Inositol 1,4,5-Trisphosphate-induced Calcium Release and Guanine Nucleotide-binding Protein-mediated Periodic Calcium Rises in Golden Hamster Eggs

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Abstract. Periodic increases in intracellular free calcium occur upon fertilization of golden hamster eggs (Miyazaki et al. 1986. *Dev. Biol.* 118:259–267). To investigate the underlying mechanism, inositol 1,4,5-trisphosphate (IP_3) and guanine nucleotides were microinjected into the egg while Ca^{2+} transients were monitored by aequorin luminescence and/or hyperpolarization in the membrane potential, which indicates the exact timing and spatial distribution of the Ca^{2+} rise. Injection of IP_3 induced an immediate Ca^{2+} transient of 13–18 s in the entire egg. The critical concentration of IP_3 was 80 nM in the injection pipette (2 nM in the egg, assuming uniform distribution); the effect was all-or-none. The Ca^{2+} rise occurred even in Ca-free external medium. Injection of 5 mM GTP or 0.33 mM guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) (calculated intracellular concentration, 200 or 12 μM, respectively) caused a similar Ca^{2+} transient with a delay of 160–200 s. More than 50 μM GTPγS produced recurring and attenuating Ca^{2+} transients in a local area of the cytoplasm, with an initial delay of 25–40 s and intervals of 45–60 s. In Ca-free medium the first one to two Ca^{2+} transients occurred but succeeding ones were absent. Preinjection of guanosine-5′-*O*-(2-thiodiphosphate) inhibited the occurrence of both GTPγS-induced and sperm-induced Ca^{2+} transients in a dose-dependent manner. Neither pertussis nor cholera toxins had effect. It was proposed that sperm–egg interaction activates a GTP-binding protein that stimulates production of IP_3, causing the first one to two Ca releases from internal stores, and also stimulates a pathway for elevation of Ca^{2+} permeability in the plasma membrane, thereby sustaining the repeated Ca^{2+} releases.

A dramatic, transient increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) occurs at the early stage of fertilization in various eggs. The biological significance of the Ca^{2+} rise is to induce cortical granule exocytosis of the egg for polyspermy block, and possibly to trigger other events at egg activation (5, 12). In golden hamster eggs, sperm–egg interaction produces transient but periodic Ca^{2+} rises, as previously demonstrated with Ca-sensitive microelectrodes (8) and the Ca^{2+}-dependent luminescent protein, aequorin (15). The first two to three Ca^{2+} transients take the form of a propagating wave starting from the sperm attachment site; later Ca^{2+} transients occur almost synchronously in the whole egg and recur at fairly constant intervals of 40–120 s (15). The initiation of Ca^{2+} transients has been shown to be related to the activation of eggs (9).

The aim of the present study was to investigate the mechanism involved in the signal transduction of sperm–egg interaction in the plasma membrane that causes the increase in $[Ca^{2+}]_i$ in the hamster egg. In this respect, recent studies of the sea urchin egg support the idea that signal transduction involves the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2), which is mediated by phosphodiesterase and is regulated by a GTP-binding protein (G protein)[1] (23). In fact, the metabolism of phosphatidylinositides is stimulated by insemination (25). Inositol 1,4,5-trisphosphate (IP_3), one of the products of this process, is able to induce the release of Ca^{2+} from intracellular stores of the sea urchin egg (3, 18). Microinjection of IP_3 results in cortical granule exocytosis and fertilization membrane elevation (23, 28). Injection of guanosine-5′-*O*-(3-thiotriphosphate)(GTPγS), a hydrolysis-resistant analog of GTP, also induces exocytosis; this response as well as sperm-induced exocytosis is blocked by preinjection of guanosine-5′-*O*-(2-thiodiphosphate)(GDPβS) (23), suggesting that a G protein is involved in the regulation of IP_3 production. The G protein has been partially purified from sea urchin egg membranes (20). These findings indicated the importance of investigating the

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1. *Abbreviations used in this paper*: CTX, cholera toxin; GDPβS, guanosine-5′-*O*-(2-thiodiphosphate); G protein, guanine nucleotide-binding protein; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); HR, hyperpolarizing response; HS, hyperpolarizing shift of the resting potential; IP_3, D-myo-inositol 1,4,5-trisphosphate; IAP, islet-activating protein (pertussis toxin); PIP_2, phosphatidylinositol 4,5-bisphosphate.

transduction mechanism in mammalian egg fertilization, and examining how the periodic Ca^{2+} rises are produced in relation to IP_3 or a G protein. In the present experiments, IP_3 and guanine nucleotides were microinjected into unfertilized or fertilized hamster eggs while an increase in $[Ca^{2+}]_i$ was monitored by aequorin luminescence and/or by hyperpolarization in the membrane potential.

Materials and Methods

Egg and Sperm

Mature eggs were collected from the oviducts of superovulated female golden hamsters injected with pregnant mare's serum gonadotropin and human chorionic gonadotropin. The surrounding cumulus cells and zona pellucida were removed by sequential treatment with 0.05% hyaluronidase (1–1.5 min at 22–24°C) (P-L Biochemicals Inc., Milwaukee, WI) and 0.07% trypsin (1.5–2 min) (Gibco, Grand Island, NY). Zona-free eggs were transferred to a 0.4-ml drop of a modified Krebs-Ringer solution (BWW medium; see reference 1) in a 35-mm plastic petri dish and covered with paraffin oil. The dish was pretreated with an aqueous solution of poly-L-lysine (50 μg/ml) to make eggs stick to the bottom of the dish. The dish was mounted on a microscope stage heated to 32–34°C. Spermatozoa obtained from the cauda epididymides were allowed to undergo the acrosome reaction by incubation at 37°C for 4–5 h. For insemination, a very small amount of sperm suspension (2–3 μl) was added to the dish containing the experimental eggs. Since multiple sperm enter a zona-free egg, the number of sperm attaching to the egg was restricted as much as possible. More details have been described previously (7, 15).

