%. Under these conditions of permeabilization, the inositol phosphates formed from hydrolysis of the membrane phosphoinositides diffuse from the cells into the medium (11).

Results

Activation of phosphoinositide hydrolysis by sodium agents and calcium ionophore. To determine whether agents that promote sodium entry initiate phosphoinositide hydrolysis in chick heart cells, cells prelabeled with [^{3}H]inositol were exposed to veratridine, batrachotoxin, or gramicidin for 30 min and assayed for [^{3}H]InsP. Each of the sodium agents increased [^{3}H]InsP significantly above the basal value (Table 1). The responses to veratridine and batrachotoxin were dose dependent, with batrachotoxin having the greatest effect. Tetrodotoxin inhibited the [^{3}H]InsP response to veratridine, confirming that the action of this agent occurred via a specific interaction with the sodium channel. The calcium ionophore ionomycin also promoted the accumulation of [^{3}H]InsP (Table 1).

To determine whether the effect of the sodium agents was on the hydrolysis of the polyphosphoinositide, PIP_2, we monitored production of tritiated InsP, InsP_2, and InsP_3 following addition of 100 μM veratridine. Since [^{3}H]InsP_3 accumulates rapidly, we used a shorter exposure time (2 min) in this experiment. Veratridine induced significant elevations of [^{3}H]InsP_3 and [^{3}H]InsP_2 (Fig. 1), although [^{3}H]InsP was not yet elevated at this early time point (data not shown). The elevation of [^{3}H]InsP_3 indicates that the primary effect of veratridine is on the

TABLE 1

Effects of sodium agents and ionomycin on [^{3}H]InsP accumulation in embryonic chick heart cells

The cells labeled with [^{3}H]inositol were exposed to the test agents for 30 min. Data are the per cent of control (basal) accumulation of [^{3}H]InsP, mean ± standard error, with the number of determinations given in parentheses.

Treatment	[^{3}H]InsP (% basal)
10 μM Veratridine	119 ± 7 (4)
30 μM Veratridine	179 ± 9 (4)[a]
100 μM Veratridine	193 ± 7 (18)[a]
100 μM Veratridine + 0.5 μM TTX[b]	125 ± 5 (7)
100 μM Veratridine + 15 μM TTX	104 ± 11 (4)
0.1 μM Batrachotoxin	135 ± 3 (3)[a]
1 μM Batrachotoxin	256 ± 12 (3)[a]
1 μM Gramicidin	160 ± 15 (4)[a]
10 μM Ionomycin	291 ± 30 (7)[a]

[a] Significantly different from basal ($p < 0.05$, Newman-Keuls test applied to the [^{3}H]InsP cpm data before normalization to controls).
[b] TTX, tetrodotoxin.

