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Extracellular Ca^{2+} entry and Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive stores function at fertilization in oocytes of the marine bivalve *Mytilus edulis*

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SUMMARY

An oocyte of the marine bivalve *Mytilus edulis*, which is arrested at metaphase I, reinitiates meiosis at fertilization. The fertilized oocyte shows increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) comprising three different phases: an initial large $[Ca^{2+}]_i$ transient, a subsequent low but sustained $[Ca^{2+}]_i$ elevation, and repetitive small $[Ca^{2+}]_i$ transients. In this study, we have investigated the sources and mechanisms of the sperm-induced $[Ca^{2+}]_i$ increases. Application of methoxyverapamil (D-600), an inhibitor of voltage-dependent Ca^{2+} influx, suppressed the initial $[Ca^{2+}]_i$ transient but did not affect the following two phases of $[Ca^{2+}]_i$ changes. Injection of heparin, an antagonist of the inositol 1,4,5-trisphosphate (IP_3) receptor, inhibited the later two phases without much affecting the initial transient. Combined application of D-600 and heparin almost completely abolished the three phases of the sperm-induced $[Ca^{2+}]_i$ changes. Furthermore, Ca^{2+} influx caused by seawater containing excess K^+ was blocked by D-600 but not by heparin, and IP_3-induced Ca^{2+} release caused by photolysis of injected 'caged' derivatives of IP_3 was blocked by heparin but not by D-600. These results strongly suggest that two types of Ca^{2+} mobilization systems, the extracellular Ca^{2+} entry responsible for an initial $[Ca^{2+}]_i$ transient and the IP_3 receptor-mediated Ca^{2+} release responsible for the following two phases of $[Ca^{2+}]_i$ changes, function at fertilization of *Mytilus* oocytes.

Key words: calcium, *Mytilus*, oocyte, fertilization, inositol triphosphate, heparin, methoxyverapamil

INTRODUCTION

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is an essential factor regulating many cellular events. Activation of oocytes or eggs at fertilization is convenient for investigating the mechanisms of changes in $[Ca^{2+}]_i$, since it is accompanied by different types of $[Ca^{2+}]_i$ changes: a single $[Ca^{2+}]_i$ increase and repetitive $[Ca^{2+}]_i$ increases ($[Ca^{2+}]_i$ oscillations) (Miyazaki et al., 1993; Whitaker and Swann, 1993). Furthermore, there are also phylogenetic differences, since such $[Ca^{2+}]_i$ increases are thought to derive from different sources in protostomes and deuterostomes (Jaffe, 1985).

$[Ca^{2+}]_i$ oscillations at fertilization of hamster oocytes are blocked by preinjection of monoclonal antibody of the inositol 1,4,5-trisphosphate (IP_3) receptor (Miyazaki et al., 1992). In frogs, a $[Ca^{2+}]_i$ increase at fertilization is similarly inhibited by preinjection of heparin (Galione et al., 1993; Nuccitelli et al., 1993), a competitive inhibitor of the IP_3 receptor (Ghosh et al., 1988). In contrast, sole application of heparin or antagonists of the ryanodine receptor, such as ruthenium red and 8-amino-cADPR, cannot block a sperm-induced $[Ca^{2+}]_i$ increase in sea urchin eggs, but a combination of heparin and ruthenium red or heparin and 8-amino-cADPR can inhibit the increase (Galione et al., 1993; Lee et al., 1993). These results suggest that the $[Ca^{2+}]_i$ increases in these deuterostomes derive mainly from Ca^{2+} released from intracellular stores, and that they are regulated by two types of mechanisms: IP_3-induced Ca^{2+} release (IICR) mediated by the IP_3 receptor, and Ca^{2+}-induced Ca^{2+} release (CICR) mediated by the ryanodine receptor, in a species-specific manner (Berridge, 1993a,b; Miyazaki et al., 1993; Whitaker and Swann, 1993).

