Detection and functional characterization of ryanodine receptors from sea urchin eggs

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1. Immunoblot analysis, [^{3}H]ryanodine binding, and planar lipid bilayer techniques were used to identify and characterize the functional properties of ryanodine receptors (RyRs) from *Lytechinus pictus* and *Strongylocentrotus purpuratus* sea urchin eggs.

2. An antibody against mammalian skeletal RyRs identified an ~400 kDa band in the cortical microsomes of sea urchin eggs while a cardiac-specific RyR antibody failed to recognize this protein. [^{3}H]Ryanodine binding to cortical microsomes revealed the presence of a high-affinity ($K_d = 13$ nM), saturable (maximal density of receptor sites, $B_{max} = 1{\cdot}56$ pmol (mg protein)$^{-1}$) binding site that exhibited a biphasic response to Ca^{2+}.

3. Upon reconstitution of cortical microsomes into lipid bilayers, only sparse and unstable openings of a high-conductance cation channel were detected. Addition of crude sea urchin egg homogenate to the cytosolic (*cis* side) of the channel increased the frequency of openings and stabilized channel activity. The homogenate-activated channels were Ca^{2+} sensitive, selective for Ca^{2+} over Cs^+, and driven by ryanodine into a long-lived subconductance state that represented ~40% of the full conductance level. Homogenate dialysed in membranes with a molecular weight cut-off ⩽ 2000 lacked the capacity to increase the frequency of RyR openings and to stabilize channel activity.

4. Direct application of cyclic adenosine diphosphoribose (cADPR) or photolysis of NPE-cADPR ('caged' cADPR) by ultraviolet laser pulses produced transient activation of sea urchin egg RyRs. Calmodulin (CaM) failed to activate reconstituted RyRs; however, channel activity was inhibited by the CaM blocker trifluoroperazine, suggesting that CaM was necessary but not sufficient to sustain RyR activity.

5. These findings suggest that a functional Ca^{2+} release unit in sea urchin eggs is a complex of several molecules, one of which corresponds to a protein functionally similar to mammalian RyRs.

Cyclic adenosine diphosphoribose (cADPR), an endogenous metabolite of nicotinamide adenine dinucleotide, was first identified as an agent capable of releasing Ca^{2+} from intracellular stores in sea urchin eggs (Lee, Walseth, Bratt, Hayes & Clapper, 1989). More recently, cADPR has also been shown to mobilize Ca^{2+} in several mammalian cells, including those from pancreatic islets, intestinal longitudinal muscle, sympathetic neurons, dorsal root ganglion, liver and brain (Galione, 1994). In all of these cells, cADPR either generates or amplifies an intracellular Ca^{2+} wave that sets in motion a series of events that culminates in egg fertilization, hormone secretion, muscle contraction, neurotransmitter release, etc.

The molecular mechanism by which cADPR mobilizes intracellular Ca^{2+} has not been clearly established. cADPR-induced Ca^{2+} release is insensitive to heparin and inositol 1,4,5-trisphosphate but sensitive to caffeine and ryanodine (Galione, Lee & Busa, 1991), two classical modulators of sarcoplasmic reticulum (SR) Ca^{2+} release channels (ryanodine receptors, RyRs; Meissner, 1994). It would seem therefore that RyRs are the molecular target of cADPR. However, in sea urchin eggs, cADPR crosslinks with ~140 and 100 kDa proteins (Walseth, Aarhus, Kerr & Lee, 1993), not with the expected ~500 kDa RyR monomer detected in cardiac and skeletal muscle (Meissner, 1994). Furthermore, calmodulin (CaM), which regulates mammalian RyRs but is not necessary to sustain channel activity (Tripathy, Xu, Mann & Meissner, 1995), is an indispensable component of cADPR-induced Ca^{2+} release in sea urchin eggs (Lee, Aarhus, Graeff, Gurnack & Walseth, 1994). Conversely, while cADPR is a clear Ca^{2+} mobilizing agent in sea urchin eggs, it produces little (Sitsapesan, McGarry & Williams, 1994) or no effect

(Fruen, Mickelson, Shomer, Velez & Louis, 1994; Guo, Laflamme & Becker, 1996) in cardiac muscle.

We have reconstituted cortical microsomes of sea urchin eggs into lipid bilayers in an attempt to identify the molecular target of cADPR and characterize its mechanism of action. We found that cADPR activates a cation channel that is similar to cardiac and skeletal RyRs in several elementary properties including unitary channel conductance, Ca^{2+} selectivity, subconductance states and ryanodine sensitivity. However, there was also a strict dependence on accessory components to sustain the activity of this channel, a condition not seen with cardiac and skeletal RyRs. Thus, a functional cADPR-dependent Ca^{2+} release unit in sea urchin eggs seems to be a complex of several molecules, one of which corresponds to a protein with elementary properties similar to those of mammalian RyRs.

METHODS

Preparation of cortical reticulum membranes and total homogenate from sea urchin eggs

Cortical reticular membranes, a honeycomb network of internal membranes that associates with the plasma membrane, were isolated from unfertilized *Lytechinus pictus* or *Strongylocentrotus purpuratus* sea urchin eggs using a modification of the procedure of McPherson, McPherson, Mathews, Campbell & Longo (1992). Briefly, eggs suspended in complete sea water (486 mM NaCl, 10 mM KCl, 26 mM $MgCl_2$, 30 mM $MgSO_4$, 10 mM $CaCl_2$, 2·4 mM $NaHCO_3$, 10 mM Hepes, pH 8·0) were allowed to sediment by gravity and homogenized (1:10, v/v) in iced sea water C (SWC; 500 mM NaCl, 10 mM KCl, 3 mM $NaHCO_3$, 30 mM EGTA, 60 mM NaOH, 200 μM benzamidine, 2 μM leupeptin, pH 8·0). A portion of this total homogenate was supplemented with 5 mM K_2ATP and 26 mM $CaCl_2$ to bring [free Mg^{2+}] and [free Ca^{2+}] to 1·5 mM and ~30 μM, respectively. After titration to pH 7·4, the supplemented total homogenate was stored at −70 °C in small aliquots until used. The remaining homogenate was diluted 1:5 (v/v) in SWC and spun in a tabletop centrifuge at 2000 r.p.m. for 2 min. The pellet was resuspended in 0·5 volumes of SWC and centrifuged again until the resuspension volume was 2 ml. The last pellet, corresponding to cortical microsomes, was suspended in 1 ml of modified sea water C (MSWC; same as above except that NaOH was replaced by 30 mM Tris (pH 8·0) and EGTA was decreased to 1 mM) and stored in small aliquots at − 70 °C until used.