Microinjection

A glass micropipette was beveled on a rotating plate with an alumina abrasive film in order to form a sharp tip where the long axis of the opening was 2–2.5 μm. The pipette was back-filled under pressure with silicon oil (100 centistokes) and connected to a microinjector (IM-4B; Narishige Scientific Instrument Laboratory, Tokyo, Japan). Then the desired volume of injection solution was sucked up in the tip and capped with oil. The inner volume of each pipette was precalibrated as the function of the length from the tip by pushing out the aqueous solution into an oil pool and then measuring the diameter of the drop. The micropipette was inserted into an egg by tapping the micromanipulator, and then the cap of oil and aqueous solution were injected by pressure in 1–2 s near the center of the egg cytoplasm. When two or three kinds of solutions were injected each solution was separated by silicon oil in a single pipette. The volume injected was 2–8 pl for each solution, which was 1–4% of the total egg volume of ~200 pl (mean egg diameter, 72 μm). Since there was no way to know the exact concentration of injected substance at its site of action, the intracellular concentration was tentatively calculated under the assumption of even distribution in the cytoplasm. In the present paper, the calculated intracellular concentration of substance X is expressed as [X].

Injection Solutions

The following substances were injected: IP_3 (Amersham International, Buchinghamshire, England); GTPγS and GDPβS (Boehringer Mannheim Biochemicals, Mannheim, West Germany); GTP, ATP, ADP, and cholera toxin (CTX) (Sigma Chemical Co., St. Louis, MO); islet-activating protein (IAP) (pertussis toxin, gift from Dr. M. Ui, University of Tokyo, Tokyo, Japan). Each substance was dissolved and diluted with the basal injection medium, which consisted of 100 μM EGTA and 10 mM Hepes buffered at pH 7.0. KCl was omitted in this medium to avoid contamination of Ca^{2+}. Injection of the basal medium alone (up to 25 pl) produced no Ca^{2+} rise in the egg. The pH was readjusted at 7.0 before use, when guanine or adenine nucleotides were dissolved at concentrations >10 mM. IAP (125 μg/ml) and CTX (1 mg/ml) were preactivated by incubation for 20–30 min at 32–37°C in the presence of 5 mM ATP and 5 mM dithiothreitol (DTT) (for IAP), or in the presence of 50 mM DTT (for CTX), and then diluted with the basal medium.

External Solutions

The primary medium was BWW (1), used in the following compositions (in millimolars): NaCl, 94.6; KCl, 4.8; $CaCl_2$, 1.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; Na lactate, 22; Na pyruvate, 0.5; glucose, 5.6; $NaHCO_3$, 25.1. The solution was equilibrated with 5% CO_2 at pH 7.4. In some experiments $CaCl_2$ was omitted (Ca-free medium); 0.5 mM EGTA was added to the Ca-free medium in several cases. 4 mg/ml BSA was added to all solutions before use.

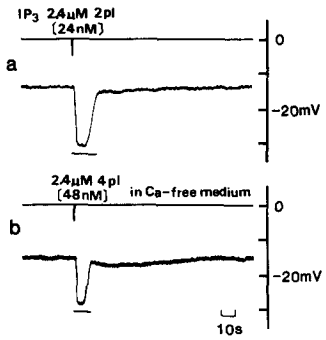

Figure 1. HR induced by injection of 2 pl of 2.4 μM IP_3 in two eggs bathed in normal BWW medium (*a*) or in Ca-free medium (*b*). The calculated intracellular concentration of IP_3 is given in a square bracket. Injection was performed at the moment indicated by the vertical bar on the top trace. The horizontal bar under the response indicates the time during which aequorin luminescence was accumulated, as shown in Fig. 2.

Electrical Recordings

Since a Ca^{2+} transient in the hamster egg always generates a hyperpolarizing response (HR) due to a Ca-activated K conductance (8, 17), an increase in $[Ca^{2+}]_i$ was monitored by recording the membrane potential on a pen recorder. A 4-M K-acetate-filled microelectrode was inserted into the egg after introduction of the injection pipette. Constant current pulses were applied through the electrode by means of a bridge circuit, and thereby a change in the membrane conductance was monitored, based on the linear current–voltage relationship (17).

Experiments with Aequorin

In some experiments aequorin was injected several minutes before the injection of IP_3 or GTPγS to directly confirm a Ca^{2+} rise. The aequorin solution consisted of 9 mg/ml purified aequorin (gift from Dr. O. Shimomura, Marine Biological Laboratory, Woods Hole, MA), 100 μM EGTA, 20 mM KCl, and 7 mM morpholino propane sulfonic acid (MOPS) at pH 7.2. Volumes injected were 14–18 pl.

Luminescence generated by the intracellular Ca^{2+}–aequorin reaction was visualized as light spots on the TV monitor screen using a supersensitive TV camera system (C1966-20; Hamamatsu Photonics, Hamamatsu, Japan) (for details of the photon-counting imaging see reference 15). Total light spots during a Ca^{2+} transient were accumulated by means of an image processor and then photographed.

Microscopic Observation

The experimental egg was observed with a phase-contrast inverted microscope. The timing of injection and of the attachment of each sperm to the egg surface was electrically marked by the observer on the record. Subsequent development of the egg was not observed because the egg was damaged upon withdrawal of the micropipettes.

Results

Injection of IP_3

In Fig. 1 *a*, 2 pl of 2.4-μM IP_3 was injected into an aequorin-loaded unfertilized egg. The calculated value of intracellular concentration $[IP_3]$ was 24 nM. An HR began immediately after the injection. The HR persisted for 18 s (horizontal bar in Fig. 1 *a*), during which time all light spots of aequorin luminescence were accumulated on the TV monitor screen (Fig. 2 *a*). It was clearly demonstrated that microinjection (1% of egg volume) of IP_3 induced a Ca^{2+}