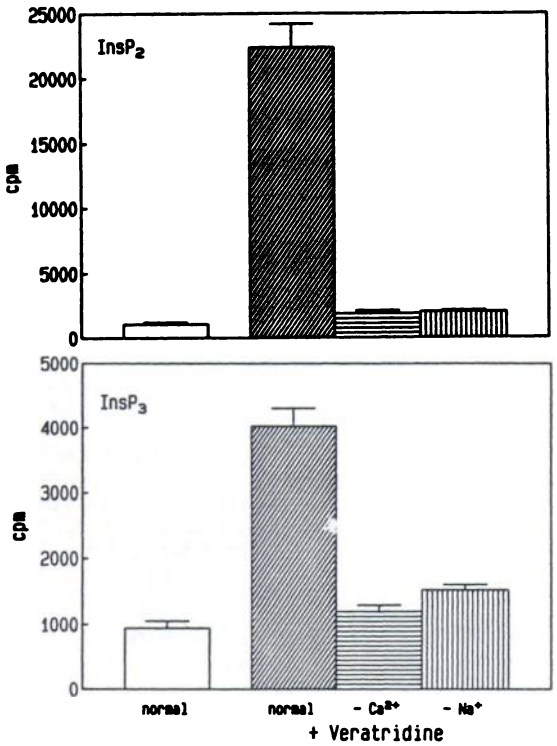

Fig. 1. Effect of 100 μM veratridine on [^{3}H]InsP$_2$ and [^{3}H]InsP$_3$ formation. Drug exposures were for 2 min. □, cells in the absence of veratridine in normal buffer; cells in the presence of veratridine in normal (▨), calcium-free (■), or sodium-free (■) buffer are also shown. The normal buffer contained 1.8 mM $CaCl_2$ and 120 mM NaCl. The low calcium buffer contained no added calcium and 1 mM EGTA (final calcium concentration ~3 nM). The low sodium buffer contained no added sodium (NaCl replaced isosmotically with sucrose). Each *bar* represents the mean ± standard error for n = 3 or 4.

hydrolysis of PIP_2, as is the case with muscarinic stimulation in this tissue (11). [^{3}H]InsP$_2$ could be formed by either the dephosphorylation of [^{3}H]InsP$_3$ or as a result of phosphatidylinositol 4-phosphate hydrolysis.

The muscarinic antagonist atropine (1 μM) and the α_1-adrenergic antagonist prazosin (1 μM) had no effect on the response to veratridine (data not shown); this rules out the possibility that the response to veratridine results from endogenously released acetylcholine or norephinephrine.

Veratridine has been shown to stimulate calcium as well as sodium influx in chick heart cells, apparently via Na/Ca exchange (13, 14). To determine whether sodium or calcium influx was required for inositol phosphate formation in response to veratridine, we tested this agent in buffers either without calcium (no added calcium and 1 mM EGTA) or without sodium (NaCl isosmotically replaced with sucrose). Removal of either calcium or sodium from the buffer virtually abolished the elevations in [^{3}H]InsP$_2$ and [^{3}H]InsP$_3$ produced by veratridine (Fig. 1).

The effect of the sodium agents and ionomycin on intracellular calcium. To directly demonstrate that the sodium agents raise the cytoplasmic calcium concentration, veratridine, batrachotoxin, and gramicidin were added to freshly dissociated cells that had been loaded with the fluorescent calcium indicator, fura-2. Basal calcium concentrations averaged 94 ± 4 nM (mean ± SE, n = 28). Addition of veratridine to the cells resulted in a substantial increase in fluorescence (Fig. 2); cytoplasmic calcium concentrations of 862 ± 103 nM (n = 11) and 1310 ± 60 nM (n = 6) were achieved with 100 μM and 1 mM veratridine, respectively, and the EC_{50} for veratridine was about 40 μM. The effect of veratridine also developed more quickly with higher drug concentrations; for 100 μM and 1 mM veratridine, peak calcium concentrations were reached in 120 and 60 sec, respectively. The response to veratridine depended upon the presence of both extracellular calcium and extracellular sodium (Fig. 2) and was blocked by 0.5 μM tetrodotoxin. The effect of batrachotoxin on cytoplasmic calcium (Fig. 2) was somewhat different from the effect of veratridine in that the response developed more slowly and kept increasing during the drug exposure; the response to batrachotoxin never reached a final plateau. Cytoplasmic calcium concentrations in excess of 1.5 μM were achieved with 1 μM batrachotoxin within 10 min. Gramicidin also rapidly increased the cytoplasmic calcium concentration (Fig. 2); the effect was even more immediate than that of veratridine.

Adding 10 μM ionomycin to the cells rapidly increased the cytoplasmic calcium levels as well (not shown), with concentrations in excess of 4 μM calcium achieved within 40 sec. Enough calcium was let into the cells by ionomycin that the fura-2 soon became saturated with calcium; thus, we could not determine the final calcium concentration reached in response to ionomycin, only that it was higher than that achieved with the sodium agents.