Dialysis of total homogenate was performed as follows: 2 ml of unsupplemented total homogenate was dialysed for 4 h against 2 l of SWC at 4 °C. Dialysis was conducted using a 3 ml dialysis cassette with a cellulose film of molecular weight cut-off (MWCO) ⩽ 2000 (Cat. No. 66225, Pierce, Rockford, IL, USA). At the end of the 4 h dialysis, the dialysed homogenate was supplemented with ATP and $CaCl_2$ as described above and stored in small aliquots at −70 °C.

[^{3}H]Ryanodine binding

[^{3}H]Ryanodine (60 Ci mmol^{-1}, Dupont NEN) was incubated with cortical microsomes (0·01–1·0 mg ml^{-1}) in medium containing 0·2 M KCl, 20 mM Mops (pH 7·2), 1 mM EGTA, and different amounts of $CaCl_2$ to set [free Ca^{2+}] in the range of 0·08–100 μM. The incubation took place in a volume of 0·1 ml at 36 °C for 90 min. After incubation, bound and free [^{3}H]ryanodine were separated by rapid filtration onto Whatman GF/B filters. The filters were washed twice with cold distilled water and placed in a liquid scintillation cocktail to measure radioactivity in a β-counter. Non-specific binding was determined in the presence of 100 μM unlabelled ryanodine and has been subtracted from all reported values. Unless otherwise indicated, data represent the mean ± S.E.M. with $n \geqslant 3$. Mathematical fitting of data was accomplished with the computer program Origin (v4.0, Microcal Inc., Northampton, MA, USA).

Reconstitution of single RyRs in planar lipid bilayers and activation by photolysis of caged cADPR

Single channel recordings of sea urchin egg RyRs were performed by fusing cortical microsomes to a Mueller-Rudin type phospholipid bilayer as described previously (El-Hayek, Lokuta, Arevalo & Valdivia, 1995; Lokuta, Rogers, Lederer & Valdivia, 1995). Cortical microsomes (~10 μg) were added to an aqueous chamber (the *cis* chamber) connected to the head stage of a 200A Axopatch amplifier (Axon Instruments). The *trans* side was held at virtual ground. The *cis* and *trans* chambers (0·8 ml each) were initially filled with 50 mM caesium methanesulphonate and 10 mM Mops (pH 7·2). After bilayer formation, an asymmetrical caesium methanesulphonate gradient (300 mM *cis*/50 mM *trans*) was established. A Ca^{2+}–EGTA admixture was then added to the *cis* chamber from a 100-fold stock to reach the [free Ca^{2+}] specified in the text. After addition of cortical microsomes, voltage steps from 0 to −40 mV were applied at random. If channel openings were detected, Cs^+ in the *trans* chamber was raised to 300 mM to collapse the chemical gradient and to avoid further vesicle insertion. Photolysis of caged cADPR was accomplished with a Q-switched, Nd:YAG laser (model GCR-12, Spectra-Physics, Mountain View, CA, USA), as described for caged Ca^{2+} (Valdivia, Kaplan, Ellis-Davies & Lederer, 1995). The laser beam (at 354 nm wavelength) was focused onto a 400 μm outer diameter, fused-silica fibre optic. The end of the fibre optic was positioned with a micromanipulator ~400 μm in front of the bilayer aperture to photolyse the caged compound in the region between the end of the fibre optic and the bilayer cup. Therefore, only a small fraction of the caged compound was photolysed with each flash and the procedure could be repeated several times during the course of a single experiment. Channel activity was recorded and analysed with Axon Instruments software and hardware (pCLAMP v6.1, Digidata 1200 AD/DA interface). The probability of a single channel being open (P_o) was calculated from current amplitude histograms using the equation: $P_o = I_{open}/(I_{open} + I_{closed})$, where I_{open} and I_{closed} were the binned currents of the channel in the open and closed states, respectively. Records were filtered at 1·5–2·0 kHz and digitized at 4–5 kHz.

SDS–PAGE and Western blot analysis of RyRs

Rabbit skeletal, sea urchin egg and pig cardiac microsomal proteins were separated by SDS–PAGE in 7% acrylamide gels, transferred to a nitrocellulose membrane, and probed with either a rabbit monoclonal skeletal RyR antibody (XA7B6, Upstate Biotech Incorporated, Goldey, CO, USA) or a mouse monoclonal cardiac RyR antibody (MA3-916, Affinity Bioreagents, Inc., Goldey, CO, USA). Secondary goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antibodies were then applied and detection of protein–antibody complexes was accomplished via chemiluminescence as described previously (Lokuta, Meyers, Sanders, Fishman & Valdivia, 1997).

RESULTS

Immunoblot analysis and [^{3}H]ryanodine binding to cortical microsomes of sea urchin eggs

Figure 1*A* shows that a monoclonal antibody raised against a cytosolic segment of the rabbit skeletal RyR recognized an ~450 kDa protein in rabbit skeletal SR (lane 1) and an ~400 kDa protein in the cortical microsomes of *L. pictus* eggs (lane 2). RyR from pig cardiac SR did not cross-react with this antibody (lane 3). Conversely, an antibody against mammalian cardiac RyRs failed to recognize high molecular weight proteins in skeletal SR and in sea urchin eggs. Thus, a high molecular weight protein of sea urchin eggs cross-reacts only with the skeletal RyR antibody. Figure 1*B* shows that high-affinity ($K_d = 13 \pm 5$ nM) and saturable (maximal density of receptor sites, $B_{max} = 1{\cdot}5 \pm 0{\cdot}5$ pmol (mg protein)$^{-1}$) [^{3}H]ryanodine binding was detectable in the cortical microsomes. We therefore used [^{3}H]ryanodine binding to test for the effect of [Ca^{2+}] on sea urchin egg RyRs. All three mammalian RyR isoforms (skeletal or *ryr*1, cardiac or *ryr*2, and brain or *ryr*3) possess a high-affinity Ca^{2+} binding site that *activates* the channel and a low-affinity Ca^{2+} binding site that *inactivates* (or closes) the channel (Meissner, 1994). However, skeletal RyR inactivates at a [Ca^{2+}] ($ED_{50} = \sim 280$ μM) lower than cardiac and brain RyR ($ED_{50} = \sim 4$ mM) (El-Hayek *et al.* 1995; Xu, Mann & Meissner, 1996; Meissner, Rios, Tripathy & Pasek, 1997). Figure 1*C* shows that the Ca^{2+} dependence of [^{3}H]ryanodine binding to sea urchin egg microsomes was biphasic with ED_{50} values for Ca^{2+} activation and inactivation of 0·2 and 60 μM, respectively. These values are lower than those of any known RyR isoform, but the bell-shaped [^{3}H]ryanodine binding curve bears more resemblance to that exhibited by skeletal RyRs. Taken together, the immunological and binding data suggest that a RyR is present and functional in sea urchin egg cortical microsomes and that it has a Ca^{2+} dependence more similar to skeletal-type RyRs than to other RyR isoforms.

