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C L Fraser, P Sarnacki

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Research Article

The role of inositol 1,4,5-trisphosphate (1,4,5-IP3) in regulating cytosolic Ca++ by stimulating Ca++ release from intracellular organelles is well established. However, other modes of intracellular Ca++ regulation by 1,4,5-IP3 have not been determined. To determine if 1,4,5-IP3 may regulate cell cytosolic Ca++ by acting on plasma membrane bound Na(+)-Ca++ exchanger, we investigated Ca++ transport in synaptosomes using 45Ca++ as tracer. In the presence of either an inhibitor of voltage gated Na+ channels (tetrodotoxin) or the K+ ionophore (valinomycin), Ca++ uptake was significantly inhibited (P less than 0.05) by 1,4,5-IP3 in a concentration dependent manner, with half-maximal inhibition occurring at submicromolar concentrations between 10(-9) M and 10(-10) M 1,4,5-IP3. Similarly, Ca++ efflux by the exchanger was significantly inhibited 40% by 1,4,5-IP3. The inhibitory effect of 1,4,5-IP3 on the Na(+)-Ca++ exchanger was observed in the presence of Ca++ channel blockers, and in vesicles pretreated with caffeine to deplete the 1,4,5-IP3-sensitive stores of Ca++. These results suggest that during signal transduction in brain, 1,4,5-IP3 may increase cytosolic [Ca++] in part by inhibiting the Na(+)-Ca++ exchanger and thus, Ca++ efflux from cell.

Inositol 1,4,5-Trisphosphate May Regulate Rat Brain Ca_i^{++} by Inhibiting Membrane Bound Na^+-Ca^{++} Exchanger

Cosmo L. Fraser and Philip Sarnacki
Divisions of Nephrology and Geriatrics, Department of Medicine, Veterans Affairs Medical Center, San Francisco, California 94121; and University of California at San Francisco, 94143

Abstract

The role of inositol 1,4,5-trisphosphate (1,4,5-IP3) in regulating cytosolic Ca^{++} by stimulating Ca^{++} release from intracellular organelles is well established. However, other modes of intracellular Ca^{++} regulation by 1,4,5-IP3 have not been determined. To determine if 1,4,5-IP3 may regulate cell cytosolic Ca^{++} by acting on plasma membrane bound Na^+-Ca^{++} exchanger, we investigated Ca^{++} transport in synaptosomes using $^{45}Ca^{++}$ as tracer. In the presence of either an inhibitor of voltage gated Na^+ channels (tetrodotoxin) or the K^+ ionophore (valinomycin), Ca^{++} uptake was significantly inhibited ($P < 0.05$) by 1,4,5-IP3 in a concentration dependent manner, with half-maximal inhibition occurring at submicromolar concentrations between 10^{-9} M and 10^{-10} M 1,4,5-IP3. Similarly, Ca^{++} efflux by the exchanger was significantly inhibited 40% by 1,4,5-IP3. The inhibitory effect of 1,4,5-IP3 on the Na^+-Ca^{++} exchanger was observed in the presence of Ca^{++} channel blockers, and in vesicles pretreated with caffeine to deplete the 1,4,5-IP3-sensitive stores of Ca^{++}. These results suggest that during signal transduction in brain, 1,4,5-IP3 may increase cytosolic [Ca^{++}] in part by inhibiting the Na^+-Ca^{++} exchanger and thus, Ca^{++} efflux from cell. (*J. Clin. Invest.* 1990. 86:2169–2173.) Key words: synaptosomes • calcium transport • cytosolic calcium • inositol phosphates • caffeine