Direct activation of phospholipase C by calcium. To test the effect of increasing calcium on inositol phosphate accumulation, cells labeled with [^{3}H]inositol were permeabilized with saponin and the calcium concentration was varied by adjusting the $CaCl_2$ content of the medium. The calcium-dependent accumulation of total [^{3}H]inositol phosphates is shown in Fig. 3A. The EC_{50} for calcium was about 200 nM calcium and a maximum response occurred at about 1 μM. These data suggest that calcium concentrations in the range achieved by the sodium agents can directly activate the phospholipase C responsible for the cleavage of the membrane polyphosphoinositides. Separation of the phosphoinositide products formed following 2-min exposures to either 3, 300, or 1000 nM concentrations of calcium confirmed that production of [^{3}H]InsP$_3$ and [^{3}H]InsP$_2$ was increased by raising calcium concentration (Table 2).

To test for possible synergistic effects between calcium and the putative G-protein that activates phospholipase C in the chick heart cells, we manipulated the permeabilized cell calcium concentration in the presence of 30 μM GTPγS. At all calcium concentrations, GTPγS substantially increased inositol phosphate accumulation, which is consistent with the proposed participation of a G-protein in the regulation of phospholipase C. The response to GTPγS was potentiated as calcium was increased between 3 nM and 5 μM (Fig. 3B).

Discussion

In the present study, we show that agents that promote sodium influx across the plasma membrane (veratridine, batrachotoxin, gramicidin) induce the hydrolysis of membrane phosphoinositides as monitored by the production of [^{3}H]InsP in embryonic chick heart cells. These results extend those reported for these and related agents in brain slices, synaptosomes, and atrial slices (3–5). To determine the route of [^{3}H]

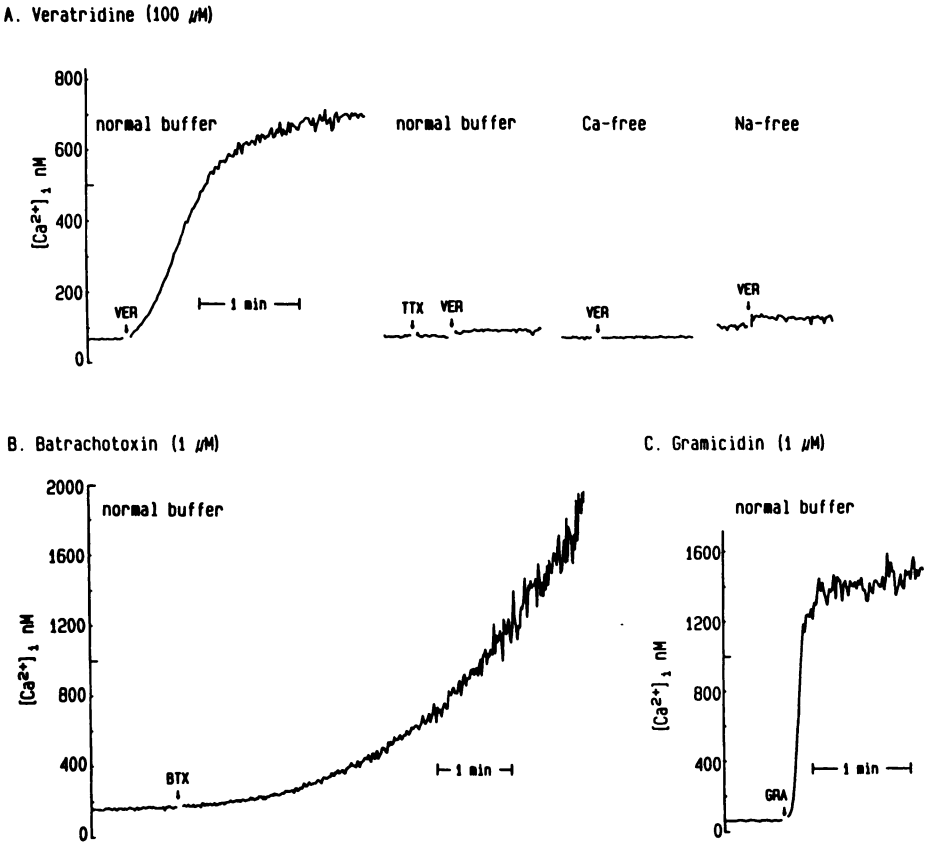

Fig. 2. The effect of the sodium agents on cytoplasmic calcium concentrations in chick heart cells determined with fura-2. A. Effect of 100 μM veratridine (*VER*) in normal buffer, normal buffer also containing 0.5 μM tetrodotoxin (*TTX*), calcium-free buffer, and sodium-free buffer. Buffers are as described in the legend to Fig. 1. B. Effect of 1 μM batrachotoxin (BTX) in normal buffer. C. Effect of 1 μM gramicidin (*GRA*) in normal buffer.