Single channel activity in sea urchin egg cortical microsomes

We next attempted to record single RyR channel activity from sea urchin eggs by fusing cortical microsomes to planar lipid bilayers. We used recording conditions considered standard for studies of cardiac or skeletal RyRs (El-Hayek *et al.* 1995; Lokuta *et al.* 1995). Microsomes were added to

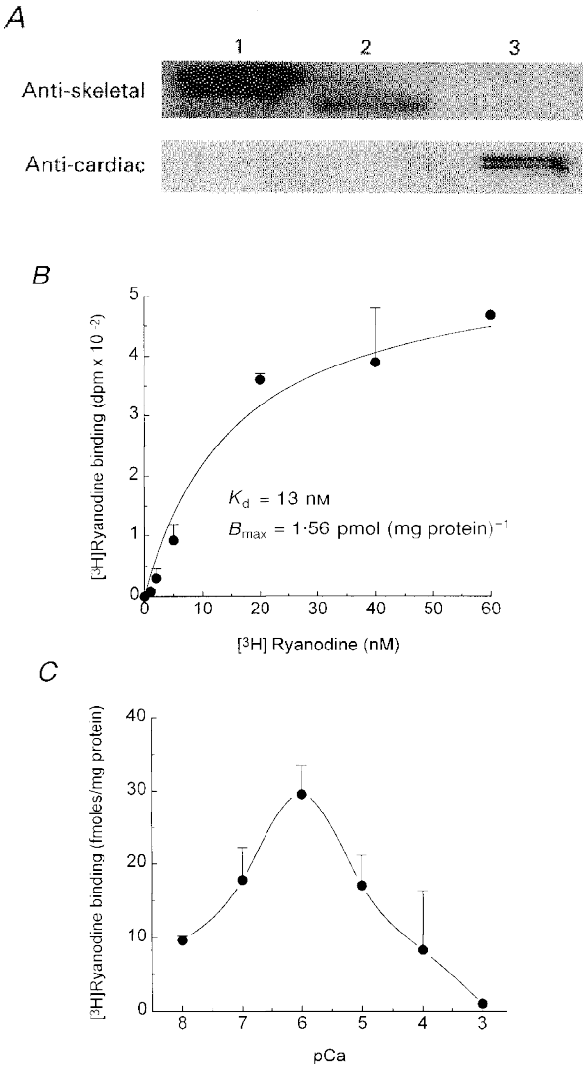

Figure 1. Immunoblot analysis and [^{3}H]ryanodine binding to cortical microsomes of sea urchin eggs

A, Western blot analysis with RyR antibodies. Cortical microsomal proteins and cardiac and skeletal SR microsomes were separated by SDS–PAGE, transferred to a nitrocellulose membrane, and probed with a rabbit monoclonal skeletal RyR antibody and cardiac RyR antibody, as indicated. Lane 1, 30 μg rabbit skeletal microsomes; Lane 2, 50 μg sea urchin egg microsomes; Lane 3, 30 μg pig cardiac microsomes. *B*, [^{3}H]ryanodine saturation binding curve. Between 1 and 10 μg of *L. pictus* cortical microsomes were incubated with the indicated concentration of [^{3}H]ryanodine as indicated in Methods. Non-specific binding has been subtracted from each data point. Data were fitted with the equation: $B = B_{max}[[^3H]ryanodine]/(K_d + [[^3H]ryanodine])$, where B corresponds to specific binding of [^{3}H]ryanodine, B_{max} is the maximal density of receptor sites and K_d is the apparent dissociation constant of the [^{3}H]ryanodine–RyR complex. *C*, Ca^{2+} dependence of [^{3}H]ryanodine binding to sea urchin egg microsomes. Binding conditions were as in *B* except that 1 mM EGTA and varying concentrations of $CaCl_2$ were added to the medium to bring [free Ca^{2+}] to the specified level. [^{3}H]Ryanodine concentration was 7 nM.

the *cis* side and channel activity recorded with symmetrical ionic composition in the *cis* and *trans* sides (300 mM caesium methanesulphonate, 10 mM Mops, pH 7·2). Cs^+ was chosen as charge carrier instead of Ca^{2+} to avoid inactivation caused by large Ca^{2+} gradients, to increase the signal-to-noise ratio (Cs^+/Ca^{2+} conductance ratio, $G_{Cs}/G_{Ca} \approx 2$), and to block K^+ channels (Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1988). Methanesulphonate was used to block Cl^- channels. Figure 2*A* shows traces of a Cs^+-conducting sea urchin egg channel. Under voltage clamp conditions, only brief and sporadic channel openings were detected (traces labelled Control), which occurred most frequently after abrupt