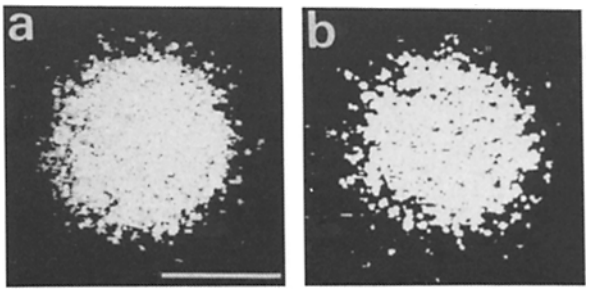

Figure 2. Aequorin luminescence during the IP_3-induced Ca^{2+} transient in the eggs from which Fig. 1 was obtained (*a* and *b* correspond to Fig. 1, *a* and *b*, respectively). All light spots on the TV monitor screen were accumulated with the image processor (see Materials and Methods) during the time indicated by the horizontal bar in Fig. 1. Bar, 50 μm.

rise in the entire egg. The Ca^{2+} rise spread over the entire egg within 1 s after the appearance of the first light spot near the tip of the injection pipette (not shown). A similar Ca^{2+} transient was observed upon injection of IP_3 in Ca-free medium (Figs. 1 *b* and 2 *b*) or in Ca-free medium containing 0.5 mM EGTA (not shown). Therefore, the IP_3-induced Ca^{2+} rise is thought to be due to Ca release from intracellular binding sites or stores, although a partial contribution of Ca influx across the plasma membrane is not completely excluded. Figs. 1 and 2 also indicate that the HR due to a Ca-activated K conductance is useful as a simple way of monitoring a Ca^{2+} transient with exact timing after microinjection.

In six cases presented in Fig. 3, the IP_3 concentration in the injection solution was changed 10,000 times from 0.024

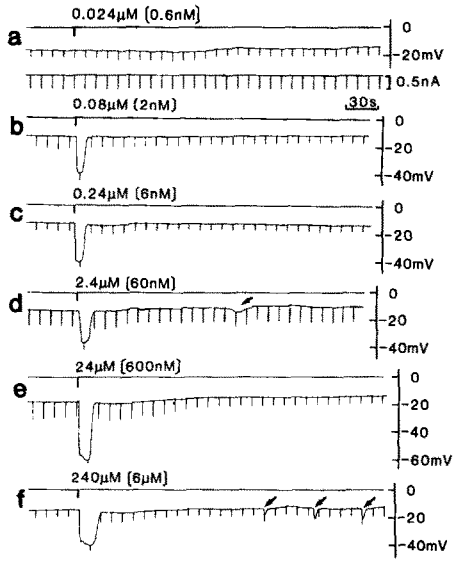

Figure 3. The HR (i.e., Ca^{2+} transient) in six eggs in response to injection of 5 pl of IP_3 solutions with different concentrations indicated. The calculated intracellular concentration of IP_3 is given in a square bracket. Arrows indicate small hyperpolarizations. As shown in the bottom trace of *a*, constant current pulses of 0.5 nA and 300 ms duration were continuously applied in all cases in this figure and those in Figs. 6–10 as well.

Table I. Dose-Response Relation In IP_3-injected Eggs

IP_3 in pipette	Volume injected	$[IP_3]$	HR*	Δg‡
μM	*pl*	*nM*		*nS*
240	5	6,000	+++	70
	5	6,000	+++	68
24	5	600	+	67
2.4	8	96	+	45
	8	96	+	–
	6	72	+	52
	5	60	+	45
	4	48	+	–
	4	48	+	–
	2	24	+	–
	2	24	+	67
	1	12	+	41
0.24	5	6	+	50
	5	6	+	41
	4	5	+	40
	3	4	+	–
	2	2	+	38
0.08	5	2	+	38
	5	2	+	35
	5	2	+	32
	4	2	–	
	4	2	–	
	3	1	–	
	2	0.8	–	
0.024	8	1	–	
	5	0.6	–	
	5	0.6	–	
	4	0.5	–	
0	25	0	–	
	18	0	–	

* The occurrence of an HR is indicated by + and that of repeated small hyperpolarizations by +++.
‡ Conductance increase at the peak of the HR: subtraction of the membrane conductance just before or after the HR from that at the peak. The measurement was missed in the cases in which the applied current pulse deviated from the peak of the HR.

μM while the volume injected was constant (5 pl). Fig. 3, *a* and *b* indicate that the critical $[IP_3]$ for the induction of an HR is between 0.6 and 2 nM. The initiation of the HR after injection occurred within 2 s, even with the minimum effective dose of IP_3. Usually, only a single HR was induced at $[IP_3]$ between 2 and 600 nM. A small hyperpolarization sometimes appeared 2–2.5 min after the termination of the HR at relatively higher $[IP_3]$ (see Fig. 3 *d*, arrow). At 6 μM the small hyperpolarization was repeated with an interval of ~40 s (Fig. 3 *f*, arrows).

The main HR appeared to be little affected by an increase in $[IP_3]$, except that its duration was a little longer at the higher $[IP_3]$ (Fig. 3). The amplitude of HRs was simply not compared, because poorer sealing of micropipettes should result in lower membrane resistance and hence smaller HRs. Therefore, the conductance increase at the peak of an HR (Δg) was calculated (see Table I), since it is considered to be a relatively quantitative indicator of an increase in $[Ca^{2+}]_i$ (7). Table I represents results from all of the IP_3-injected eggs. The critical $[IP_3]$ was precisely shown to be 2 nM. The Δg increased only about twofold while $[IP_3]$ was increased 3,000 times. Thus, the IP_3-induced Ca^{2+} rise was

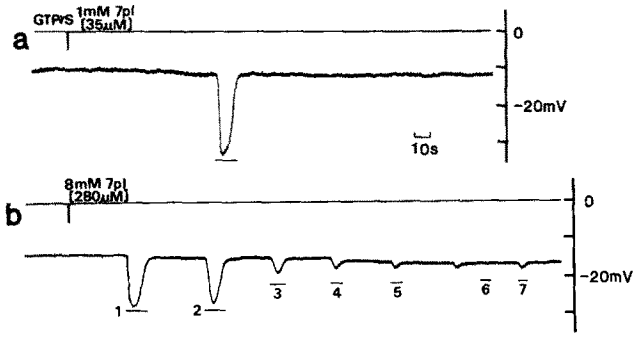

Figure 4. An HR (*a*) or periodic HRs (*b*) induced by injection of GTPγS. Each horizontal bar indicates the time for accumulation of aequorin luminescence shown in Fig. 5.

of an all-or-none nature. Furthermore, an HR with a similar Δg was generated, even if the volume injected was 1% (or less) of the egg volume. IP_3 seems to trigger a propagating Ca release such as that seen upon insemination (15).

