Inositol Trisphosphate Induces Calcium Release from Nonmitochondrial Stores in Sea Urchin Egg Homogenates

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This study presents evidence that inositol trisphosphate (IP₃) releases Ca²⁺ from intracellular stores in sea urchin eggs. First, high voltage discharge was used to transiently permeabilize eggs and introduce IP₃; the resultant induction of cortical reactions (a well characterized Ca²⁺-dependent event) provided indirect evidence that IP₃ released Ca²⁺ from intracellular stores. Next, Ca²⁺ uptake and release from egg homogenates and homogenate fractions were monitored by both Ca²⁺-sensitive minielectrodes and the fluorescent Ca²⁺ indicator quin-2. Both assay methods showed Ca²⁺ release upon IP₃ addition, with a half-maximal response at 50-60 nM IP₃ and maximal Ca²⁺ release at ~1 µM IP₃. Homogenates were 300-fold more sensitive to IP₃ than IP₆, and Ca²⁺ release was 95% inhibited by the Ca²⁺ antagonist TMB-8 (3 mM). Fractionation by density gradient centrifugation showed that activities for Ca²⁺ sequestration and IP₃ responsiveness co-purified with endoplasmic reticulum microsomes. Following an initial IP₃ addition, homogenates were refractory (desensitized) to additional IP₃. However, if homogenates were centrifuged and the vesicles resuspended in media lacking IP₃, they would respond to added IP₃, therefore, showing that desensitization is most likely due to the presence of IP₃. This study also shows that the mechanism of IP₃ action is inherent to the microsomes and ions present in the medium used, with no cytoplasmic factors being required. The stability of this microsome preparation and the purification obtained with density gradient centrifugation make this a promising system with which to further characterize the mechanism of IP₃ action.

When sea urchin eggs are fertilized, development is activated by the combined effects of a transient increase in intracellular Ca²⁺ and a long-duration increase in intracellular pH (reviews by Refs. 1-3). The Ca²⁺ transient is produced by Ca²⁺ released from intracellular stores (1-3); however, identifying the mechanism by which sperm attachment at the cell surface releases Ca²⁺ from stores within the egg has proved elusive. Only recently has research on phosphatidylinositide metabolism provided a likely mechanism (review by Ref. 4). First it was reported that several cell types which mobilize intracellular Ca²⁺ in response to peptide hormones or other cell surface stimuli also hydrolyze phosphatidylinositol 4,5-bisphosphate to produce diglyceride and inositol trisphosphate (IP₃) (4). Subsequently, IP₃ was shown to release Ca²⁺ from internal stores in a variety of cells (5-10).

Recent studies with sea urchin eggs have reported a 40% increase in phosphatidylinositol 4,5-bisphosphate concentration by 15 s after fertilization of Strongylocentrotus purpuratus eggs (11), thus linking phosphatidylinositolide metabolism with fertilization. Also, IP₃ injected into Lytechinus pictus eggs induced cortical reactions (12), and since the egg cortical reaction is a Ca²⁺-mediated event, this injection experiment provides indirect evidence that IP₃ mediates Ca²⁺ release in these eggs.

We report here the first direct evidence that IP₃ releases Ca²⁺ from intracellular stores in sea urchin eggs. Ca²⁺ uptake by and release from egg homogenates was monitored by both quin-2 and a Ca²⁺-sensitive minielectrode. Ca²⁺ uptake was ATP dependent, and IP₃ induced the release of Ca²⁺. Percoll density gradients were used to fractionate components of these homogenates and showed co-purification of IP₃ responsiveness, ATP-dependent Ca²⁺ sequestration, and glucose-6-phosphatase activity (a marker enzyme for the endoplasmic reticulum); and all three activities were clearly separated from cytchrome c oxidase activity (a mitochondrial enzyme). Therefore, IP₃ induces Ca²⁺ release from a nonmitochondrial store which is most likely the endoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Animal Maintenance and Gamete Handling
Gametes from Lytechinus pictus and Strongylocentrotus purpuratus were collected by intracoelomic injection of 0.5 M KCl. Eggs were spawned directly into reagent grade artificial seawater (ASW, described below), filtered through 95-µm (S. purpuratus) or 125-µm (L. pictus) Nitex screen cloth (Tetko, Inc.), dejellied by brief exposure to pH 5 ASW, and used within 6 h. An aliquot of each egg preparation was tested for fertilizability, and only preparations with ≥70% of the eggs showing an elevation of fertilization envelopes were used.

Media Used
The ASW used for experiments consisted of 460 mM NaCl, 27 mM MgCl₂, 28 mM MgSO₄, 10 mM CaCl₂, 10 mM KCl, and 2.5 mM NaHCO₃. For Ca²⁺-free artificial sea water (OCSW), the Ca²⁺ was deleted from ASW, and NaCl was increased to 470 mM. The pH was adjusted to 8.0 for both ASW and OCSW. For experiments with egg homogenates, an intracellular medium (IM) was used that duplicates the intracellular ionic and osmotic content of sea urchin eggs (13, 14). This IM was modified from Suprynowicz and Mazia (14) and consisted of 250 mM potassium gluconate, 250 mM N-methylglucama...
mine, 20 mM HEPES, 5 mM NaCl, and 1.0 mM MnCl₂. The pH was adjusted to 7.2 by the addition of acetic acid.