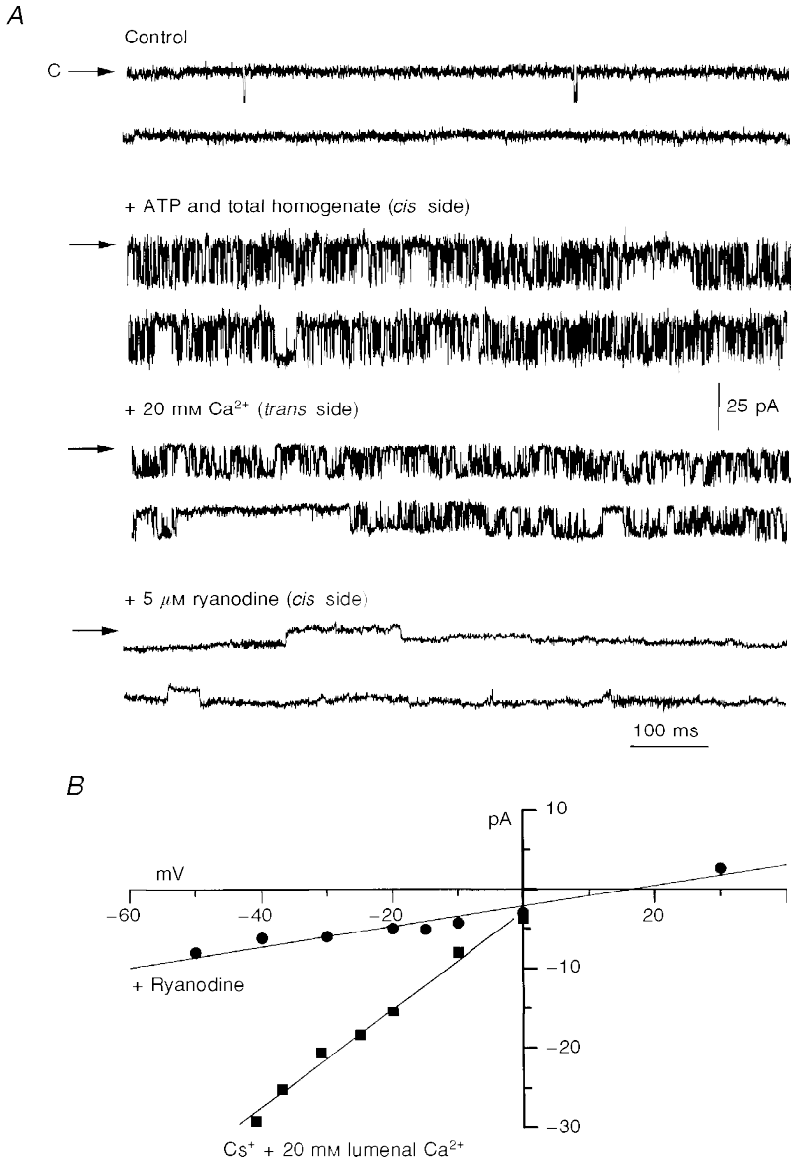

Figure 2. Reconstitution of sea urchin egg RyR channels in planar lipid bilayers

All traces were recorded at a holding potential of −25 mV. At this voltage, Cs^+ flows from the *trans* (lumenal) to the *cis* (cytosolic) chamber and channel openings correspond to downward deflections of the baseline current. *A*, traces labelled Control: only brief and sparse openings were observed under our standard recording conditions (symmetrical 300 mM caesium methanesulphonate, 10 mM Mops, pH 7·2). C indicates the closed state. + ATP and total homogenate (*cis* side): the same channel after addition of 10 μl of sea urchin egg total homogenate (0·1 mg protein ml^{-1}) supplemented with 5 mM ATP. For this particular channel, P_o increased from ⩽ 0·01 to ~0·6. This is one of the most dramatic responses. Typical and consistent responses to homogenate addition were an increase in P_o from ⩽ 0·01 to 0·2–0·5 ($n = 9$). + 20 mM Ca^{2+} (*trans* side): addition of 20 mM $CaCl_2$ to the *trans* (lumenal) side decreased single channel conductance; + 5 μM ryanodine: ryanodine induced the appearance of a long-lived subconductance state. The transition to this modified state was not reversible within the duration of the experiment (~20 min). *B*, current–voltage relation for the RyR channel before and after addition of ryanodine.

voltage steps from positive to negative holding potentials. However, addition to the *cis* (cytosolic) chamber of total egg homogenate, supplemented with 5 mM ATP as described in Methods, elicited a remarkable increase of channel activity (second row of traces). The combined addition of ATP and total homogenate stabilized channel openings and allowed steady-state recordings for relatively long times (20–40 min).

The following four crucial observations strongly suggested that the homogenate-activated channels of sea urchin eggs corresponded to functional counterparts of mammalian RyRs. (1) Addition of 20 mM $CaCl_2$ to the *trans* (lumenal) solution decreased the amplitude of single channel openings (Fig. 2*A*, third row of traces) and shifted E_{rev}, the reversal potential, from 0 to ~+10 mV (Fig. 2*B*). The change in current amplitude was expected from the higher affinity and longer dwelling time of Ca^{2+} in the channel's pore, while the shift in E_{rev} indicated that the channel was selective for Ca^{2+} over Cs^+ (Smith *et al.* 1988). (2) The slope conductance for this channel in 300 mM symmetrical Cs^+ plus 20 mM Ca^{2+} (Fig. 2*B*) was ~600 pS, similar to that of skeletal and cardiac RyRs under comparable conditions (Smith *et al.* 1988; Meissner, 1994). (3) Addition of 5 μM ryanodine to the *cis* solution profoundly modified the amplitude and kinetics of the channel (Fig. 2*A*, bottom row of traces). Ryanodine 'locked' the channel into a long-lived conductance state that represented ~40% of the full-conductance openings (Fig. 2*B*). These kinetic modifications are the signature effects of ryanodine on skeletal and cardiac RyRs (Rousseau, Smith & Meissner, 1987; Lindsay, Tinker & Williams, 1994). (4) Lowering $[Ca^{2+}]$ at the cytosolic (*cis*) side of the channel markedly decreased channel activity (Fig. 4), as expected from the Ca^{2+} dependence of open probability (P_o) for cardiac and skeletal RyRs (Chu, Fill, Stefani & Entman, 1993) and from the Ca^{2+} dependence of [^{3}H]ryanodine binding in sea urchin eggs (Fig. 1*C*). Thus, several elementary properties observed in cardiac and skeletal RyRs are also present in, and may be used as markers for detection of, sea urchin egg RyRs.