Introduction

The regulation of cytosolic Ca^{++} by inositol 1,4,5-trisphosphate (1,4,5-IP3)[1] has been shown to be primarily due to the release of Ca^{++} from intracellular microsomes (1–3). However, recent evidence indicates that 1,4,5,-IP3 may also increase cytosolic Ca^{++} by changing flux from extracellular Ca^{++} stores (4), though the mechanism is poorly understood (5). Injection of 1,4,5-IP3 into cells has produced a rise in intracellular Ca^{++} which is due to both an immediate Ca^{++} release from an intracellular pool, and a more prolonged phase attributed to calcium influx from extracellular space (6–11). However, only one study thus far has shown that 1,4,5-IP3 stimulated Ca^{++} influx may be mediated by a membrane bound receptor (12). Recently, 1,4,5-IP3 was shown to increase the permeability of plasma membrane vesicles to Ca^{++} (13), and to increase Ca^{++} current through membrane patches of lymphocytes (14). In other studies, 1,4,5-IP3 enhanced Ca^{++} entry occurred only when it was coinjected with 1,3,4,5-IP4 (6, 8, 9). In spite of these reports, mechanisms by which 1,4,5-IP3 may increase cytosolic Ca^{++}, other than by Ca^{++} release from intracellular organelles, are unclear.

In cells, cytosolic [Ca^{++}] is regulated in part by the Na^+-Ca^{++} exchanger (15). In excitable tissues, the exchanger operates in a bidirectional manner (16–18), capable of participating physiologically and importantly in both Ca^{++} influx and efflux (17–21). Calcium influx occurs when the cell membrane is depolarized, and efflux of Ca^{++} occurs when the membrane is repolarized (15, 22). The bidirectionality of the Na^+-Ca^{++} exchanger in excitable tissues is operative in both synaptosomes and synaptic plasma membranes (18, 23, 24). In non-excitable tissues, the exchanger functions primarily to efflux Ca^{++} from cells (25). Because it appears that a primary role of 1,4,5-IP3 is to regulate cytosolic Ca^{++}, we hypothesized that 1,4,5-IP3 may control the function of the Na^+-Ca^{++} exchanger.

Methods

Isolation of synaptosomes

Synaptosomes were isolated from 200 g male Sprague-Dawley rats as we previously described (26). In brief, the rats were decapitated and their cerebral cortex removed and immediately placed in 10 ml of ice-cold isolation media containing 320 mM sucrose, 0.2 mM K-EDTA, 5 mM Tris-HCl, pH 7.4 at 0–4°C. The brains were chopped finely with scissors, washed with the isolation media and homogenized in a glass Dounce homogenizer (clearance 1 mm). The crude synaptosomal-mitochondrial pellet was obtained after several centrifugations at 1,300 *g* for 3 min and a final spin at 18,000 *g* for 10 min. The purified synaptosomal preparation was then obtained by differential centrifugation on a discontinuous Ficoll gradient for 60 min (26, 27). The synaptosomal protein (containing ~ 30–40% inverted vesicles) is diluted in isolation media to a final concentration of 8–10 mg/ml and used for transport studies.

Calcium transport assay

Uptake studies. Calcium uptake studies were carried out by the Na-Ca exchange mechanism as previously described (23). Half a milliliter aliquot of synaptosomal protein (~ 5 mg protein) was brought up to 2 ml in a preequilibration media (140 mM NaCl, 1 mM $MgSO_4$, 10 mM glucose, 5 mM Hepes-Tris, pH 7.4) and allowed to incubate for 10 min

Address correspondence to Cosmo L. Fraser, M.D., Veterans Affairs Medical Center, Division of Nephrology (111J), 4150 Clement St., San Francisco, CA 94121.

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1. *Abbreviations used in this paper:* 1,4,5-IP3, 1,4,5-trisphosphate; TTX, tetrodotoxin.

at 37°C. The suspension was then spun at 20,000 *g* for 5 min and the resulting pellet was resuspended in the same media and spun at the same speed for 5 min. At the end of the second spin, the final pellet was resuspended in 400 μl of the preequilibration media and kept on ice (0–49°C) until transport studies were commenced.