For certain experiments, EGTA and Ca²⁺ were added to OCaSW, IM, or egg homogenates to produce media containing a range of free Ca²⁺ concentrations. The resultant free Ca²⁺ levels were computed with the program Chelator (18). The stability of the Ca²⁺ pools used for EGTA were given by Fabiato and Fabiato (15) and those for gluconate were from Martell and Smith (16). IM and IM were prepared by alkaline hydrolysis of their respective phosphatidylinositides using the protocol of Grad0 of Minnesota. IPS, IP₃, and IPS were prepared by alkaline hydrolysis of the other half. The medium would induce Ca²⁺ release from intracellular stores (as approximations of either 1 mM (OCaSW) or 10 mM (IM) and various free Ca²⁺ concentrations). These stocks were prepared from CaCl₂, EGTA, and NaOH or KOH. The concentrations of Na⁺ and K⁺ were kept constant by the addition of NaCl or KCl as needed, and free Mg²⁺ was kept constant by the addition of MgCl₂. After adding these reagents, the pH was adjusted to 8.00 ± 0.02 for OCaSW or 7.10 ± 0.02 for IM or egg homogenates. The EGTA solutions in OCaSW were buffered with 10 mM Tris.

Preparation of Phosphoinositides

The inositol polyphosphonates used in this study were generously supplied to us by Drs. T. F. Walseath and N. D. Goldberg, University of Minnesota. IP₃, IP₄, and IP₅ were prepared by alkaline hydrolysis of their respective phosphatidylinositides using the protocol of Grad0 and Ballou (17). They were then purified by high pressure liquid chromatography on an AG MP-1 anion-exchange resin eluted with trifluoroacetic acid under conditions similar to those used by Axelson et al. (18) to separate nucleotides.

Permeabilization of Eggs by High Voltage Discharge (HVD)

L. pictus eggs were transiently permeabilized by brief pulses of HVD to determine whether IP₃ introduced from the extracellular medium would induce Ca²⁺ release from intracellular stores (as assayed by the induction of cortical reactions). The HVD chamber consisted of two stainless-steel plate electrodes separated by 1 cm in a rectangular chamber of 3-mm depth and 6-mm width. Unless indicated otherwise, each aliquot of eggs was exposed to 10 pulses of 500 V/m each. The pulses had a half-time of 7–10 ms and a current of 14 A.

For each determination, the eggs in 0.4 ml of a 1% egg suspension were washed once with 1 ml of OCaSW, once with 1 ml of OCaSW containing CaEGTA (to produce the desired free Ca²⁺ concentration), and then resuspended in 0.34 ml of the same CaEGTA buffer in OCaSW. IP₃ (7 mM) was added to one-half of this egg suspension, and the other half was used as a control without IP₃. Both were subjected to an identical HVD treatment. Nomarski optics (magnification, 10 x) was used to assay for the percentage of eggs showing cortical reactions, and 100 eggs were counted for each determination.

Protease inhibitors (0.5–1.0 mM phenylmethylsulfonyl fluoride and 0.02 for IM or egg homogenates. The Ca:EGTA solutions in OCaSW or CaK₂EGTA and yEGTA (for IM) to produce final EGTA concentrations of either 1 mM (OCaSW) or 10 mM (IM) and various free Ca²⁺ concentrations. These stocks were prepared from CaCl₂, EGTA, and NaOH or KOH. The concentrations of Na⁺ and K⁺ were kept constant by the addition of NaCl or KCl as needed, and free Mg²⁺ was kept constant by the addition of MgCl₂. After adding these reagents, the pH was adjusted to 8.00 ± 0.02 for OCaSW or 7.10 ± 0.02 for IM or egg homogenates. The EGTA solutions in OCaSW were buffered with 10 mM Tris.

Egg Homogenate Preparation

Unfertilized eggs were dejellied, sequentially washed in OCaSW and IM, and resuspended to 3–25% (v/v) in IM. Unless indicated otherwise, all homogenates contained ATP (0.4 mM) and an ATP-regenerating system consisting of phosphocreatine (4 mM) and creatine phosphokinase (2 units/ml) to provide energy for the Ca²⁺ transport. Protease inhibitors (0.5–1.0 mM phenylmethylsulfonyl fluoride and 2.5 mM benzamidine) were added, and the eggs were homogenized with a Dounce-type glass tissue homogenizer with a size “A” glass pestle. The homogenates were centrifuged for 1 min (13,000 × g, 4 °C) in a Microfuge (model 235A, Fisher Scientific Co.) to remove large particles (e.g., plasma membrane fragments, nuclei, and yolk platelets), and the supernatant was saved. For the Percoll gradients, 25% homogenates were utilized; all remaining experiments used a 3–9% homogenate. Protein content was assayed with the BioRad protein assay (Bio-Rad); 4% homogenates of L. pictus eggs contained 3.7 mg of protein/ml (S.D. = 0.7, n = 15), and 8% homogenates of S. purpuratus eggs contained 4.6 mg/ml (S.D. = 0.3, n = 2). S. purpuratus homogenates showed ≥50% decreases in both Ca²⁺-pumping activity and responsiveness to IP₃ by 5 h from egg homogenization, whereas L. pictus homogenates usually showed no loss during this time. Therefore, L. pictus was used for most experiments reported here.

Monitoring Ca²⁺ Fluxes

Ca²⁺ uptake by and release from Ca²⁺ stores in egg homogenates was monitored by measuring changes in the Ca²⁺ concentration of the medium. Two methods were used to measure these Ca²⁺ levels: a Ca²⁺-sensitive minielectrode and the fluorescent Ca²⁺-sensitive molecule, quin-2.