InsP production, we examined each of the inositol phosphate metabolites. Veratridine led to the early production of [^{3}H]$InsP_3$ and [^{3}H]$InsP_2$ prior to any significant increase in [^{3}H]InsP. These results are evidence for the stimulation of PIP_2 hydrolysis (with concomitant $InsP_3$ and diacylglycerol production) by the sodium agents.

The action of veratridine on [^{3}H]InsP formation was blocked by tetrodotoxin, and the veratridine-stimulated accumulation of [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ required extracellular sodium, confirming that the effect of this agent involves sodium influx. Removing extracellular calcium also blocked the response to veratridine. This is consistent with the hypothesis by Gurwitz and Sokolovsky (5) that calcium influx stimulated by the sodium agents activates phospholipase C to hydrolyze membrane phosphoinositides. Another result consistent with this hypothesis was that ionomycin, a calcium ionophore, also stimulated the hydrolysis of membrane phosphoinositides. To confirm that the sodium agents increased cytoplasmic calcium, we monitored the effects of the sodium agents on cytoplasmic calcium in cells containing the fluorescent calcium indicator fura-2. Veratridine at 100 μM increased the cytoplasmic calcium from 94 to 860 nM. Batrachotoxin and gramicidin also significantly increased cytoplasmic calcium. In some cases, cytoplasmic calcium concentrations in excess of 1 μM were achieved. The same treatments that blocked [^{3}H]InsP, [^{3}H]$InsP_2$,and [^{3}H]$InsP_3$ production by veratridine (tetrodotoxin, absence of calcium, absence of sodium) also blocked the calcium response to veratridine. These data indicate that the calcium response occurs secondary to sodium entry, probably via Na/Ca exchange (13, 14), and further implicates calcium as the causative agent for the hydrolysis of PIP_2.

Direct evidence that the phospholipase C that stimulates [^{3}H]$InsP_3$ formation can be activated by calcium was obtained by permeabilizing the chick heart cells with saponin and monitoring the production of phosphoinositide metabolites in response to different calcium concentrations (determined by using various EGTA-calcium combinations). The production of labeled InsP, $InsP_2$, and $InsP_3$ by the permeabilized cell preparation exhibited a dose dependency to calcium with an EC_{50} of about 200 nM. Interestingly, the basal cytoplasmic calcium concentration in the chick heart cells is about 90 nM; thus, the phospholipase C enzyme is poised to respond to increases in cytoplasmic calcium.

Our conclusion that an increase in cytoplasmic calcium can activate polyphosphoinositide cleavage by phospholipase C is consistent with previous observations made on a variety of cell preparations. The calcium ionophore A23187, for example, stimulates the hydrolysis of PIP_2 in embryonic chick myoblasts (15), WRK-1 rat mammary tumor cells (16), and rat lacrimal acinar cells (17). Similar effects have also been noted with potassium depolarization which may induce calcium entry into cells by opening voltage-dependent calcium channels (4, 5, 18–23). Furthermore, permeabilized pancreatic acinar cells hydrolyze PIP_2 in response to calcium concentrations in the same calcium range reported here (24), and calcium-sensitive $InsP_3$ formation has been demonstrated for intact liver cells in which the cytoplasmic calcium was varied between 50 and 200 nM by loading the cells with high concentrations of quin-2 (25). Purified phospholipase C can also exhibit calcium sensitivity (26–29).