Conductance states in sea urchin egg RyRs

In addition to the predominant ~600 pS conductance level, two subconducting states were occasionally observed in the homogenate-activated sea urchin egg RyR (Fig. 3). In the standard recording solution, these substates had chord conductances of ~300 pS (Fig. 3*B*) and ~150 pS (Fig. 3*C*), which corresponded to approximately $\frac{1}{2}$ and $\frac{1}{4}$ of the main conducting level, respectively (Fig. 3*A*). The relative fractional conductance of the substates (labelled $F_{¼}$ and $F_{½}$ in Fig. 3) were conserved in Cs^+- and Ca^{2+}-conducting channels. Direct transitions between full and subconducting states (Fig. 3*D*) surpassed transitions between closed and subconducting states (first segment of Fig. 3*C*). P_o of the fully open and subconducting levels fluctuated under stationary conditions. Particularly, $P_o[F_{½}]$, the open

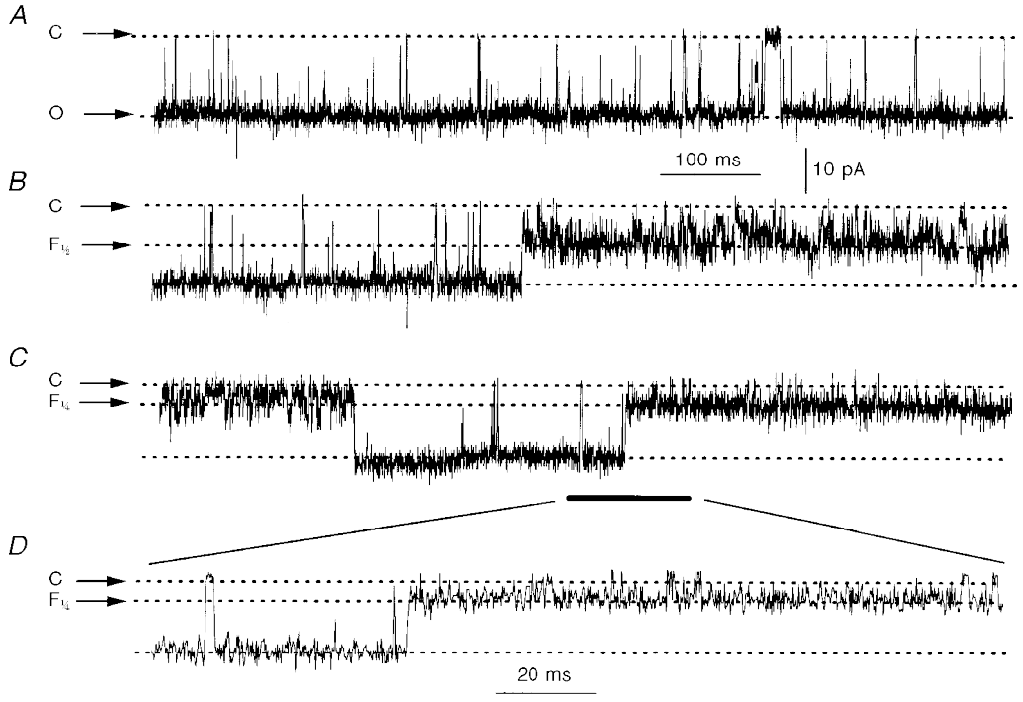

Figure 3. Conductance states in homogenate-activated sea urchin egg RyRs

Single channel openings to the full (~600 pS), most frequently encountered conducting state of the sea urchin egg RyR (*A*), or to a conducting state with current amplitude value corresponding to $\frac{1}{2}$ ($F_{½}$; *B*) or to $\frac{1}{4}$ ($F_{¼}$; *C*) of the fully open channel. *D*, expansion of the recording segment in *C* indicated by bar. Holding potential for all traces = −25 mV. The dashed lines indicate the current levels of the various conducting states; C, closed; O, open. Traces were digitized at 4 kHz and filtered at 1·5 kHz.

probability of the ~300 pS conductance state, differed markedly from $(P_o[F]/2)^2$, the probability of two independent channels opening to the fully conducting state simultaneously. This argued against $F_{½}$ being the unitary current of an independently gated channel and suggested instead that it corresponded to a true substate of the sea urchin egg RyR. These current amplitude transitions are reminiscent of the subconducting states observed in cardiac and skeletal RyRs (Liu, Lai, Rousseau, Jones & Meissner, 1989; Ding & Kasai, 1996).

Effect of cADPR on sea urchin egg RyRs

In sea urchin egg microsomes, the response to cADPR appears to be conditioned to the presence of accessory proteins (Lee *et al.* 1994). We therefore investigated whether reconstituted RyRs retained their capacity to respond to cADPR. Addition of up to 10 μM cADPR to the cytosolic (*cis*) side was unable to activate sea urchin egg RyRs ($P_o = \leqslant 0{\cdot}01$ before and after addition, $n = 3$) (results not shown). We thus resorted again to adding total homogenate to produce a steady level of activity on which to test the effect of cADPR. The top traces in Fig. 4*A* show a homogenate-activated RyR in the presence of [free Ca^{2+}] of 10 μM at the cytosolic side (*cis* side) of the channel. Ca^{2+} elicited a level of activity that was constant over time (lower trace). Upon lowering [free Ca^{2+}] to 0·1 μM, P_o decreased to $\leqslant 0{\cdot}01$, indicating that Ca^{2+} is a central regulatory element of this channel, as it is for cardiac and skeletal RyRs (Smith *et al.* 1988; Chu *et al.* 1993; Meissner, 1994).