Ca²⁺ Minielectrodes—Ca²⁺ electrodes were prepared using a protocol similar to that of Prentki (19). Pieces of Teflon tubing (1.5 mm, inner diameter, 4 cm long) were dipped into a solution containing a 10-µl aliquot of the premixed Ca²⁺ electrode cocktail (Fluka Chemical Corp.) and 60 µl of 12% (w/v) polyvinyl chloride dissolved in tetrahydrofuran. The thin membrane formed at the tip of the tubing was allowed to dry, and the tubing was then filled with a Ca²⁺ buffer containing 10-⁰ mM free Ca²⁺ (prepared according to Ref. 20) and stored in this buffer until used (≥12 h). A silver wire was inserted into the Ca²⁺ electrode buffer and connected to a Corning pH/ion meter (model 135). The mini-reference electrode used (model MI-401) was purchased from Microelectrodes, Inc. Assays were conducted with 0.5- or 0.6-ml aliquots of homogenate in a Flexiglas temperature-controlled chamber (17 °C) and stirred with a magnetic stirring bar. The Ca²⁺ calibration solution was prepared by adding CaCl₂ and K₂EGTA to IM to give 10 mM EGTA and various free Ca²⁺ concentrations. Benzamidine interfered with the Ca²⁺ electrode and was omitted from all Ca²⁺ electrode assays.

Quin-2—The fluorescence was measured at 393 nm excitation (2-mm slit) and 492 nm emission (10-nm slit) with a fluorescence spectrophotometer (model 650-10S, Perkin-Elmer) used in ratio mode. Samples were stirred with a magnetic stirring bar, the temperature was maintained at 17 °C, and 0.7-ml aliquots of homogenate were used. The Ca²⁺ concentration of the homogenate was calculated from the relationship,

\[ [\text{Ca}^2+] = K_d (F - F_{\text{min}})/(F_{\text{max}} - F) \]

where \( F \) is the fluorescence measured during an experiment, \( F_{\text{min}} \) and \( F_{\text{max}} \) are fluorescence values for minimal and maximal Ca²⁺ concentrations (measured at the end of each experiment by adding EGTA and then saturating Ca²⁺, respectively, to each homogenate), and \( K_d \) is the dissociation constant for quin-2 (21).

Quantitation of Ca²⁺ Released from Vesicles

After each experiment, the relationship between Ca²⁺ electrode or quin-2 response and changes in Ca²⁺ concentration were determined by adding known amounts of Ca²⁺ to an aliquot of the same homogenate (containing the same reagents). Since the quin-2 response to Ca²⁺ was nonlinear (see inset to Fig. 3), a series of Ca²⁺ additions was always made, with the homogenate starting at the same free Ca²⁺ level as in the experiment being calibrated. This calibration procedure allowed an accurate determination of Ca²⁺ release in the presence of reagents that altered the quin-2 response (3,3′,4,4′-tetrachlorofluorescein and TMB-8) and in homogenates containing differing vesicle or protein content (e.g., fractions from Percoll gradients). Also, for quin-2 experiments, the vesicle content and/or quin-2 concentration was adjusted so that Ca²⁺ release never saturated the quin-2 signal. The Ca²⁺ contamination in IP₃ and apyrase preparations were determined by a similar procedure, with each reagent being added to quin-2 in distilled water (pH buffered at 7.1 with 30 mM HEPES), and the resultant response was calibrated with known amounts of Ca²⁺. The respective Ca²⁺ contents were 0.06 nmol of Ca²⁺/nmol of IP₃ and 0.07 nmol of Ca²⁺/unit of apyrase, and both Ca²⁺ contents were insignificant for the experiments reported here.

Vesicle Purification by Percoll Density Gradient Centrifugation

Percoll was diluted into 1.3 × concentrated IM to produce final concentrations of 25% Percoll and normal strength IM. Since ATP interfered with the assay for glucose-6-phosphatase, one gradient was prepared without ATP or quin-2 and was used for the enzyme assays. A second gradient (containing 0.5 mM ATP, 4 mM phosphocreatine, 2 units/ml creatine phosphokinase, and 10 µM quin-2) was used for...
the Ca\(^{2+}\)-pumping and IP\(_3\) responsiveness assays. A third gradient (without ATP and quin-2) was layered with density marker beads designed to calibrate Percoll gradients (Sigma). All three gradients contained 2.6 mM benzamidine.

The egg homogenate (25%) was prepared as previously described and was divided into 2 aliquots, with ATP, phosphocreatine, and creatine phosphokinase added to only 1 aliquot. Then 1 ml of each homogenate (±ATP) was layered onto 9 ml of its respective Percoll gradient (±ATP), and the density beads (suspended in 1 ml of IM) were layered onto the third gradient. The gradients were centrifuged for 50 min (25,000 × g, 10 °C), photographed, and 1-ml fractions were recovered by upward displacement with 80% sucrose.

Ca\(^{2+}\)-pumping and IP\(_3\)-induced Ca\(^{2+}\) release were assayed with quin-2 as previously described. Glucose-6-phosphatase was assayed (as an endoplasmic reticulum marker) by measuring the release of inorganic phosphate (P\(_i\)) from glucose 6-phosphate according to the procedure of Morre (22). The reaction was stopped after 2 h (at room temperature) by the addition of trichloroacetic acid, and the P\(_i\) was quantitated (23). The medium (lacking vesicles) produced some color in this P\(_i\) assay; therefore, blanks were prepared for each fraction by adding an aliquot of the same fraction to incubation medium that already contained trichloroacetic acid and would, therefore, prevent any P\(_i\) production by the vesicles. Cytochrome c oxidase was assayed (as a mitochondrial marker) following the procedure of Smith (24).