Uptake was initiated by adding 5 μl of synaptosomal suspension (~ 50 μg protein) to 95 5 μl of an external media consisting of 140 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 10 μM $CaCl_2$, 0.1 μCi $^{45}Ca^{++}$ (40,000 cpm/20 μl), 5 mM Hepes-Tris, pH 7.4 at 25°C. Depending on the experimental protocol a number of compounds (4,5-IP2, 1,4,5-IP3, 1,3,4-IP3, 1,3,4,5-IP4, caffeine, D600, diltiazem) were added to the external media before transport studies were begun. After the desired period of incubation, uptake was terminated by adding to the uptake media 2 ml of ice-cold 150 mM KCl solution (stop solution). The mixture is immediately vacuum filtered through a 0.45-μm pore size cellulose acetate filter and washed twice with 2 ml of the cold stop solution as previously described (24). The filters were dissolved in ScintiVerse scintillant and counted by a model 2000CA counter (Packard Instrument Co., Inc., Downers Grove, IL). Each time point was done in triplicate and the value obtained at time zero, which we attributed to binding of calcium to protein and filter, was subtracted from each of the points of observation (23). As we described previously, nonspecific binding to the filter and synaptosomal protein was < 0.05% (26).

Efflux studies. For calcium efflux studies the vesicles are loaded by the Na-Ca^{++} exchanger for 10 min as discussed above. At the end of this period, the loaded vesicles are spun at 20,000 *g* for 10 min and resuspended to a protein concentration of 10 mg/ml in the external media 140 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 10 μM $CaCl_2$, 0.1 μCi $^{45}Ca^{++}$ 40,000 cpm/20 μl, 5 mM Hepes-Tris, pH 7.4) and kept on ice until efflux studies were commenced. Efflux was promptly started by diluting 5 μl of the calcium loaded vesicles in 95 μl of a calcium-free efflux media consisting of 140 mM NaCl, 1 mM $MgSO_4$, 10 mM glucose, 5 mM Hepes-Tris, pH 7.4 at 25°C with appropriate additions of the test compounds. At the appropriate period of efflux the reaction was terminated by adding 2 ml of ice-cold stop solution to 100 μl of the transport mixture (23). The mixture was then filtered as above, washed twice with the stop solution and counted. The initial time point was obtained at the instant before starting the efflux studies and this was taken as the maximum value of calcium where comparison was made over time.

Inositol 1,4,5-trisphosphate transport assay

To determine if synaptosomes take up 1,4,5-IP3, vesicles were preequilibrated in a media containing 140 mM NaCl, 1 mM $MgSO_4$, 10 mM glucose, 5 mM Hepes-Tris, pH 7.4, and 0.1 M 1,4,5-IP3 to saturate the 1,4,5,-IP3 receptor sites. ^{3}H-1,4,5-IP3 was then used as tracer in an external media which contained 140 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 10 μM $CaCl_2$, 0.1 mM 1,4,5-IP3, 5 mM Hepes-Tris, pH 7.4 at 25°C. Except for the additions of cold 1,4,5-IP3, both the preequilibration and external media were identical to those used for Ca^{++} uptake studies, since we wanted to determine if 1,4,5-IP3 crosses synaptosomal membrane under the conditions of our experiments. Additionally, in the external medium $^{45}Ca^{++}$ was replaced with ^{3}H-1,4,5-IP3. ^{3}H-1,4,5-IP3 uptake was terminated and counted in ScintiVerse scintilant as discussed above at the appropriate time of incubation.

Materials

$^{45}Ca^{++}$, ^{3}H-1,4,5-IP3 were obtained from New England Nuclear (Boston, MA). Inositol 1,3,4-trisphosphate was obtained from Calbiochem-Behring Corp. (San Diego, CA). D-Myoinositol 4,5-bisphosphate and inositol 1,3,4,5-tetrakisphosphate were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). ScintiVerse scintilant DB obtained from Fisher Scientific Co. (Santa Clara, CA). Caffeine and methoxyverapamil (D600) were obtained from Sigma Chemical Co. (St. Louis, MO), diltiazem from Marion Laboratory (Kansas City, MO), nifedipine from Pfizer Pharmaceutical (Brooklyn, NY). All other chemicals were of reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Results