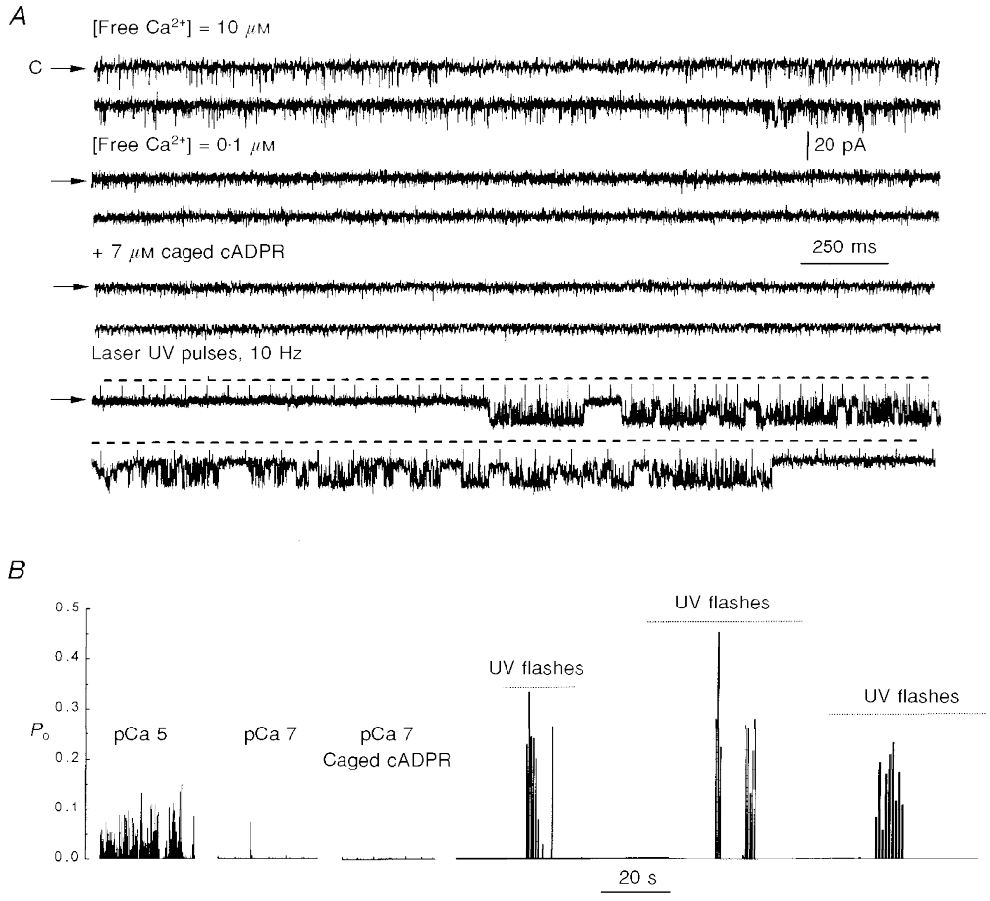

Figure 4. Activation of RyRs by photolysis of caged cADPR

Sea urchin egg RyRs were reconstituted in lipid bilayers as described in Fig. 2, except that Ca^{2+} in the *trans* side was omitted and 1 mM EGTA and varying amounts of $CaCl_2$ were added to the *cis* side to set cytosolic [free Ca^{2+}] to 10 μM (top traces in *A*) or 0·1 μM (second row of traces in *A*). *A*, in the presence of sea urchin egg homogenate, 10 μM [free Ca^{2+}] in the cytosolic (*cis*) side elicited bursts of channel activity (first row of traces), which disappeared when *cis* [free Ca^{2+}] was lowered to 0·1 μM (second row of traces). After a period of recording at [free Ca^{2+}] = 0·1 μM, 7 μM caged cADPR was added to the *cis* side (third row of traces). No activity was detected during this period. A train of UV laser flashes was applied at 10 Hz frequency, shown by the horizontal dashed lines (bottom traces). The upward deflections of the baseline current are electrical artifacts created by the laser pulse. *B*, diary of activity of the RyR. Continuous records in control and after additions were divided into intervals of 30 s; P_o in each interval is plotted as a bar of length 0–1 with level 0 corresponding to no activity. The dotted line underneath the label UV flashes indicates the time in which laser UV flashes were applied at a frequency of 10 Hz. Three representative trains of channel activity elicited by UV pulses are shown ($n = 6$).

Addition of 7 μM caged cADPR to the cytosolic side of the channel did not produce any noticeable effect on channel activity. Similarly, sham flashes (flashes of high energy in the absence of caged cADPR) did not elicit channel openings (not shown). However, trains of ultraviolet laser flashes in the presence of caged cADPR elicited bursts of channel activity, the appearance of which correlated with the photolytic stimulus (bottom traces and panel *B*). Thus, whole homogenate supplements the RyR with accessory components that render the channel functional and responsive to cADPR.

Effect of calmodulin blockers on RyR channel activity

Since CaM has been reported as an indispensable component for activation of sea urchin egg RyRs (Lee *et al.* 1994; Tanaka & Tashjian, 1995) we tested whether CaM could replace total homogenate in its ability to activate RyRs. Under control conditions, only sporadic channel openings were detected (Fig. 5). Addition of CaM (10 μM) to the *cis* solution failed to increase channel activity (+ Calmodulin). In contrast, the frequency of channel openings increased remarkably when total homogenate was added, making apparent the presence of two channels in the bilayer (+ Total homogenate). Addition of the CaM blocker trifluoperazine (Meissner, 1986) to homogenate-activated channels reduced channel activity considerably (bottom traces). For the experiment presented in Fig. 5, nP_o, the open probability of a single channel multiplied by the number of observable channels (n), was $< 0{\cdot}01$ both in control and after CaM, 0·81 after total homogenate, and 0·08 after trifluoperazine. These results suggest that CaM is necessary but not sufficient to elicit channel activity of the sea urchin egg RyR in planar lipid bilayers.

Dialysis renders total homogenate ineffective to activate RyRs

The previous experiments suggested that neither cADPR nor CaM were the primary factors in total homogenate that promote and stabilize the activity of sea urchin egg RyRs. To shed light on the nature of the factor(s) in total homogenate which activate RyRs, we subjected total homogenate to 4 h of dialysis in cellulose membranes with molecular weight cut-off $\leqslant 2000$. We reasoned that if the activating factor corresponded to a membranous or cytosolic protein with molecular weight $\geqslant 2000$, then the dialysed homogenate should retain its capacity to activate RyRs. Figure 6*A* shows that *cis* addition of 32 μg of undialysed total homogenate evoked numerous channel openings in an otherwise sluggish RyR ($P_o \leqslant 0{\cdot}01$ and 0·05 before and after addition, respectively, $n = 3$), consistent with the results presented above. In contrast, a similar addition of dialysed homogenate was unable to increase channel