In protostomes, $[Ca^{2+}]_i$ increases in the oocytes at fertilization have been detected in some bivalves (Guerrier et al., 1993; Deguchi and Osanai, 1994a,b) and a polychaete (Eckberg and Miller, 1995), although precise mechanisms of the increases are still unknown. In the bivalve *Mactra*, in which spawned oocytes are arrested at prophase I until fertilization, sperm-induced $[Ca^{2+}]_i$ increases are immediately abolished by removal of external Ca^{2+} after fertilization (Deguchi and Osanai, 1994b), suggesting that the increases result mainly from extracellular Ca^{2+} entry. On the other hand, the same treatment fails to abolish sperm-induced $[Ca^{2+}]_i$ increases in another type of bivalve including *Mytilus*, in which spawned oocytes are arrested at metaphase I until fertilization (Deguchi and Osanai, 1994a), indicating that some intracellular Ca^{2+} release system at fertilization exists in these oocytes. Thus,

Ca^{2+} sources at fertilization seem to be different in prophase I type and metaphase I type protostomes.

The aim of the present study is to elucidate the sources and mechanisms of the sperm-induced $[Ca^{2+}]_i$ increases in the metaphase I type protostome *Mytilus*. For this purpose, we injected naturally spawned metaphase I-arrested oocytes with fluorescent Ca^{2+} indicators and assessed the effects of some activators and inhibitors on Ca^{2+} mobilization. The results obtained strongly suggest that (1) in *Mytilus* oocytes, the extracellular Ca^{2+} influx mechanism occurs via Ca^{2+} channels and the Ca^{2+} release mechanism via IP_3-sensitive receptors, and that (2) both types of mechanisms actually function at fertilization.

MATERIALS AND METHODS

Gametes

Adult specimens of the marine mussel *Mytilus edulis* were collected in Mutsu Bay, Aomori, from November to December, and kept in running seawater at 5-15°C. All experiments were performed during the breeding season from December to April. In order to obtain mature oocytes arrested at metaphase I and sperm with high motility, spawning was induced by placing the mussels in warm seawater (at 25-30°C). The spawning mussels were then transferred into natural filtered seawater (NSW) at 16-20°C. The spawned oocytes were washed two or three times with NSW before use. Sperm were further diluted with NSW just before insemination.

Solutions

Ca^{2+}-free seawater (Osanai, 1975) was generally supplemented with 10 mM EGTA. Excess K^+ seawater was a mixture of 0.52 M KCl and NSW (1:4). Ca^{2+}-free seawater containing EGTA and excess K^+ seawater were adjusted to pH 8.3, approximately corresponding to the pH value in NSW used in this study. Stock solutions of 20 or 40 mM methoxyverapamil (D-600, Sigma), 20 mM nifedipine (Sigma) and 20 mM nitrendipine (RBI) were prepared in DMSO:ethanol (1:3) and diluted 1:200 in NSW or in excess K^+ seawater. Caffeine (Sigma or Wako) was directly prepared in NSW, to a final concentration of 50 mM.

Measurement of $[Ca^{2+}]_i$ with Fura-2

The methods of microinjection, fluorescence measurement and calibration for the fluorescent Ca^{2+} indicator Fura-2 (Dojindo) have been described in detail (Deguchi and Osanai, 1994a). Briefly, Fura-2 dissolved at 2.5 mM in an injection buffer containing 100 mM potassium aspartate and 10 mM HEPES (pH 7.0) was injected into the cytoplasm of oocytes by water pressure. The Fura-2-injected oocyte was placed in a measurement chamber and excited at 340 nm and 380 nm, and the emitted light from the whole oocyte was collected after passing through a bandpass filter (510 nm). Changes in the fluorescence intensities at 340 nm (F340) and 380 nm (F380) with time were continuously recorded on a floppy disk. From in vitro calibration, a fluorescence ratio (F340/F380) of 0.4 roughly corresponds to an absolute Ca^{2+} concentration of 30 nM; a ratio of 0.8 to 170 nM Ca^{2+}; a ratio of 1.2 to 320 nM Ca^{2+}; a ratio of 1.6 to 480 nM Ca^{2+}; and a ratio of 2.0 to 640 nM Ca^{2+}. However, recordings are presented as changes in F340/F380.

In some experiments, Fura-2 was injected together with one or more of the following: 8, 20 or 50 mg/ml heparin (M_r: 3×10^3, Sigma), 50 mg/ml de-N-sulfated heparin (Sigma), and 5 or 10 mM ruthenium red (Sigma or Wako). Intracellular concentrations of the injected compounds (1-2.5% of the original concentrations in injection pipettes) were roughly estimated from the fluorescence intensities excited at 360 nm (F360), a Ca^{2+}-insensitive wavelength of the dye (Grynkiewicz et al., 1985).