Addition of GTPγS to permeabilized chick heart cells increases the hydrolysis of membrane polyphosphoinositides ap-

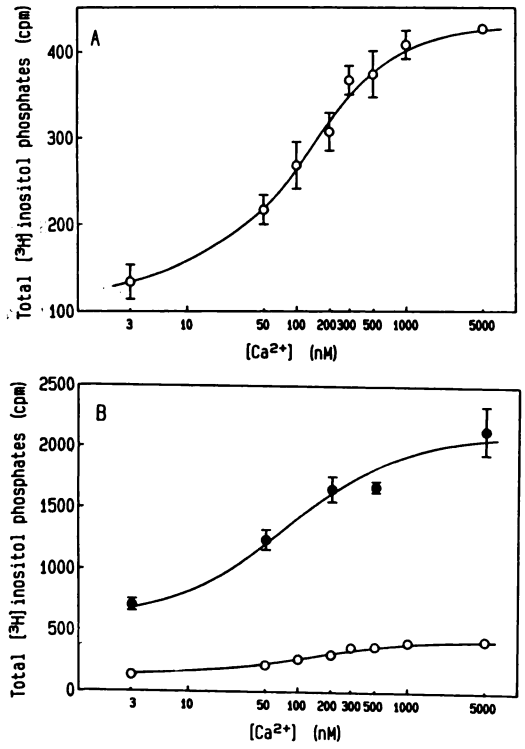

Fig. 3. The effect of calcium on phosphoinositide metabolism in permeabilized chick heart cells. A. The saponin-treated chick heart cells were exposed to calcium concentrations from 3 to 5000 nM (established with various EGTA-calcium combinations) for 30 min. The data for [^{3}H]InsP, [^{3}H]InsP$_2$, and [^{3}H]InsP$_3$ were pooled. B. Effect of 30 μM GTPγS on accumulation of inositol phosphates in the permeabilized cells. The data shown are the pooled inositol phosphate response in the presence of GTPγS (●) and in the absence of GTPγS (○; data from A regraphed for comparison).

TABLE 2

Production of [^{3}H]InsP$_2$ and [^{3}H]InsP$_3$ at different calcium concentrations in permeabilized cells

Saponin-permeabilized chick heart cells were exposed to 3, 300, or 1000 nM free calcium concentration for 2 min. Data are cpm in the [^{3}H]InsP$_2$ and [^{3}H]InsP$_3$ fractions, mean ± standard error, for n = 3–5. For both [^{3}H]InsP$_2$ and [^{3}H]InsP$_3$, the data at 300 and 1000 nM are significantly larger than the values obtained at lower calcium concentrations (Newman-Keuls test, $p < 0.05$).

[Ca^{2+}] (nM)	[^{3}H]InsP$_2$	[^{3}H]InsP$_3$
3	296 ± 8	193 ± 22
300	397 ± 37	352 ± 25
1000	492 ± 23	497 ± 40

proximately 10-fold (11), an effect similar to that observed in other preparations (30–34). This is apparently due to the activation of a GTP-binding protein (G-protein) that couples to and activates phospholipase C. The extent to which GTPγS stimulated inositol phosphate production was sensitive to calcium within the range of calcium concentrations achieved in the cytoplasm by the sodium agents. The effect of calcium on PIP$_2$ hydrolysis may therefore be at the level of the G-protein that regulates the phospholipase C (32, 33). Although the calcium sensitivity of inositol phosphate accumulation in the absence of GTPγS suggests an effect of calcium directly on the phospholipase, this "basal" activity might in fact be due to stimulation of the G-protein by endogenous GTP remaining in the permeabilized cells.

To summarize, our results demonstrate that sodium agents elevate intracellular calcium in chick heart cells. The increased calcium concentration in the cytoplasm appears to be sufficient to activate phospholipase C and increase hydrolysis of the membrane polyphosphoinositides to produce InsP$_3$ and, presumably, diacylglycerol. One implication of our results is that phospholipase C might be activated, *in vivo*, by contractile-associated calcium transients. Cytoplasmic calcium concentrations achieved in muscle cells during contraction are similar to or even higher than the levels achieved with sodium agents in the present study (35, 36). The finding that the phospholipase C of this tissue is calcium sensitive also suggests that pathological conditions or drug treatments that lead to elevations in cytoplasmic calcium might activate the phosphoinositide hydrolysis pathway in the heart.

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