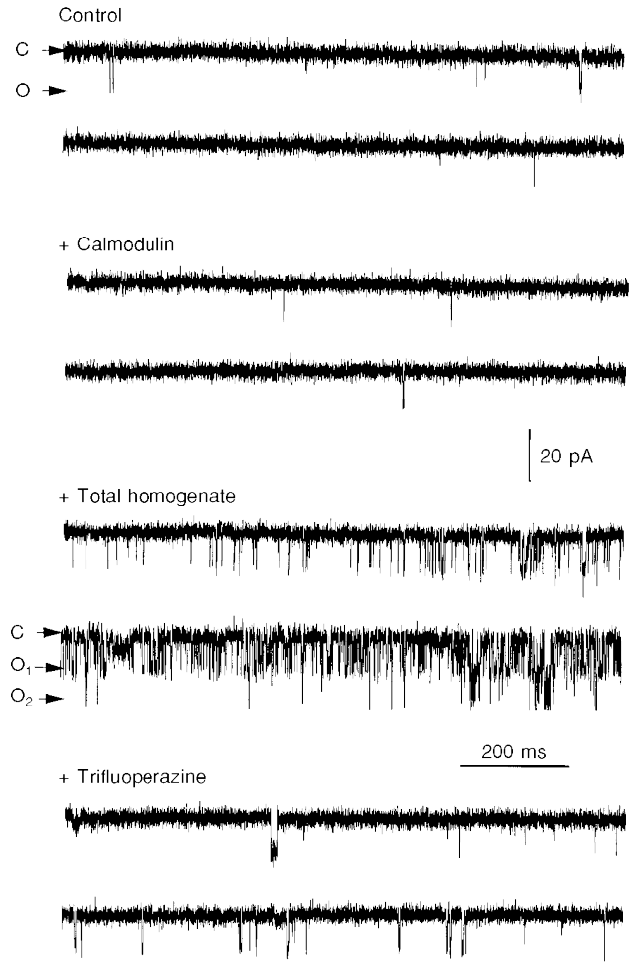

Figure 5. Effect of CaM and a CaM blocker on sea urchin egg RyRs

The [free Ca^{2+}] in the *cis* (cytosolic) side of the channel was 10 μM (1 mM EGTA and 0·997 mM $CaCl_2$). Sporadic openings elicited by voltage steps from 0 to −30 mV were obtained in the absence (Control) and the presence of 10 μM CaM added to the *cis* side (+ Calmodulin). No change in channel activity was detected following addition of CaM. In contrast, channel activation occurred after addition of 10 μl of sea urchin egg total homogenate (+ Total homogenate), which revealed the presence of two channels in this experiment (open states are labelled O_1 and O_2). Channel activation was partially reversed by addition of 20 μM trifluoperazine to the *cis* side (+ Trifluoperazine). All traces were taken from the same experiment, which was repeated twice with essentially qualitatively identical results.

activity or to stabilize full and long lasting openings (Fig. 6*B*). Instead, channel activity in the presence of dialysed homogenate was irregular, and openings fluctuated rapidly between states of different fractional conductance. These results suggested that the activating factor(s) in total homogenate corresponded to molecule(s) with molecular weight $\leqslant 2000$.

DISCUSSION

We used immunological, radioligand binding and single channel experiments to provide evidence that a high molecular weight protein of sea urchin eggs, displaying key functional properties of mammalian RyRs (including sensitivity to ryanodine), is part of the Ca^{2+} release unit sensitive to cADPR. Although extensive data obtained in whole and fragmented cells (Galione, 1994) support RyRs as the effectors of cADPR-induced Ca^{2+} release, this is the first study that directly links cADPR with single sea urchin egg RyRs.

A skeletal (but not a cardiac) RyR antibody cross-reacted with an ~400 kDa protein in sea urchin eggs (Fig. 1*A*). The molecular weight of this protein was similar to that found by McPherson *et al.* (1992) using a skeletal RyR antibody with cortical microsomes of *L. variegatus* eggs. Thus, either the sea urchin egg RyR exhibits different mobility in SDS–PAGE, or it represents a new RyR isoform that is smaller than the typical mammalian RyR isoforms. Although immunological cross-reactivity between two proteins is far from guaranteeing similarity in structure/function, the Ca^{2+} dependence of the [^{3}H]ryanodine binding curve in cortical microsomes (Fig. 1*C*) was nonetheless bell-shaped like that of skeletal RyRs (Meissner, 1994), or at least more so than that of cardiac or brain RyRs (El-Hayek *et al.* 1995; Meissner *et al.* 1997). If [^{3}H]ryanodine binding is to be

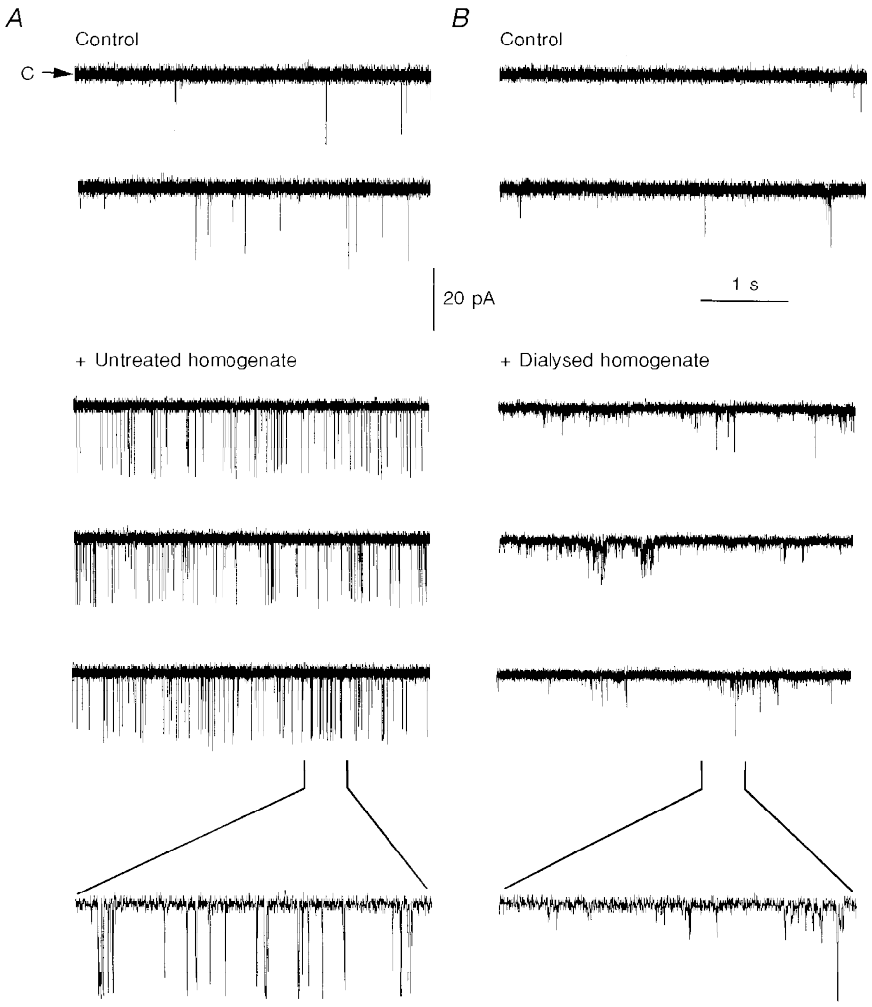

Figure 6. RyR activity in the presence of untreated *versus* dialysed total homogenate

Sea urchin egg RyR activity was recorded before (labelled Control) and after the addition of 32 μg of untreated homogenate (*A*) or 35 μg of dialysed homogenate (*B*), prepared as described in Methods. The bottom trace is an expanded 400 ms segment of the trace indicated by the brackets. The results are representative of $n = 3$ and 6 channels for untreated and dialysed homogenate, respectively.

taken as an index of the activity of RyRs (Meissner, 1994), the bell-shaped curve suggests that Ca^{2+}, over a very narrow range, may act as a trigger and a blocker of cADPR-sensitive Ca^{2+} stores.