Injection of GTP or GTPγS

GTP or GTPγS was injected into unfertilized eggs to investigate the involvement of a G protein in the regulation of Ca^{2+} rise(s). GTPγS was mainly used since it is hydrolysis resistant. In Fig. 4 *a*, injection of 7 pl of 1 mM GTPγS produced an HR that corresponded to a Ca^{2+} rise in the entire egg (Fig. 5 *a*, *1-2*). A remarkable feature of the Ca^{2+} rise induced by GTPγS was the delay after injection (102 s in Fig. 4 *a*). Another feature was the repeated occurrence of Ca^{2+} transients at higher [GTPγS], as shown in Fig. 4 *b*, where 7 pl of 8 mM GTPγS was injected. The first two HRs were associated with the Ca^{2+} rise in the entire egg (Fig. 5 *b*, *1* and *2*). Succeeding HRs were much smaller and progressively became even smaller. The luminescence intensity during each HR became lower, but a Ca^{2+} rise was substantially recognized in a partial area of the egg (Fig. 5 *b*, *3-5* and *7*) when light spots during a small HR were compared with those taken at the pause between small HRs (horizontal bar and photograph No. *6* in Figs. 4 *b* and 5 *b*, respectively).

Fig. 6 shows HRs induced by various concentrations of GTPγS. The minimum effective [GTPγS] was 12 μM with injection solution of 0.33 mM GTPγS. The critical injections at 12 μM were associated with a long delay of 160–190 s before the induction of HRs (Fig. 6 *a*). The delay decreased to 30 s with increasing [GTPγS]. A delay of 25 s remained, even when 8 mM GTPγS solution was injected close to the cell's cortex (when the tip of the pipette happened to be situated at the edge of the egg). A [GTPγS] >50 μM produced periodic HRs with fairly constant intervals of 45–60 s (Fig. 6, *c* and *d*). This pattern of repeated HRs was similar to that induced by sperm (7, 16, see Fig. 9 *a* in this paper), except for the remarkable attenuation beginning from the third or fourth HR. Each HR in the series was discrete in its occurrence but was not an all-or-none event in terms of size. At least the first HR seemed to be of an all-or-none nature: the increase in Δg was within twofold over a 30-fold range of [GTPγS].

The [GTPγS] of 350 μM generated periodic HRs with an interval of 33–45 s (Fig. 6 *e*) whereas comparable injection

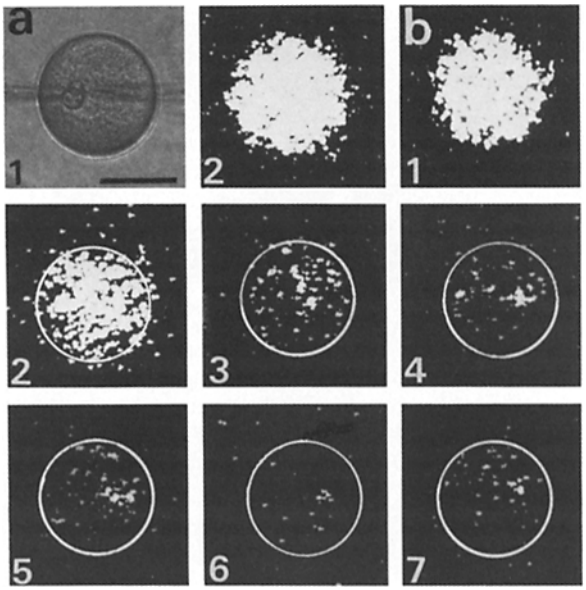

Figure 5. Spatial distribution of aequorin luminescence during each Ca^{2+} transient upon injection of GTPγS in the cases of Fig. 4. (*a*) The egg for Fig. 4 *a*, showing the bright field image (*1*) and the aequorin luminescence (*2*). In photograph *1*, two oil drops are seen in the cytoplasm: the smaller one is the injected cap of oil in the pipette and the larger one corresponds to the oil between aequorin and GTPγS solutions in the pipette (see Materials and Methods). The injection pipette is seen on the left and the recording electrode is on the right. (*b*) The egg for Fig. 4 *b*. All light spots were accumulated during the period indicated by the bar with corresponding numbers in Fig. 4 *b*. Photograph *6* shows background light level between two Ca^{2+} transients, accumulated for a time comparable to those of *5* and *7*. The contour of the egg was traced with white ink, based on the bright field image. Bar, 50 μm.

of GDPβS produced no HRs (Fig. 7 *c*). The [GTP] of 200 μM caused an HR with a delay of 200 s (Fig. 7 *a*); 200 μM GTP was apparently comparable to 12 μM GTPγS (Fig. 6 *a*). 200–400 μM GTP produced only one or two HRs. The failure of periodic Ca^{2+} transients might be due to the susceptibility of GTP to hydrolysis. These findings indicate the presence of a GTP-dependent process causing Ca^{2+} rise(s).

When 10 mM GTPγS or GTP solution was injected, an HR sometimes occurred instantaneously (Fig. 6 *e*, arrow and Fig. 7 *b*). The instantaneous HR was also induced by injection of >30 mM GDPβS in the pipette solution (Figs. 8, *c* and *d* and 9, *b–d*) and even by >20 mM ATP or ADP in the pipette (not shown). With lower concentrations, injection of 8 mM GTPγS or 5 mM GTP solution produced only the HR(s) with a substantial delay (Figs. 6 *f* and 7 *a*), and injection of 10 mM GDPβS solution (Figs. 7 *c* and 8 *a*) or 10 mM ATP or ADP solution caused no HRs. The occurrence of the instantaneous HR was dependent on the concentration in the injection solution of these phosphates, but it was rather independent of the volume injected: the HR may be caused by the fact that a local area in the cytoplasm near the tip of the pipette is transiently exposed to phosphates with high concentrations at the moment of injection. Aequorin measurements showed these HRs to be associated with a Ca^{2+} rise; these HRs were evoked even in Ca-free medium (not shown).