Measurement of $[Ca^{2+}]_i$ with Calcium Green

Calcium Green (Molecular Probes), a visible excitation wavelength Ca^{2+} indicator, was used in experiments for caged compounds, which require UV light irradiation to be photoactivated (Walker et al., 1987; Peres et al., 1991). Calcium Green (dissolved at 1 mM in an injection buffer) was injected into oocytes alone or together with one or more of the following: 12, 50 or 300 μM *myo*-inositol 1,4,5-trisphosphate $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester (caged IP_3, Calbiochem), 300 μM 1-(α-glycerophosphoryl)-*myo*-inositol 4,5-bisphosphate $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester (caged $GPIP_2$, Calbiochem), 500 μM adenosine-5′-triphosphate P^3-1-(2-nitrophenyl)ethyl ester (caged ATP, Dojindo), 8 or 20 mg/ml heparin and 20 mg/ml de-N-sulfated heparin.

The Calcium Green-injected oocyte was illuminated with blue light (475-490 nm) and the emission fluorescence (530 nm) was collected. Although the fluorescence intensity (F) in the unstimulated oocyte will be influenced both by the injection volume and by the resting $[Ca^{2+}]_i$ level, the F value was considered to be mainly affected by the intracellular dye concentration, since injection of a mixture of 500 nM Calcium Green and 1 mM Fura-2 produced almost constant F/F360 ratios in individual oocytes (data not shown). Thus, the Calcium Green signals were corrected for variations of dye concentration by normalizing the recorded fluorescence (F) against the initial fluorescence (F_0), and the data of $(F-F_0)/F_0$ were used to show $[Ca^{2+}]_i$ changes from baseline levels (Rakow and Shen, 1990; Stricker et al., 1994). Assuming that resting $[Ca^{2+}]_i$ levels were always the same in all oocytes, estimated intracellular concentrations of the injected compounds were 1-3% of the original concentrations in injection pipettes.

Insemination, solution change and UV irradiation during $[Ca^{2+}]_i$ measurement

Unless stated otherwise, fluorescence measurements were started 10-20 minutes after injection of Ca^{2+} indicators. The experimental oocyte in a chamber was inseminated, exposed to various agents or irradiated with UV light after steady levels of F340/F380 or F had been confirmed. For insemination, 30-50 μl of sperm suspension diluted with NSW was added to the chamber (final concentration: 10^4-10^5 sperm/ml). The time of fertilization was defined as the time of the first detectable increase in F340/F380 or F (Deguchi and Osanai, 1994a). To examine the effects of externally applied chemicals (e.g. D-600) on $[Ca^{2+}]_i$ changes, new medium (approx. 1.5 ml) containing the final concentrations of the chemicals was added after withdrawal of the original medium (residue: approx. 50 μl). For photoactivation of caged compounds, the oocyte was globally irradiated with UV light (300-400 nm) for 5 seconds and the blue light for Calcium Green excitation was withdrawn during the period. The gap during the $[Ca^{2+}]_i$ measurement (see Figs 7, 8) corresponds to the irradiation time. The intensity of UV light was not measured, but the same UV irradiation was enough to induce the elevation of fertilization membrane of sea urchin eggs injected with 25 μM caged IP_3 (R. Deguchi, unpublished data).

Cytological observation

Each experimental oocyte was checked for the presence or absence of polar body formation, which occurred, if at all, at 10-20 minutes after insemination or artificial stimulation. In some experiments, each oocyte was drawn from the chamber before the first cleavage and cultured in one well of a 96-well culture plate for observation of the subsequent mitotic process.

RESULTS

Initial $[Ca^{2+}]_i$ transient at fertilization derives mainly from extracellular Ca^{2+} entry

At normal fertilization in NSW, a *Mytilus* oocyte shows $[Ca^{2+}]_i$ increases comprising three different phases (Deguchi and Osanai, 1994a). Insemination immediately causes an initial

large $[Ca^{2+}]_i$ transient, followed by a period of low but sustained $[Ca^{2+}]_i$ elevation. The elevated $[Ca^{2+}]_i$ returns nearly to the resting level at 12-20 minutes after fertilization and is then followed by repetitive small $[Ca^{2+}]_i$ increases, the number of which varies considerably in different oocytes. Similar $[Ca^{2+}]_i$ changes were observed in oocytes preincubated with 0.5% DMSO:ethanol (1:3) for 5 minutes and then inseminated (*n*=8, Fig. 1A), indicating that the vehicle alone has no effect on the sperm-induced $[Ca^{2+}]_i$ increases.