Light scattering at 695 nm was measured (in a spectrophotometer) as an indicator of vesicle content.

Materials

Quin-2 was obtained from Calbiochem-Behring, and 3,3',4,5-tetrachlorosalicylaldehyde was from Eastman Kodak. ATP (dipotassium), phosphocreatine (disodium), creatine phosphokinase (Type I), and apyrase (Grade III) were obtained from Sigma.

RESULTS

Eggs Permeabilized by HVD

HVD was used to transiently permeabilize eggs and allow IP\(_3\) to enter from the extracellular medium. Fig. 1 shows that eggs permeabilized in seawater containing 7 μM IP\(_3\) and 10\(^{-7}\) to 5 × 10\(^{-6}\) M free Ca\(^{2+}\) produced 60–70% cortical reactions, whereas eggs permeabilized in the same media lacking IP\(_3\) produced ≤2% cortical reactions. This suggests that during the permeabilization, IP\(_3\) entry from the medium induced Ca\(^{2+}\) release from internal stores and triggered the cortical reaction. The slight decrease in cortical reactions as free Ca\(^{2+}\) was lowered from 10\(^{-7}\) to 10\(^{-8}\) is probably due to EGTA buffering the intracellular Ca\(^{2+}\) released by IP\(_3\). Since HVD allows EGTA to enter along with IP\(_3\), the increased free EGTA concentration at 10\(^{-7}\) M Ca\(^{2+}\) could be sufficient to partially inhibit the IP\(_3\)-induced Ca\(^{2+}\) increase.

The cortical reaction in most eggs did not propagate and occurred at localized regions corresponding to the cathode and anode sides of each cell. The percentage of cortical reacted eggs decreased as [IP\(_3\)] in the medium was lowered. Thus, at 5 × 10\(^{-6}\) M free Ca\(^{2+}\) the percentage dropped from 60% to 55%, and then to 31% as [IP\(_3\)] was reduced from 7 to 3.5 μM and then to 1.8 μM, respectively. The size of the surface area covered by the localized cortical reaction also correlated with the [IP\(_3\)], with >50% of the egg surface covered at 7 μM IP\(_3\) and progressively smaller areas with lower [IP\(_3\)]. The high concentrations of both IP\(_3\) (7 μM) and Ca\(^{2+}\) (10\(^{-4}\) M in the absence of IP\(_3\)) required to initiate cortical reactions by this procedure could be explained by the transient nature of the permeabilization.

Ca\(^{2+}\) Transport and IP\(_3\)-induced Release in Egg Homogenates

Since the previous experiments with intact eggs provide only indirect evidence for IP\(_3\)-induced Ca\(^{2+}\) release in sea urchin eggs, the system was simplified by using egg homogenates or vesicles isolated from egg homogenates to directly assay Ca\(^{2+}\) uptake by and release from intracellular stores. The Ca\(^{2+}\) concentration of the medium was assayed as an indicator of Ca\(^{2+}\) uptake or release; thus with this approach, a decreased Ca\(^{2+}\) concentration in the medium corresponds to Ca\(^{2+}\) uptake by vesicular components in the homogenate, and, conversely, an increased medium Ca\(^{2+}\) concentration represents an efflux.

Fig. 2 shows that the rate of Ca\(^{2+}\) sequestration by an L. pictus egg homogenate (as monitored by quin-2) is greatly increased by the addition of 0.4 mM ATP. Creatine phosphokinase (2 units/ml) and phosphocreatine (4 mM) were also added as an ATP regeneration system, to maintain constant ATP levels. The low initial rate of Ca\(^{2+}\) sequestration prior

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**FIG. 1.** Cortical reactions initiated by IP\(_3\) in eggs permeabilized by high voltage discharge. Unfertilized eggs of L. pictus were permeabilized by HVD as described under "Experimental Procedures." In experiments at 10\(^{-4}\)–10\(^{-3}\) M Ca\(^{2+}\), 1 mM EGTA and sufficient Ca\(^{2+}\) was added to produce the indicated free Ca\(^{2+}\) levels; the experiments at 10\(^{-4}\) M Ca\(^{2+}\) were done with ASW lacking EGTA and containing 10\(^{-4}\) M total Ca\(^{2+}\). Triangles represent experiments containing 7 μM IP\(_3\), and circles represent controls without IP\(_3\). Eggs showing either partial or complete elevations of the fertilization envelope were scored as having undergone a cortical reaction. Experiments were done in triplicate (S.E. indicated).

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**FIG. 2.** ATP requirement for Ca\(^{2+}\) uptake and retention by Ca\(^{2+}\) stores. Quin-2 (10 μM) was used to monitor the extravesicular Ca\(^{2+}\) concentration in 0.2-ml aliquots of a 3% L. pictus egg homogenate. Three experiments utilizing aliquots of the same initial homogenate are shown, and each aliquot was treated identically (—) until the addition of either IP\(_3\) (— —) or apyrase (— ■—). Following apyrase addition, IP\(_3\) was added at either 5 min (— —) or at 25 min (— ■—). At the arrow marked ATP, 0.4 mM ATP plus 2 units/ml creatine phosphokinase and 4 mM phosphocreatine were added. Other arrows represent the addition of 5 nmol of Ca\(^{2+}\) (Ca), 330 nM IP\(_3\) (IP\(_3\)), or 2 units/ml apyrase (Apyr). The ordinate indicates the approximate free Ca\(^{2+}\) concentrations in the medium and was calibrated as indicated under "Experimental Procedures." The addition of 5 nmol of Ca\(^{2+}\) to 0.9 ml of homogenate produced a free Ca\(^{2+}\) change of ~200 nM, which is much less than the 0.5 μM change that would occur if no Ca\(^{2+}\) buffers were present. However, quin-2, gluconate, and egg components all bind Ca\(^{2+}\) and contribute to the buffered response observed here.
to ATP addition was not due to mitochondria since it was not inhibited by the addition of 2.5 μM 3,3′,4′,5-tetrachlorosalicylanilide (a mitochondria uncoupler) and is probably due to a low endogenous concentration of ATP present in the initial homogenate. After the homogenate had sequestered Ca\(^{2+}\) to an equilibrium level (~30 nM), an aliquot of CaCl\(_2\) (5 nmol) was added to demonstrate the responsiveness of quin-2.