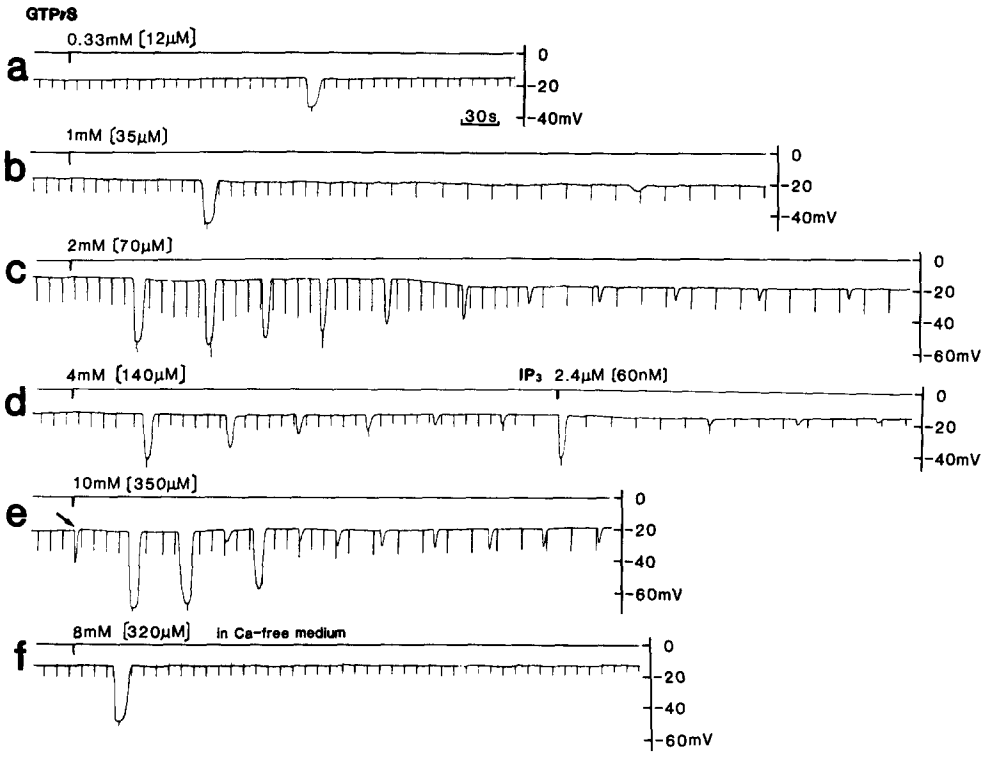

Figure 6. (*a–e*) HR(s) induced by injection of 7 pl GTPγS solution with different concentrations. In *d*, 5 pl of 2.4 μM IP_3 was subsequently injected. (*f*) Injection of 7 pl of 8 mM GTPγS after perfusion of Ca-free medium. Explanations are in the text.

The instantaneous Ca^{2+} transient, probably due to intracellular Ca release, appears to be based on another mechanism, different from that for Ca^{2+} transient(s) produced by relatively low concentrations of GTP or GTPγS with a certain delay. After the instantaneous HR, only GTPγS or GTP could produce succeeding HR(s).

Repeated Ca^{2+} Transients

In relation to the periodic Ca^{2+} transients induced by GTPγS, some additional findings should be noted. First, the resting potential gradually shifted slightly in the hyperpolarizing direction when periodic HRs appeared (see Figs. 4 *b* and 6, *c–e*), whereas the membrane was rather gradually depolarized during recording in the case without periodic HRs (see Figs. 3, 6 *a*, and 7 *c*). The hyperpolarizing shift of the resting potential (HS) has been observed during the repetitive occurrence of HRs upon insemination (see Fig. 9 *a*) and has been shown to reflect continuous Ca^{2+} influx across the plasma membrane (7).

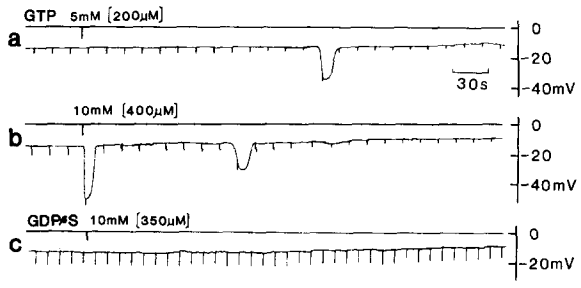

Figure 7. (*a* and *b*) Injection of 8 pl of 5 mM GTP (*a*) or 10 mM GTP (*b*); (*c*) injection of 7 pl of 10 mM GDPβS.

Second, injection of GTPγS in Ca-free medium produced only one or two HRs. For example (Fig. 6 *f*), injection of 8 mM GTPγS in the pipette (320 μM in the egg), a concentration lower than that eliciting the instantaneous HR, evoked the first HR with a delay of 30 s but failed to produce any additional HR. Of six eggs studied, five eggs showed only the first HR and one egg showed the first two HRs. This result is consistent with the previous finding that the periodic HRs upon insemination are abolished by perfusion of Ca-free medium (7). The HR was rarely generated by GTPγS injection when eggs were kept in Ca-free medium longer than 10 min (necessary for the attachment to the bottom of the dish) and then impaled by micropipettes. These eggs became leaky; the membrane resistance decreased remarkably ~1 min after injection of GTPγS. Therefore, records from six eggs described above, including those presented in Fig. 6 *f*, were obtained by the following procedure: eggs were impaled by pipettes in normal medium, kept in the stream of perfused Ca-free medium for 4 min, and then injected with GTPγS. Although at least the first or first two Ca^{2+} transients are due to internal Ca release, the occurrence of succeeding Ca^{2+} rises requires the presence of Ca^{2+} in the external medium (see Discussion).