To determine the effect of 1,4,5-IP3 on Ca^{++} influx, Ca^{++} uptake by the Na^+-Ca^{++} exchanger was measured in synaptosomes equilibrated with 140 mM NaCl, 1 mM $MgSO_4$, 10 mM glucose, 5 mM Hepes-Tris, pH 7.4 and diluted in an external media containing 140 mM KCl, mM $MgSO_4$, 10 mM glucose, 1 μM tetrodotoxin (TTX), 10 μM $CaCl_2$, 0.1 μCi $^{45}Ca^{++}$ (100,000 cpm/100 μl), 5 mM Hepes-Tris, pH 7.4 at 25°C (23). In some studies membrane potential of the vesicles was fixed at a potential near 0 mV with 25 μM valinomycin and equal $[K^+]$ on both sides (Fig. 1 *B*), instead of TTX (inhibitor of the voltage gated Na^+ channel in nerve cells). Under these conditions, Ca^{++} flux takes place predominantly by the Na^+-Ca^{++} exchanger and not by the Ca^{++} or Na^+ channels. In the K^+/Val experiment (Fig. 1 *B*), the external media contained 100 mM choline chloride and 40 mM KCl instead of 140 mM KCl (where K^+ is replaced by the cation choline, which is imper-

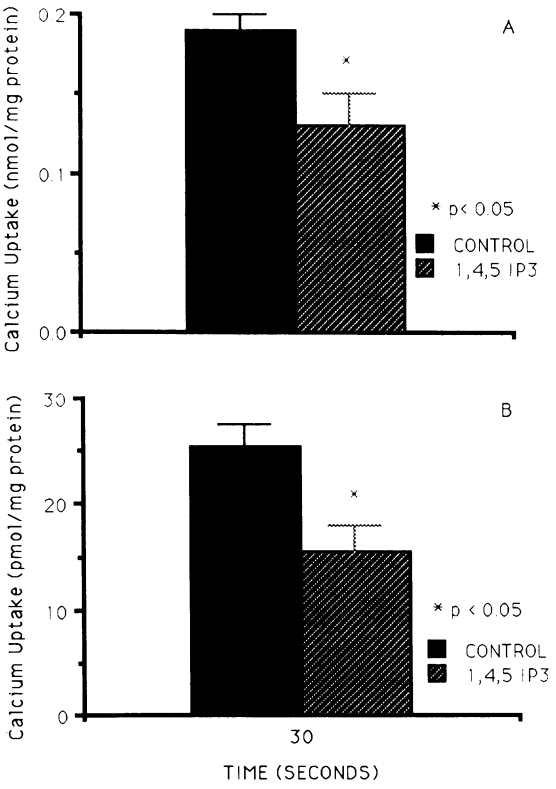

Figure 1. (*A*) Effect of 1,4,5-IP3 on Ca^{++} uptake by the Na^+-Ca^{++} exchanger in rat brain synaptosomes was studied in the presence of 1 μM TTX to inhibit the Na^+ flux through voltage-dependent Na^+ channels. In the presence of 10^{-7} M 1,4,5-IP3, uptake was significantly ($P < 0.025$) less than in its absence. (*B*) Effect of 1,4,5-IP3 on Ca^{++} uptake by the Na^+-Ca^{++} exchanger in rat brain synaptosomes which were maintained at a fixed voltage with 25 μM valinomycin. 10^{-7} M 1,4,5-IP3 significantly ($P < 0.05$) inhibited uptake by 40%. Data are expressed as the mean±SE. Significant differences between groups were determined by two-tailed paired Student's *t* test analysis. Five experiments were performed in each group, and each time point was done in triplicate.

meant and does not disturb the Na^+ gradient) and the loading media contained 100 mM NaCl and 40 mM KCl instead of 140 mM NaCl.

In the presence of 1 μM TTX, 10^{-7} M 1,4,5-IP3 significantly ($P < 0.05$) inhibited uptake by the exchanger from 0.19±0.02 to 0.13±0.01 nmol/mg protein (Fig. 1 *A*). Similarly in the presence of 25 μM valinomycin, 10^{-7} M 1,4,5-IP3 inhibited uptake by 40% (from 25.5±2.0 to 15.4±2.5 pmol/mg protein) (Fig. 1 *B*). The apparent inhibition of Ca^{++} transport with valinomycin is principally reflective of the decreased Na^+ gradient in this experiment. No significant Ca^{++} uptake was observed when equal [Na^+] was on both sides of the membrane in either experiment. The magnitude of 1,4,5-IP3 inhibition of the exchanger in the presence of TTX is concentration dependent, with half-maximal inhibition occurring between 10^{-9} and 10^{-10} M (Fig. 2).