After the added Ca\(^{2+}\) had been sequestered, addition of IP\(_3\) (330 nM) produced a rapid release of Ca\(^{2+}\) which lasted for about 3–4 min. A comparison of the quin-2 response to the change induced by the Ca\(^{2+}\) pulse added earlier shows that IP\(_3\) induced the release of more than 5 nmol of Ca\(^{2+}\) from the homogenate. The released Ca\(^{2+}\) was then resequestered. The resequestration process depends critically on the availability of ATP, since very little was observed when ATP was removed from the medium by the addition of apyrase, an ATP-hydrolyzing enzyme, as shown in Fig. 2.

The vesicles also require ATP to retain their sequestered Ca\(^{2+}\) since removal of ATP by apyrase produced a slow but sustained release of Ca\(^{2+}\). Addition of IP\(_3\) at various times after the apyrase produced the typically fast release of Ca\(^{2+}\) so that the sum of the Ca\(^{2+}\) released by apyrase followed by IP\(_3\) is nearly the same as that released in the control experiment by IP\(_3\) alone. The respective amounts of Ca\(^{2+}\) released (per mg of protein) were: IP\(_3\) alone, ~4 nM; apyrase followed by IP\(_3\) at 3 min, ~4.1 nM; and apyrase followed by IP\(_3\) at 23 min, ~3.6 nM. The Ca\(^{2+}\) release was quantitated by adding known amounts of Ca\(^{2+}\) to aliquots of homogenate ± apyrase as described under "Experimental Procedures." This experiment indicates that the Ca\(^{2+}\) release by apyrase is probably from the same store as that released by IP\(_3\) and also demonstrates that the Ca\(^{2+}\)-release mechanism activated by IP\(_3\) does not require ATP.

The ATP requirement for retention of sequestered Ca\(^{2+}\) indicates that these vesicles are inherently leaky to Ca\(^{2+}\), and a dynamic relationship between Ca\(^{2+}\) leakage and sequestration maintains the Ca\(^{2+}\) gradient between the vesicles and extravascular medium. This suggests the possibility that IP\(_3\) could release Ca\(^{2+}\) by inhibiting the Ca\(^{2+}\) pump, although the slow kinetics of Ca\(^{2+}\) release by apyrase argues against this mechanism.

Ca\(^{2+}\) uptake and release from homogenates was also monitored with a Ca\(^{2+}\)-sensitive electrode (data not shown). Both _L. pictus_ and _S. purpuratus_ egg homogenates pumped Ca\(^{2+}\) to the detection limit of the electrode (~100 nM Ca\(^{2+}\)), released Ca\(^{2+}\) in response to IP\(_3\), and resequestered the released Ca\(^{2+}\).

At near maximal IP\(_3\)_s (300–600 nM), the measured Ca\(^{2+}\) release from _L. pictus_ homogenates (4.1–5.5 nmol of Ca\(^{2+}/mg\) of protein, n = 4) was similar to that measured with quin-2 (2.6–5.5 nmol of Ca\(^{2+}/mg\) of protein, n = 14), whereas _S. purpuratus_ released 2.2–4.4 nmol of Ca\(^{2+}/mg\) of protein (n = 3) when assayed with the Ca\(^{2+}\) electrode.

### Steady State Concentration to Which Homogenates Pumped Ca\(^{2+}\)

**Ca\(^{2+}\) Electrode**—The Ca\(^{2+}\) electrodes were calibrated with Ca\(^{2+}\)-EGTA-buffered IM in the presence of homogenates and indicated that the minimum detection limit of the Ca\(^{2+}\) electrode is about 100 nM. In four experiments with two preparations of _4% L. pictus_ homogenates, the steady state free Ca\(^{2+}\) concentration to which the homogenates pumped was found to be below the detection limit of the Ca\(^{2+}\) electrode, thus indicating the vesicular components in these homogenates can pump Ca\(^{2+}\) to ≤100 nM free Ca\(^{2+}\).

**Quin-2**—Quin-2 has a lower detection limit for Ca\(^{2+}\) than does the Ca\(^{2+}\) electrodes, and since it also has a faster response time, quin-2 was used for the remaining experiments. The effective _K_E for quin-2 was determined to be 170 nM in IM at pH 7.1. This _K_E was the same in the presence of homogenates and was unaffected by benzamidine (2.5 mM), phenylmethylsulfonyl fluoride (0.5 mM), ATP (0.4–0.5 mM), phosphocreatine (4 mM), or creatine phosphokinase (2 units/ml), which are the concentrations of these reagents routinely added to _L. pictus_ homogenates. This _K_E is somewhat higher than the value of 115 nM reported for quin-2 in a medium designed for mammalian cells (21) and probably reflects the higher ionic strength of the IM used here. The steady state Ca\(^{2+}\) concentration to which 4% _L. pictus_ homogenates pumped was found to range from 20–30 nM in fresh homogenates to 40–50 nM in homogenates assayed 6–24 h later. This free Ca\(^{2+}\) is consistent with the ≤100 nM determined by Ca\(^{2+}\) electrodes.