To determine whether voltage-dependent Ca^{2+} influx is involved in the sperm-induced $[Ca^{2+}]_i$ increases, we first tested the effect of its inhibitor, D-600. Incubation with 100 μM D-600 for 5 minutes followed by insemination greatly suppressed an initial $[Ca^{2+}]_i$ transient without much affecting the subsequent two phases of $[Ca^{2+}]_i$ changes (*n*=7, Fig. 1B). Similar results were obtained when a higher concentration (200 μM) of D-600 was used (*n*=6, data not shown). Later addition of 100 μM D-600 (at 90-300 seconds after normal fertilization in NSW) also failed to inhibit the subsequent $[Ca^{2+}]_i$ changes (*n*=5, data not shown). Two other Ca^{2+} channel blockers tested, nifedipine and nitrendipine, also suppressed the initial $[Ca^{2+}]_i$ transient when applied at 100 μM (data not shown). However, since D-600 inhibited an excess K^+-induced $[Ca^{2+}]_i$ increase most strongly (described below, see Fig. 6L,M,N), D-600 was chosen as the most suitable inhibitor of Ca^{2+} channels in *Mytilus* oocytes.

Later phases of $[Ca^{2+}]_i$ changes at fertilization derive mainly from Ca^{2+} release from IP_3-sensitive stores

To elucidate whether IICR is involved in sperm-induced $[Ca^{2+}]_i$ increases, we tested the effect of heparin, which is known to inhibit IICR by competing with IP_3 for binding to the IP_3 receptor (Ghosh et al., 1988). Injection of 50 mg/ml de-N-sulfated heparin, which does not bind to the IP_3 receptor (Ghosh et al., 1988), had no inhibitory effect on the sperm-induced $[Ca^{2+}]_i$ changes (*n*=7, Fig. 2A). Injection of 20 mg/ml heparin preserved the initial $[Ca^{2+}]_i$ transient after insemination (*n*=9, Fig. 2B), but normal later $[Ca^{2+}]_i$ changes were observed in only one out of nine oocytes. In the eight remaining oocytes, the phase of sustained $[Ca^{2+}]_i$ elevation did not persist long, nor did the subsequent repetitive small increases take place (Fig. 2B). Injection of a higher concentration of heparin (50 mg/ml) also prevented the later $[Ca^{2+}]_i$ increases without much affecting the initial transient in all cases (*n*=8). In these heparin-injected oocytes, the initial transient was sometimes (4/9 with 20 mg/ml heparin, 4/8 with 50 mg/ml heparin) followed by one or two additional large transients (Fig. 2C). Injection of a lower concentration of heparin (8 mg/ml) caused $[Ca^{2+}]_i$ changes intermediate

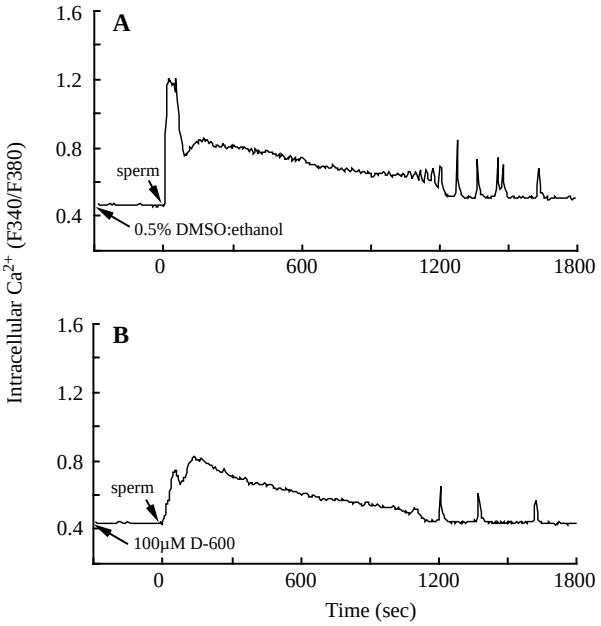

Fig. 1. Effects of D-600 on sperm-induced $[Ca^{2+}]_i$ changes. The Fura-2-injected oocyte was incubated for 5 minutes with 0.5% DMSO:ethanol (A) or 100 μM D-600 (B) before insemination. Both of the oocytes underwent polar body formation during the time in which $[Ca^{2+}]_i$ was measured and developed to early trochophores after being withdrawn from chambers and cultured in NSW.