Because the Na^+-Ca^{++} exchanger activity in synaptosomes is bidirectional, we performed studies to determine whether 1,4,5-IP3 also inhibited Ca^{++} efflux from vesicles loaded by the Na^+-Ca^{++} exchanger (23). As shown with the uptake studies, Ca^{++} efflux was significantly ($P < 0.025$) inhibited by 1,4,5-IP3 between 5 and 10 s after transport was initiated (Fig. 3). 15 s after efflux began, the effect of 1,4,5-IP3 was no longer apparent, as the amounts of Ca^{++} remaining in vesicles from both groups were no longer significantly different from each other. The equilibration of vesicular Ca^{++} in both groups at these later times indicates that the Na^+ gradient, which is the driving force for this process, was largely dissipated. This effect could also in part be due to a decreased Ca^{++} gradient across the plasma membrane after the vesicles have been essentially exhausted of Ca^{++}. The dependence of this transport process on the Na^+ gradient is further dramatized in Fig. 3, where in the absence of a Na^+ gradient ($Na_i = Na_o$), Ca^{++} was not transported out of the vesicles in either group. This suggests that the transport process is Na^+ driven and that in the absence of a Na^+ gradient the Na^+-Ca^{++} exchanger is inactivated (Fig. 3).

To determine the specificity of 1,4,5-IP3 on the exchanger, we performed Ca^{++} transport studies in the presence of each of the following compounds: 10^{-7} M 1,3,4,5-IP4; 10^{-7} M 1,3,4-IP3; 10^{-7} M 4,5-IP2. No significant differences in uptake were observed when compared to control, and no additional inhibitory effect was observed when studies were carried out in conjunction with 1,4,5-IP3 and any of these compounds. In the presence of 1,4,5-IP3 in combination with each of the following (1,3,4-IP3, 1,3,4,5-IP4, and 4,5-IP2), Ca^{++} uptake were 0.18±0.03, 0.18±0.03, 0.19±0.02, and 0.18±0.02 nmol/mg protein, respectively. We next determined whether the Ca^{++} channels played a significant role in this Ca^{++} transport process. Using the Ca^{++} channel antagonists nifedipine, diltiazem, and D600, which inhibits L-type channels (28, 29), we found the same inhibition of transport by 1,4,5-IP3 that was found in the absence of these compounds, suggesting that the L-type Ca^{++} channels were not playing a significant role in the transport of Ca^{++}.

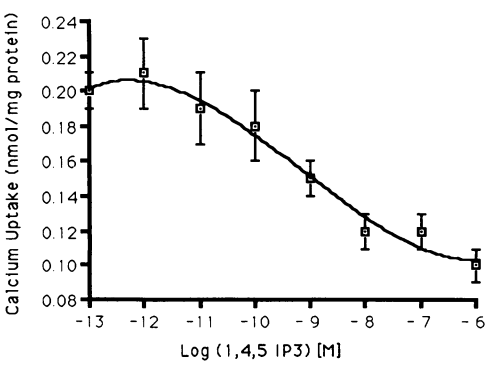

Figure 2. Effect of increasing [1,4,5-IP3] on Ca^{++} uptake by the Na^+-Ca^{++} exchanger in the presence of 1 μM TTX. Over [1,4,5-IP3] of 10^{-13}–10^{-6} M, Ca^{++} uptake went from 0.20±0.01 to 0.10±0.01 nmol/mg protein. Half-maximal inhibition is observed at [1,4,5-IP3] between 10^{-10} and 10^{-9} M. Data are expressed as the mean±SE. Five experiments were performed in each group, and each time point was done in triplicate.