### Characterization of Ca\(^{2+}\) Uptake and Release

The dose-response relationship for IP\(_3\) is shown in Fig. 3. The Ca\(^{2+}\) released from a 4% _L. pictus_ egg homogenate was maximal at 1 μM and half-maximal between 50 and 60 nM. At saturating doses of IP\(_3\), 11 nmol of Ca\(^{2+}\) was released from 0.7 ml of the 4% homogenate (2.9 nmol of Ca\(^{2+}/mg\) of protein).

The half-maximal dose is 40-fold less IP\(_3\) than the 2 μM that produced a half-maximal response with HVD-permeabilized eggs (Fig. 1); however, as indicated previously, the difference is probably due to the transient nature of the permeabilization produced by HVD using the parameters selected.

The effects of homogenate pH on Ca\(^{2+}\) pumping and IP\(_3\)-induced Ca\(^{2+}\) release were investigated next. A 4% _L. pictus_ egg homogenate (6% ATP, 2.5 mM), ATP (0.4–0.5 mM), phosphocreatine (4 mM), or creatine phosphokinase (2 units/ml), which are the concentrations of these reagents routinely added to quin-2 was used for the remaining experiments. The respective responses to IP\(_3\) were obtained from a series of experiments in which the indicated concentrations of IP\(_3\) were added to 0.7-ml aliquots of a 4% homogenate (5.4 mg of protein/ml) of _L. pictus_ eggs. The Ca\(^{2+}\) release was monitored by 50 μM quin-2. The maximal Ca\(^{2+}\) release of 11 nmol of Ca\(^{2+}/0.7\) ml of homogenate converts to 2.9 nmol of Ca\(^{2+}/mg\) of protein.

The inset shows the internal calibration procedure used to quantitate the amount of Ca\(^{2+}\) released from homogenates by IP\(_3\). The initial fluorescence in each tracing represents the free Ca\(^{2+}\) level after the pH adjustments. The rate of Ca\(^{2+}\)-pumping activity at the different pH values was determined.
by adding 1.25 nmol of Ca$^{2+}$ and measuring the proportion of this Ca$^{2+}$ that had been sequestered by 2 min. Finally, after the homogenate had sequestered this added Ca$^{2+}$, IP$_3$ (290 nM) was added, and the Ca$^{2+}$ released was assayed as previously described. The results showed little difference in either Ca$^{2+}$ pumping or IP$_3$ response between pH 7.1 and 7.5; however, both responses are reduced by about 50% at pH 6.7. The respective Ca$^{2+}$-pumping rates at pH 6.7, 7.1, and 7.5 were 0.052 ± 0.006, 0.12 ± 0.02, and 0.17 ± 0.02 nmol of Ca$^{2+}$ sequestered per min per mg of protein (S.D. indicated, n = 3), and the respective IP$_3$ responses were 2.0 ± 0.3, 4.0 ± 0.4, and 4.3 ± 0.4 nmol of Ca$^{2+}$ released per mg of protein (n = 3).

Since fertilization increases the intracellular pH of *L. pictus* eggs from about 6.8 to 7.3 (25, 26), these results indicate that both Ca$^{2+}$ pumping and IP$_3$ responsiveness may be greater in fertilized eggs.

The Ca$^{2+}$ release mechanism is highly specific for IP$_3$. Thus when IP$_3$ and IP$_2$ (6–20 μM each) were assayed for induction of Ca$^{2+}$ release from *L. pictus* homogenates, 16 μM IP$_3$ produced the same Ca$^{2+}$ release as did 60 nM IP$_3$, and 20 μM IP$_2$ produced no Ca$^{2+}$ release. Therefore, the specificity for inducing Ca$^{2+}$ release is 300-fold greater for IP$_3$ than for IP$_2$ and >300-fold greater than for IP$_1$. Also, when 43 nM IP$_3$ was added 5 min after 20 μM IP$_3$ or 5 μM IP$_2$, no inhibition of Ca$^{2+}$ release was observed; therefore, showing that neither reagent inhibits IP$_3$ action.

The Ca$^{2+}$ blocking agent TMB-8 was found to reversibly block the Ca$^{2+}$ release induced by IP$_3$. The TMB-8 experiments were monitored with quin-2, and addition of 3 mM TMB-8 to 4% *L. pictus* homogenates inhibited 95% of the Ca$^{2+}$ release induced by 290 nM IP$_3$, with half-maximal inhibition being observed at about 1 mM TMB-8. The reversibility of the TMB-8 inhibition was tested by a dilution experiment. Ca$^{2+}$ release (induced by 290 nM IP$_3$) from a 9% homogenate was totally inhibited by 3 mM TMB-8; however, following a 3-fold dilution into IIM lacking TMB-8 (thus producing a final TMB-8 concentration of 1 mM), the IP$_3$ addition produced 0.91 ± 0.04 nmol of Ca$^{2+}$/mg of protein. This was nearly the same as the 1.09 ± 0.04 nmol of Ca$^{2+}$ released from controls diluted from a 9% homogenate not previously exposed to TM-8 and to which 1 mM TMB-8 was added. Other controls assayed in the absence of TMB-8 released 3.3 ± 0.1 nmol of Ca$^{2+}$. Experiments were conducted in triplicate, standard deviations are indicated, and TMB-8 was added at least 4 min before IP$_3$ responsiveness was assayed.