Third, the periodic small HRs induced by GTPγS could be interrupted by an interposed HR induced by IP_3 injection (Fig. 6 *d*). The extra HR induced by IP_3 was much larger than the small HRs and nearly as large as the first HR induced by GTPγS. The small HR, therefore, corresponded to a local Ca^{2+} rise insufficient to develop a Ca release throughout the egg, although Ca stores had been reloaded and were ready to release Ca. The small HRs were interrupted by the IP_3-induced HR, reappeared ~2 min later, and then recovered periodicity gradually (Fig. 6 *d*). Thus, once a Ca release over the whole egg took place in response

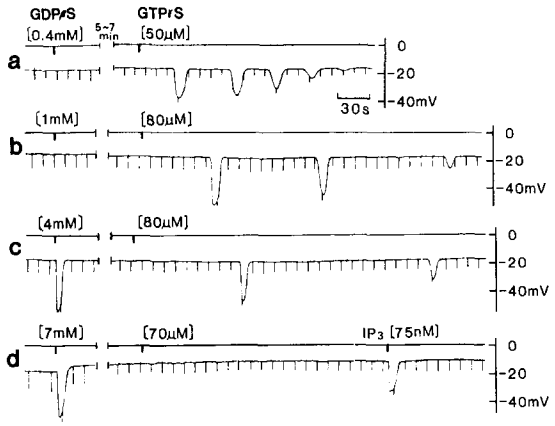

Figure 8. Preinjection of increasing amounts of GDPβS, followed by injection of 2 mM GTPγS solution. Records were interrupted for 5–7 min between the two injections, during which time no response occurred. The concentrations of GDPβS in the injection pipette were (in mM): 10 (*a*), 30 (*b*), 100 (*c*), and 200 (*d*). In *d*, 2.4 μM IP_3 was injected after the application of GTPγS. The volume injected was 5–8 pl for each solution.

to IP_3 injection, it caused an apparent refractory period in the local Ca^{2+} rise. It has been shown that the periodicity of sperm-induced HRs is reset by an extra HR induced by Ca^{2+} injection (7).

Inhibition of Ca^{2+} Transients by GDPβS

In Fig. 8, GDPβS was preinjected and then GTPγS sufficient to produce periodic HRs was injected 6.5–8.5 min later. The [GDPβS] of 0.4 mM had no effect (Fig. 8 *a*), but 1–4 mM GDPβS prolonged both the delay and intervals of GTPγS-induced HRs (Fig. 8, *b* and *c*), like the case of lower [GTPγS] shown in Fig. 6. GDPβS itself often generated a single instantaneous HR when it was >30 mM in the injection pipette, as already described. 7 mM GDPβS in the egg completely blocked GTPγS-induced HRs but did not affect the IP_3-induced HR (Fig. 8 *d*) (n = 6), indicating that the blocking site of GDPβS precedes IP_3-induced Ca release. Probably, GDPβS inactivates a G protein by competing with GTPγS at the guanine nucleotide-binding site.

In Fig. 9, GDPβS was preinjected and then sperm suspension was applied to make sperm attach to the egg 8.5–12.5 min later. Fig. 9 *a* shows a control record without injection of GDPβS. Upon insemination of zona-free hamster eggs, the first HR appears at about the time when flagellar motion of the first attached sperm stops (Fig. 9 *a*, 1 st; see reference 17 for detailed analysis), i.e., when sperm and egg are likely to fuse (29). Although zona-free eggs always become polyspermic, even a single sperm induces a series of multiple Ca^{2+} transients (15). HRs are repeated as the characteristics of inseminated eggs (7, 15, 16), and the recurring HRs don't always coincide with the stopping of flagellar motion of additional sperm (see sts in Fig. 9 *a*). The additional sperm fuse and enter the egg irrespective of preceding Ca^{2+} rises. When GDPβS was preinjected, 1 mM GDPβS in the egg had no effect on sperm-induced HRs (Fig. 9 *b*; see legend). 5 mM GDPβS inhibited the occurrence of periodic HRs except for the first two HRs (Fig. 9 *c*), and 8 mM GDPβS completely blocked HRs (Fig. 9 *d*) (n = 4). The partial inhibition or complete block of sperm-dependent Ca^{2+} rises by GDPβS was not due to a block of sperm–egg fusion, because the occurrence of sperm-egg fusion was judged by the stopping of flagellar motion of each sperm (see sts in Fig. 9, *c* and *d*). The multiple sperm that have fused the zona-free egg are usually incorporated into the egg cytoplasm (29), but the confirmation of sperm entry into GDPβS-injected eggs was not done because of the technical limitation in the present study (see Materials and Methods).