In the presence of cytosolic Ca^{2+} as the sole agonist, and using Cs^{+} as the charge carrier, only sparse and unstable channel openings are observed (Figs 2, 5 and 6). Thus, conditions considered standard for recording cardiac and skeletal RyRs do not favour the activity of sea urchin egg RyRs. Addition of total egg homogenate supplemented with ATP (see Methods) brings about robust and sustained channel activity. ATP alone cannot evoke channel activity (not shown). Thus, ATP may be a cofactor for accessory components, or it may overcome the potential inhibitory effect of free Mg^{2+} (present in the total homogenate since it is a component of the cortical membrane isolation medium). Also, cADPR and CaM, which were added separately and in tandem, could modulate but not evoke the level of sustained activity generated by adding the whole homogenate. Therefore, some unidentified factor(s) of the homogenate seem to be indispensable components of the functional Ca^{2+} release unit of sea urchin eggs. To characterize the nature of the activating factor(s), we dialysed the sea urchin homogenate in dialysis cassettes with a molecular weight cut-off $\leqslant 2000$ and found that RyRs systematically failed to display the vigorous level of activity elicited by undialysed homogenate (Fig. 6). Because dialysis presumably excluded from the homogenate substances of a molecular weight $\leqslant 2000$, this result alone strongly argues against the activating factor being a single protein. In this context, the role of membranous or cytosolic proteins which by themselves modulate Ca^{2+} release in sea urchin eggs remains to be determined. These proteins include FK506-binding protein (FKBP12), which restores cADPR sensitivity in pancreatic islets (Noguchi *et al.* 1997), and a protein akin to the cytosolic protein 'oscillin', which induces Ca^{2+} oscillations in sea urchin eggs (Parrington, Swann, Shevchenko, Sesay & Lai, 1996). Whatever the factor(s), its indispensable presence reinforces the notion that several constituents of sea urchin eggs coexist in close interdependence to form a functional Ca^{2+} release unit.

Similar to cardiac and skeletal RyRs (Liu *et al.* 1989; Ding & Kasai, 1996), sea urchin egg RyRs exhibited subconducting states which corresponded to approximately $\frac{1}{2}$ and $\frac{1}{4}$ of the full ~600 pS conducting level (Fig. 3). The molecular mechanism underlying the appearance of these substates in mammalian RyRs has not been resolved, but an advanced hypothesis is that the fully conducting state represents the simultaneous opening of four RyR monomers, each contributing $\frac{1}{4}$ of the fully conducting level (Liu *et al.* 1989; Ding & Kasai, 1996). This hypothesis is consistent with the tetrameric arrangement of the cardiac and skeletal RyR (Wagenknecht, Grassucci, Frank, Saito, Inui & Fleischer, 1989). If applicable to sea urchin egg RyRs, it would suggest that these channels are similarly arranged and, because the full conductance state predominated in the recordings, that the gating of the monomers within the tetramer displays a strong co-operative interaction. Recently, it has been postulated that the co-operative interaction in the gating activities of the skeletal (Brillantes *et al.* 1994) and cardiac (Xiao, Valdivia, Bogdanov, Valdivia, Lakatta & Cheng, 1997) RyR monomers is enhanced by the immunophilin FKBP12. Given the uncertainty in the molecular components of the Ca^{2+} release unit in sea urchin eggs, it will be of interest to test whether FKBP12 inhibitors (FK506, rapamycin) affect the gating mechanism of sea urchin egg RyRs.

Photolysis of caged cADPR produced a transient activation of sea urchin egg RyRs (Fig. 4). From the traces of channel activity, it is clear that a latency period exists before the bursts of openings are evident and that the channel closes before the train of flashes is terminated. This is well represented in the three trains of flashes shown in Fig. 4 and was consistently found in other traces. It is unlikely that this phenomenon is a consequence of cADPR diffusion from the microenvironment of the channel because Ca^{2+}, a smaller and more mobile molecule, diffuses from the site of photolysis with a time constant of ~6 s (Valdivia *et al.* 1995). Furthermore, in other experiments in which direct additions of cADPR were made, we observed a transient response of sea urchin egg RyRs as well (not shown). Thus, this phenomenon manifests several of the essential characteristics of 'adaptation'. In receptors that 'adapt', there is an attenuation of the response in the presence of a prolonged stimulus (Knox, Devreotes, Goldbeter & Segel, 1986); no further response is detected as long as the stimulus is held constant. Recovery of sensitivity begins when the stimulus is removed (Knox *et al.* 1986). Since cardiac and skeletal RyRs exhibit adaptation to agonists such as Ca^{2+} (Györke & Fill, 1993; Valdivia *et al.* 1995), it is conceivable that the termination of the RyR response to a prolonged cADPR stimulus reflects adaptation of sea urchin egg channels. However, this will not be definitively established until an accurate determination of the magnitude and time course of the cADPR levels produced by photolysis is made, and this phenomenon be discriminated from simple desensitization.

In summary, the behaviour of sea urchin egg RyRs in lipid bilayers is complex and conditional upon the presence of cytosolic constituent(s) of low molecular weight. Once a cortical membrane-embedded RyR is rendered functional by addition of accessory components, it is capable of responding to cADPR and of displaying several of the characteristic properties of its mammalian counterparts. The ability to record sustained channel activity in a system where individual components may be added or subtracted may prove an excellent assay for defining the pharmacological profile of sea urchin egg RyRs and for dissecting the mechanism by which Ca^{2+}, CaM and cADPR induce stimulus–secretion coupling in a variety of cells.

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