**Homogenates Are Refractory to a Second Dose of IP$_3$**

Following the addition of a saturating dose of IP$_3$ (490 nM), egg homogenates did not respond to a second aliquot of IP$_3$ (Fig. 4A). Also, sequential additions of a submaximal dose of IP$_3$ (70 nM) produced decreasing responses with each additional dose (Fig. 4B). However, if these homogenates were allowed to incubate for 3.2–3.5 h before additional aliquots of IP$_3$ were added, the homogenate exposed to submaximal IP$_3$ had completely recovered its responsiveness, and the homogenate initially exposed to higher IP$_3$ had recovered about half of its initial responsiveness. The time course of recovery was determined next. A 5% *L. pictus* homogenate was treated with 290 nM IP$_3$, and the recovery was monitored by assaying the responsiveness of 0.7 ml aliquots to 290 nM IP$_3$ at 1-h intervals. The assay times and Ca$^{2+}$ released per mg of protein were: initial, -2.7 nmol of Ca$^{2+}$; 1 h, -0.03 nmol; 2 h, -0.90 nmol; 3 h, -2.1 nmol; and 4 h, -2.7 nmol.

The recovery of responsiveness may be due to the removal of IP$_3$ from the medium (e.g. hydrolysis by endogenous IP$_3$ phosphatase) and subsequent refilling of responsive vesicles with Ca$^{2+}$. If this is the case, one would expect that washing of IM containing no IP$_3$ should rejuvenate the vesicles. A 5% *L. pictus* egg homogenate was first exposed to 400 nM IP$_3$ and divided into three portions. One portion was centrifuged (180,000 X g, 20 min, 10 °C) to pellet the vesicles and then resuspended into fresh IM containing no IP$_3$. Addition of 290 nM IP$_3$ induced 6.77 nmol of Ca$^{2+}$ release/mg of protein (S.D. = 0.23, n = 3). On the other hand, an identical dose of IP$_3$ added to the control portion (which had not been washed) produced only 0.08 ± 0.06 nmol/mg (n = 3) of Ca$^{2+}$ release. Similar nonresponsiveness (0.08 ± 0.01 nmol/mg) was found for vesicles pelleted but resuspended with the original medium containing IP$_3$ (instead of the fresh IM without IP$_3$). A more accurate comparison is obtained if the response of washed vesicles (6.77 nmol Ca$^{2+}$/mg of protein) is corrected for the loss of soluble proteins (57% of the total protein); such a correction yields 2.9 nmol of Ca$^{2+}$/mg of protein and does not alter the qualitative results.

These results are consistent with the continuous presence of a high concentration of IP$_3$ in the medium being the cause of the refractory response. This centrifugation and resuspension experiment also shows that soluble factors from the egg do not mediate the action of IP$_3$, and the apyrase experiment (Fig. 2) provided evidence against a requirement for ATP. Therefore, the mechanism of IP$_3$ action must utilize only components inherent to the vesicles and ions present in the medium used.

**Purification of Vesicles by Percoll Density Gradient Centrifugation**

Percoll density gradient centrifugation of egg homogenates produced two major bands of vesicles as is shown in Fig. 5A. The large pellets at the bottom of each tube (Fig. 5A) were...
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found to consist of sedimented Percoll particles and contained no vesicles. When the gradients containing homogenates were compared with the calibrating gradient containing density beads, the densities of the major two vesicle bands were determined to be about 1.023 and 1.040 g/ml, respectively. The upper band in the gradient containing ATP was much sharper than the corresponding band without ATP. Fractions collected from both gradients were analyzed for turbidity at 695 nm, which was used as a measure of the vesicle content, and the results are shown in Fig. 5B. In the absence of ATP, vesicles in the upper band were divided equally between fractions 4 and 5, while nearly all of the vesicles were concentrated in the denser fraction 5 when ATP was present. Protein content was also measured for fractions from the gradient without ATP, and three peaks were found (Fig. 5B). Two of these peaks correspond to the vesicle bands. The main protein peak was at the top of the gradient (fractions 1 and 2) and most likely represents soluble protein in the homogenate.

Fig. 5C shows that the activities for Ca²⁺ sequestration and IP₃-induced Ca²⁺ release co-purify in a region occupied by the upper vesicle band in the gradient containing ATP. It is likely that the diffused pattern of the upper band in the gradient without ATP is due to the loss of Ca²⁺ from the vesicles. As was shown earlier, the IP₃-responsive vesicles require ATP to retain their Ca²⁺ (Fig. 2). The Ca²⁺ loss in the absence of ATP would, therefore, reduce the vesicle density and result in
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spreading of the vesicles to a lower density region of the gradient.

Marker enzyme distributions were analyzed only in the gradient without ATP, since ATP interferes with one of the enzyme assays (glucose-6-phosphatase). Fig. 5D shows that activity for cytochrome c oxidase (a marker enzyme for mitochondria) was localized in the higher density band, which was completely devoid of ATP-dependent Ca²⁺ transport and IP₃-induced Ca²⁺ release activity. On the other hand, the activity for glucose-6-phosphatase (a marker enzyme for the endoplasmic reticulum) showed two peaks in the gradient, with the major peak of activity coinciding with the IP₃-induced Ca²⁺ release activity. The complete separation of the mitochondrial marker enzyme activity from the IP₃-induced Ca²⁺ release activity provides strong evidence that the latter is not mitochondrial. The co-purification with glucose-6-phosphatase suggests it is most likely a component of the endoplasmic reticulum network.