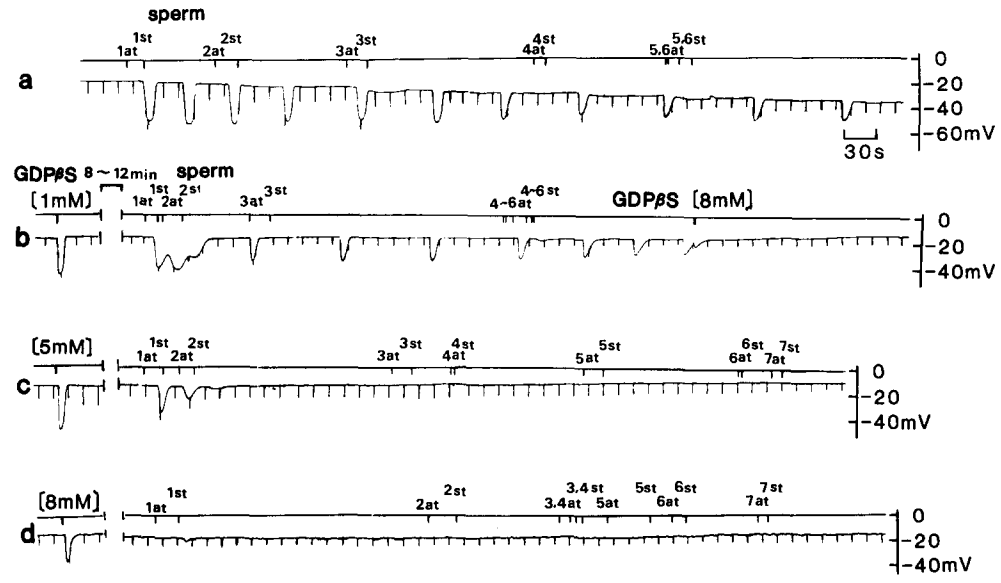

Figure 9. a: HRs upon insemination (control experiment). (*1–6 at*) Attachment of the first to sixth sperm to the egg surface. (*1–6 st*) Stop of flagellar motion of the first to sixth sperm. (*b–d*) Preinjection of GDPβS, followed by insemination. There was an interruption of 8–12 min after GDPβS injection in each record. The concentrations of GDPβS in the injection pipette were (in mM): 30 (*b*), 100 (*c*), and 200 (*d* and *b*, *right*). Injected volume was 7–8 pl. Usually each HR is discrete when recorded with a single fine electrode as in *a*, but fused HRs (as the first three HRs in *b*) were often recorded with two electrodes, one of which (injection pipette) was fairly thick. Leakage of ions in the plasma membrane may effect the recovery of a Ca^{2+} rise.

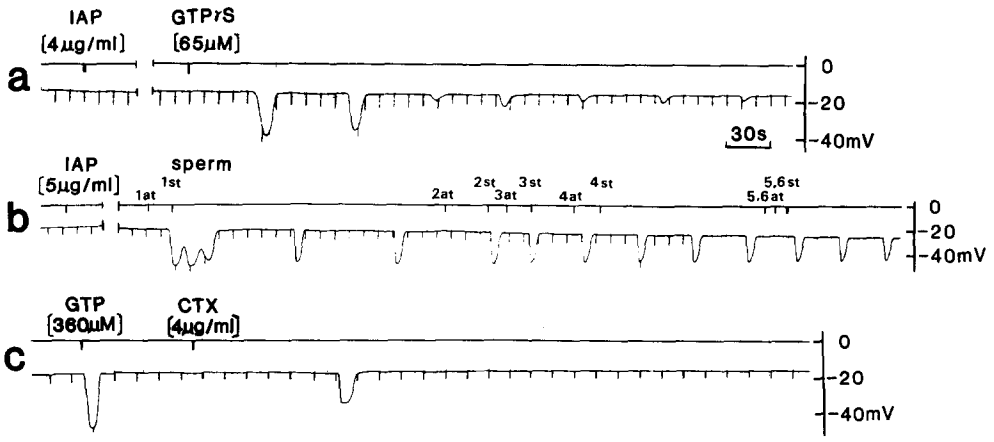

Figure 10. (*a* and *b*) Preinjection of IAP (125 μg/ml in the pipette), followed by injection of 2 mM GTPγS solution (*a*) or by insemination (*b*). There is an interruption of ~8 min after IAP injection in each record. (*c*) Injection of CTX (100 μg/ml in the pipette) 75 s after the application of GTP (10 mM in the pipette). Volume injected was 6–8 pl for each solution.

The blocking effect of 8 mM GDPβS in the egg was also observed when GDPβS was injected during the series of periodic HRs (see Fig. 9 *b*, right). The blocking of HRs by GDPβS was associated with a slight depolarizing shift of the resting potential instead of a hyperpolarizing shift (Fig. 9, *b–d*). The finding that the occurrence of both GTPγS-induced HRs and sperm-induced HRs was blocked by GDPβS with similar concentrations led to the conclusion that a G protein-mediated process was involved in formation of repeated Ca^{2+} transients during fertilization.

Injection of Pertussis Toxin and Cholera Toxin

To identify G proteins, the egg was injected with IAP and CTX, which catalyze the ADP ribosylation of G proteins (6, 26). Preinjection of purified IAP, which had been activated with DTT and ATP, neither inhibited nor stimulated the occurrence of GTPγS-induced Ca^{2+} rises (examined in nine eggs, $n = 9$) and sperm-induced Ca^{2+} rises ($n = 18$). The interval between injection of IAP and injection of GTPγS or attachment of the first sperm to the egg surface was 7–12 min (25 min in one case). The concentration of IAP in the injection solution was 1–125 μg/ml, a final concentration of 40 ng/ml–6 μg/ml in the egg. Examples are shown in Fig. 10 (*a* and *b*). No significant effect was observed when IAP was injected during the series of periodic HRs. Some eggs were incubated for 5 h in medium containing 2 μg/ml IAP and then inseminated. Periodic HRs occurred normally ($n = 4$, not shown).

CTX was injected in 33 eggs; the [CTX] in the egg ranged between 0.1 and 47 μg/ml. No HR was induced in 29 eggs. At 4 μg/ml, a single HR was induced with a delay of 40–120 s in 4 eggs, but no HR appeared in another 11 eggs. In five eggs, 1 mg/ml CTX was injected, giving 41–47 μg/ml in the egg. No HR was observed in these five eggs. In 14 eggs, 5–10 mM GTP or 8–10 mM GTPγS was injected before or after the CTX injection ([GTP], 210–420 μM; [GTPγS], 320–400 μM). Neither a facilitatory nor an inhibitory effect of CTX was observed (Fig. 10 *c*; cf. Fig. 7 *b*). No significant effect of CTX on sperm-induced HRs was observed ($n = 5$). Thus, although a stimulating effect of CTX was suggested in some cases, it can not be concluded from the present experiments that the G protein involved in fertilization is CTX sensitive.

Discussion

In the present study, IP_3- and GTPγS-induced Ca^{2+} transients were directly demonstrated in single cells with exact timing and spatial distribution of the Ca^{2+} rise. Microinjection experiment revealed that IP_3 induces a single Ca release with no measurable delay in the hamster egg and that GTPγS as well as GTP causes Ca^{2+} release(s) with a certain delay. GTPγS produces multiple Ca^{2+} rises that require external Ca^{2+}, similar to those at fertilization. Preinjection of GDPβS inhibits the occurrence of both GTPγS-induced and sperm-induced Ca^{2+} rises in a dose-dependent manner, suggesting that a G protein-mediated process is involved in Ca^{2+} release at fertilization of mammalian eggs.