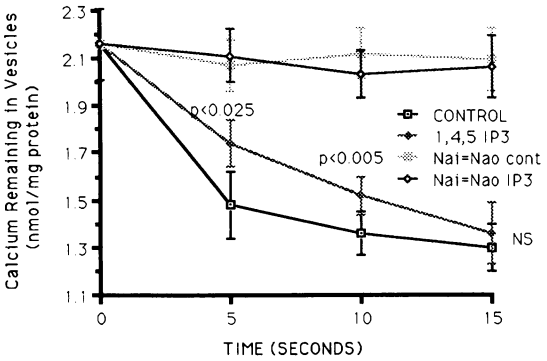

Figure 3. In the presence of Na^+ gradient and TTX, 10^{-7} M 1,4,5-IP3 significantly ($P < 0.025$) inhibits Ca^{++} efflux (third curve down) at the points indicated vs. 1,4,5-IP3 absence (bottom curve). After 15 s, the amount of Ca^{++} remaining in the vesicles was not significantly different ($0.05 < P < 0.1$) in both groups. In the absence of Na^+ gradient ($Na_i = Na_o$), no significant transport occurred±1,4,5-IP3 (top two curves). Data are expressed as the mean±SE. Significant differences between groups were determined by two-tailed paired Student's *t* test analysis. Four experiments were performed, and each time point was done in triplicate.

To investigate if the decreased Ca^{++} uptake observed with 1,4,5-IP3 was due to Ca^{++} release from internal stores, we performed the following studies. We first performed Ca^{++} efflux studies as described (23), and found Ca^{++} efflux to also be decreased with 1,4,5-IP3 (Fig. 3). This suggests that the inhibition of Ca^{++} uptake by 1,4,5-IP3 was not due to Ca^{++} release from internal stores, as a sustained increase in cytosolic Ca^{++} from internal stores would increase the [Ca^{++}] gradient across the plasma membrane and enhanced Ca^{++} efflux by 1,3,4,5-IP3. To further determine if the internal stores affected our results, we first depleted the internal stores of Ca^{++} by incubating the vesicles in a Ca^{++}-free media containing 5 mM caffeine (30, 31), then investigated 1,4,5-IP3 effect on the exchanger. Under these conditions 1,4,5-IP3 still inhibited Ca^{++} uptake as was observed in Figs. 1 and 2.

We next blocked the voltage gated Na^+ channels with 1 μM TTX, inhibited the L-type Ca^{++} channels with 25 μM D600, and depleted the intracellular stores of Ca^{++} with 5 mM caffeine. As shown in Fig. 4, despite these maneuvers, 10^{-7} M 1,4,5-IP3 still significantly ($P < 0.004$) inhibited Ca^{++} uptake by 45%. Thus, it appears likely that the inhibitory effect of 1,4,5-IP3 on Ca^{++} transport in these experiments is principally due to its effect on the Na^+-Ca^{++} exchanger.

To determine if the externally applied 1,4,5-IP3 exerted its inhibitory effects on the Na^+-Ca^{++} exchanger by binding to

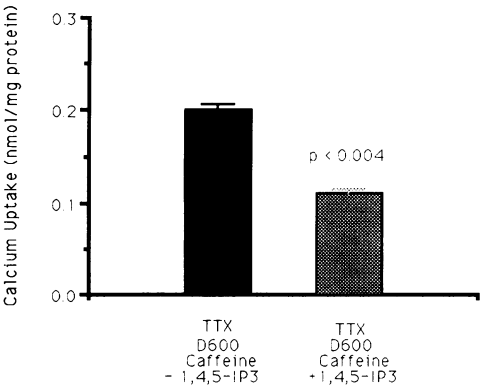

Figure 4. Graph shows the effect of 1,4,5-IP3 on Ca^{++} uptake in vesicles where the voltage gated Na^+ channels were blocked by 1 μM TTX, the Ca^{++} channels blocked by 25 μM D600, and the internal stores depleted of Ca^{++} by 5 mM caffeine. 10^{-7} M 1,4,5-IP3 significantly ($P < 0.004$) inhibited uptake by 45%. Results are obtained from the mean±SE of four experiments. Significant differences between groups were determined by two-tailed paired Student's *t* test analysis. Four experiments were performed, and each time point was done in triplicate.

external sites on synaptosomes, or if 1,4,5-IP3 must be transported into the vesicles to produce its effects, we carried out 1,4,5-IP3 transport using ^{3}H-1,4,5-IP3 as tracer . Our results showed significant binding of ^{3}H-1,4,5-IP3 to synaptosomes, but no evidence of significant active transport across the plasma membrane. In five experiments, the percent binding of ^{3}H-1,4,5-IP3 at times 0, 15, 30, 60, and 120 seconds were 0.73, 0.77, 0.75, 0.80, and 0.82, respectively.