When the recovered activities for Ca²⁺ sequestration and IP₃ responsiveness (Fig. 5C) were compared to the total applied activities (assayed as a 4% homogenate but extrapolated to 1 ml of a 25% homogenate), it was determined that 87 and 65% of the respective applied activities were recovered. Also, Fraction 5 contained 62 and 73% of the recovered sequestration and release activities but only 30% of the vesicles (as assayed by A₂₆₀ nm); therefore, this purification method enriches the active vesicles with little loss of applied activity.

**DISCUSSION**

This study presents evidence that IP₃ releases Ca²⁺ from intracellular stores in sea urchin eggs. First, HVD was used to transiently permeabilize eggs and introduce IP₃, with the resultant induction of cortical reactions (a well-characterized Ca²⁺-dependent event, Refs. 1–3) being assayed as an indicator of intracellular Ca²⁺ release. Both our HVD results (Fig. 1) and the microinjection study of Whitaker and Irvine (12) show that IP₃ introduction into eggs induces cortical reactions, thus providing similar indirect evidence that IP₃ releases Ca²⁺ from internal stores in sea urchin eggs.

To directly assay Ca²⁺ uptake by and release from intracellular stores, egg homogenates and microsomal fractions were utilized. The extravascular Ca²⁺ concentration was monitored by either a Ca²⁺ minielectrode or quin-2, and both assay methods showed increases in medium Ca²⁺ levels (closed to 100 nM), and when assayed with Ca²⁺-sequestering threshold was estimated to be about 100 nM. The homogenates were also reported to sequester Ca²⁺ in intact unfertilized Arbacia punctulata eggs assayed with the Ca²⁺-sensitive photo protein aequorin (29) but considerably below the 144 nM free Ca²⁺ reported for intact unfertilized L. pictus eggs assayed with fura 2 (30). The reason for this difference is not known.

ATP-dependent Ca²⁺ sequestration in sea urchin eggs and embryos has also been demonstrated in studies utilizing "Ca²⁺ (14, 31, 32). Silver et al. (31) demonstrated Ca²⁺ uptake by vesicles associated with the mitotic apparatus of fertilized eggs. Suprynowicz et al. (14) demonstrated Ca²⁺ uptake by fertilized eggs permeabilized by HVD and by homogenates of both fertilized and unfertilized eggs (32); in each case the Ca²⁺-sequestering threshold was estimated to be about 100 nM. The homogenates were also reported to sequester Ca²⁺ 5 times faster at pH 7.4 than at 6.8, which is similar to the 3.3-fold increase we measured between pH 6.7 and 7.5.

When egg homogenates were fractionated by Percoll density gradient centrifugation, the activities for Ca²⁺ sequestration, IP₃-induced Ca²⁺ release, and glucose-6-phosphatase (an enzyme associated with the endoplasmic reticulum) all copurified (Fig. 5) and showed clear separation from cytochrome c oxidase (an enzyme associated with the mitochondria). Therefore, the Ca²⁺ store that is responsive to IP₃ is most likely a component of the endoplasmic reticulum network. This result is supported by a study with A. punctulata eggs loaded with aequorin and whose organelles were stratified by centrifugation. When fertilized, these eggs showed Ca²⁺ to be released from a region enriched in endoplasmic reticulum but depleted for mitochondria (29).

Studies with several mammalian cell types have also shown that IP₃-responsive vesicles co-purify with endoplasmic reticulum-associated enzymes (5, 7, 8, 9).

These purification studies and the experiment where L. pictus microsomes were centrifuged and reassembled also show that no cytoplasmic factors are required for IP₃ action. The mechanism of IP₃ action must utilize only components inherent to the vesicles and ions present in the medium used. When L. pictus egg homogenates were exposed to saturating
doses of IP₃ (e.g., 430 nm in Fig. 4), Ca²⁺ was released for 1–2 min, whereupon resequestration began. However, the homogenates were refractory (desensitized) to subsequent IP₃ addition, even after the Ca²⁺ concentration had returned to its initial level. Similar desensitization to IP₃ has been reported for three insulinoma cell lines (6–8). When the desensitized egg homogenates were centrifuged and washed with fresh medium lacking IP₃, the desensitization was reversed, indicating that the continuous presence of IP₃ in the medium may be the cause. It is possible that there is more than one type of Ca²⁺-sequestering vesicle in the homogenate. In the presence of IP₃, the IP₃-responsive vesicles would not resequester Ca²⁺, and the released Ca²⁺ would be transported into another type of vesicle which is not responsive to IP₃. Reversal of desensitization would, therefore, require the removal of IP₃ and subsequent redistribution of Ca²⁺ back into responsive vesicles. Such a model would explain both the time-dependent recovery we observed and the centrifugation and washing experiment (see "Results").

This study provides the first direct evidence that IP₃ induces Ca²⁺ release from intracellular stores in sea urchin eggs and, therefore, supports the hypothesis that IP₃ mediates the sperm-induced Ca²⁺ increase that activates development. Depletion of Ca²⁺-sequestering vesicle in the homogenate. In the presence of IP₃, the IP₃-responsive vesicles would not resequester Ca²⁺, and the released Ca²⁺ would be transported into another type of vesicle which is not responsive to IP₃. Reversal of desensitization would, therefore, require the removal of IP₃ and subsequent redistribution of Ca²⁺ back into responsive vesicles. Such a model would explain both the time-dependent recovery we observed and the centrifugation and washing experiment (see "Results").

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