IP_3-induced Ca Release

Injection of IP_3 induced a Ca release in an all-or-none fashion. The critical concentration was 80 nM in the pipette solution or 2 nM in the egg. The critical [IP_3] in the egg is consistent with the value in the sea urchin egg for causing cortical vesicle exocytosis (23). However, the concentration at the site of action is uncertain, because the Ca release occurs immediately after injection. Rather, 80 nM in the injection solution may be closer to the critical concentration at the site of action. The IP_3-induced Ca^{2+} rise occurs in the entire egg, as shown by aequorin luminescence. The concurrent HR, which is mediated by Ca-activated K channels in the plasma membrane, probably reflects a Ca^{2+} rise in the subsurface area of the cytoplasm. The Ca release seems to occur not only in the peripheral area but also in the central area, since microinjection of IP_3 near the center could induce the HR within 2 s. A previous study has shown that iontophoretic injection of Ca^{2+} into the egg induces a regenerative HR in Ca-free medium (7). It is possible that a small amount of IP_3 causes a local Ca^{2+} rise and triggers a propagating Ca release based on Ca-induced Ca release, as proposed for the *Xenopus* egg by Busa et al. (2). They have demonstrated an IP_3-induced local Ca^{2+} release, a subthreshold response insufficient to trigger a conducting Ca^{2+} release (2). Attempts to investigate such local Ca^{2+} release using aequorin upon subthreshold injection of IP_3 were not performed in the present study, but are now in progress. In the sea urchin egg, Swann and Whitaker (22) have proposed

a recycling process between Ca-stimulated production of IP_3 and IP_3-induced Ca release for the propagating Ca release at fertilization.

Injection of IP_3 usually produced only a single Ca^{2+} transient, despite the ability of the hamster egg to form repeated Ca^{2+} rises. The injected IP_3 is likely to be immediately turned over. Extremely high doses of IP_3 could produce repeated small HRs. The small HR may correspond to a local Ca^{2+} release.

An instantaneous Ca release was also induced nonspecifically with adenine and guanine nucleotides in the concentration higher than 10 mM in the pipette solution. This may be attributed to the high level of phosphate and may be an unphysiological phenomenon.

G Protein-mediated Process

The critical [GTPγS] for causing a Ca^{2+} transient was 12 μM, which is also consistent with the value in the sea urchin egg (23). In this case the calculated value will be realistic, considering the substantial delay of the response. It is possible that the injected GTP diffuses to the plasma membrane, binds and activates G protein, and eventually causes the Ca^{2+} rise. This plausible process in the membrane requires at least 25 s for induction of the first Ca^{2+} rise, as suggested by sufficient application of GTPγS in the peripheral area. The first or first two Ca^{2+} transients were thought to be due to internal Ca release. The possible pathway is that G protein activates phosphodiesterase, which facilitates the breakdown of PIP_2, and resulting IP_3 mobilizes Ca^{2+} from stores, a process thought to take place in other cells (4, 13, 19, 27). Since early Ca^{2+} transients were blocked by GDPβS upon insemination, the sperm–egg interaction in the hamster possibly activates this pathway by way of G protein, as proposed for the sea urchin (23).

The supposed G protein was not identified in terms of IAP or CTX sensitivity in the present experiments. In the sea urchin egg it has been shown that microinjected CTX at high concentration (~30 μg/ml) causes exocytosis of cortical vesicles, but the effect of IAP is unclear (24). IAP- and CTX-insensitive G proteins that stimulate phosphodiesterase have been reported in other cells (13, 14).

Periodic Ca^{2+} Transients

A characteristic feature in the fertilizing hamster egg is periodic Ca^{2+} transients. GTPγS generated recurring HRs, although the amplitude attenuated remarkably. There are several similarities between GTPγS-produced HRs and sperm-induced HRs: both of them are (*a*) blocked by GDPβS, (*b*) abolished by perfusion of Ca-free medium, (*c*) associated with an HS, (*d*) generated with similar intervals of 35–60 s, and (*e*) interrupted by interposed Ca release. These similarities strongly suggest that, in addition to early Ca^{2+} transients, succeeding periodic Ca^{2+} rises at fertilization are also mediated by a G protein. GTPγS should activate the G protein persistently because of its hydrolysis resistance. Correspondingly, injection of GDPβS interrupts the series of sperm-induced HRs (Fig. 9 *b*, right). Therefore, the series of Ca^{2+} rises upon fertilization requires persistent activation, not transient activation as a trigger, of the G protein–mediated process.

What is the G protein–mediated process for the formation of repeated Ca^{2+} rises? The requirement of external Ca^{2+} suggests the contribution of Ca^{2+} influx across the plasma membrane. A previous study (7) has given evidence that continuous Ca^{2+} influx, which is reflected in the HS upon fertilization, is linked and converted to periodic Ca^{2+} releases by reloading of the intracellular stores with transported Ca^{2+}, and that the interval between Ca^{2+} releases is determined by the rate of Ca accumulation in the stores. The similarities described above support the idea that the G protein–mediated process causes continuous Ca^{2+} influx and also causes, via persistently produced IP_3, the periodical Ca^{2+} release from reloaded stores. The Ca^{2+} permeability of the plasma membrane could be mediated by the G protein through diacylglycerol and protein kinase C. The C kinase has been shown to recruit covert Ca channels in *Aplysia* neurons (21). Alternatively, Ca^{2+} permeability may be induced by inositol 1,3,4,5-tetrabisphosphate, as reported in the sea urchin egg (10, 11).

GTPγS-induced Ca^{2+} rises attenuated and occurred in the partial area, probably of the periphery, causing small HRs. Each Ca^{2+} rise may be a local Ca^{2+} release, as suggested by the interference with interposed Ca^{2+} release. Upon insemination, additional factor(s) may be involved in causing a synchronous Ca^{2+} release throughout the egg.

In summary, the present paper proposes two kinds of pathways for the G protein–mediated process: one is the production of IP_3 for causing Ca^{2+} release and the other is elevation of Ca^{2+} permeability for maintaining periodic Ca^{2+} releases.

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