Discussion

These results provide evidence for the possibility that signal transduction by 1,4,5-IP3 in brain synaptosomes may increase cytosolic Ca^{++} not only by the mobilization of Ca^{++} from intracellular stores (1–3), but also by inhibition of Ca^{++} efflux from cytosol by the Na^+-Ca^{++} exchanger.

To determine if the externally applied 1,4,5-IP3 exerted its inhibitory effects by binding to receptor sites on the bidirectional Na^+-Ca^{++} exchanger or if it had to be transported into the vesicles to produce its effects, we investigated 1,4,5-IP3 uptake using radiolabeled 1,4,5-IP3. We found that although there is significant binding of ^{3}H-1,4,5-IP3 to synaptosomes, it is not readily transported across the synaptosomal plasma membrane. This suggest that if 1,4,5-IP3 only binds to receptors on inside out vesicles ($\sim$ 30–40% vesicular preparation), the percent inhibition of Ca^{++} uptake by 1,4,5-IP3 which we observed would underestimate its true *in vivo* inhibitory action. On the other hand, if 1,4,5-IP3 binds to the exchanger on both the inside out and right side out vesicles, the degree of inhibition observed may more accurately reflect 1,4,5-IP3 *in vivo* potential. The latter point is purely speculative, however, because there is no available evidence to indicate that there are receptors for IP3 on the external surface of synaptosomes or any other cell type.

With regards to the efflux studies, it also appears that the binding of IP3 to the inside-out vesicles is responsible for the inhibition of Ca^{++} efflux. This is supported by the fact that the bidirectionality of the Na^+-Ca^{++} exchanger does not represent two exchangers oriented in two directions, but one exchanger operating in two different directions depending on the state of polarization of the plasma membrane (15–21). The caffeine studies suggest that Ca^{++} release from endoplasmic reticulum (ER) at intracellular storage sites did not contribute significantly to our results (Fig. 4). It is also unlikely that the < 5% contamination of our preparation by external ER (26) significantly affected the total Ca^{++} accumulated in the vesicles. If indeed it did, the caffeine pretreatment would also negate the external ER contribution as well.

Because we demonstrated that 1,4,5-IP3 inhibits both Ca^{++} influx and efflux by the exchanger, it is not entirely clear what the net effect of 1,4,5-IP3 action on cytosolic Ca^{++} would be in vivo. It appears quite likely, however, that since 1,4,5-IP3 is an intracellular compound, and it does not readily cross the plasma membrane, its primary action would be to first inhibit the Na^+-Ca^{++} exchanger which causes Ca^{++} efflux. The net effect of which would be to raise cytosolic Ca^{++}.

We contend that the Ca^{++} fluxes were primarily by the exchanger, because Ca^{++} transport was Na^+ dependent (Figs. 1–3), independent of membrane potential changes caused by voltage-dependent channels (Fig. 1), independent of Ca^{++} channel blockers which inhibits L-type channels (Fig. 4), and unaffected by Ca^{++} depletion from intracellular stores (Fig. 4). The effect of 1,4,5-IP3 on the exchanger also appears to be quite specific, because neither 1,3,4,5-IP4, 1,3,4,-IP3, nor 4,5-IP2 affected the ability of the exchanger to transport Ca^{++}. Our conclusion that 1,4,5-IP3 may regulate cytosolic Ca^{++} by interacting with plasma membrane Na^+-Ca^{++} exchanger, is consistent with recent observations in human neutrophils, rat hepatocytes, bovine adrenal cortex, and parotid acinar cells, where receptors for 1,4,5-IP3 located at or near the plasma membrane were isolated (32–36).

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