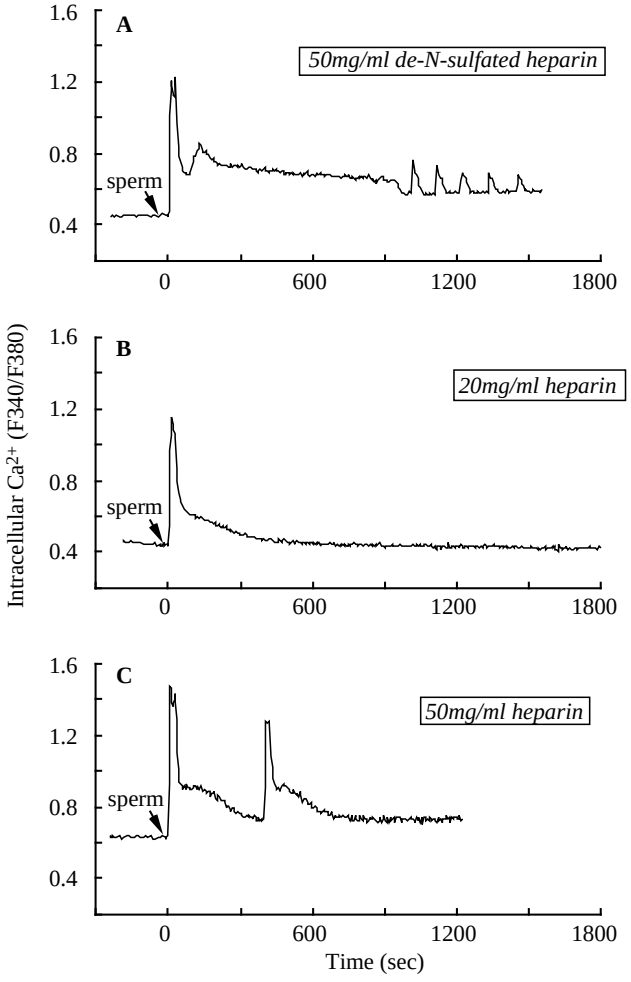

Fig. 2. Effects of heparin on sperm-induced $[Ca^{2+}]_i$ changes. The oocyte was injected with Fura-2 together with 50 mg/ml de-N-sulfated heparin (A), 20 mg/ml heparin (B) or 50 mg/ml heparin (C) and then inseminated. The estimated intracellular concentration of de-N-sulfated heparin or heparin was 690 (A), 270 (B) or 720 μg/ml (C). None of the oocytes showed polar body formation during the time of measurements.

between those observed following injection of 0 mg/ml (control) and 20 or 50 mg/ml heparin: a low sustained $[Ca^{2+}]_i$ elevation was generally followed by an initial transient, but additional large transients were superimposed on the elevated $[Ca^{2+}]_i$ in three out of eight oocytes and the subsequent repetitive small increases were obscure in four out of the eight oocytes (data not shown).

Since the oocytes injected with 20 or 50 mg/ml heparin did not proceed to polar body formation after insemination (as described below), there is a possibility that high concentrations of heparin might prevent the injected oocytes from accepting fertilizing sperm and therefore abolish later $[Ca^{2+}]_i$ changes. However, when 50 mg/ml heparin and Fura-2 were simultaneously injected into oocytes at 5-8 minutes after natural fertilization, i.e. after decondensation of single sperm nuclei had been completed, no significant $[Ca^{2+}]_i$ increase was observed (0/8, data not shown). In contrast, normal later phases of $[Ca^{2+}]_i$ changes were detected in all of the control oocytes injected with Fura-2 alone (5/5, data not shown). These results clearly indicate that a high concentration of heparin can abolish the later $[Ca^{2+}]_i$ changes even in normally fertilized oocytes.

Mytilus oocytes inseminated in the complete absence of external Ca^{2+} cannot be fertilized, presumably because of the inability of sperm to induce the acrosome reaction. However, when external Ca^{2+} is removed at 2-8 seconds after fertilization, i.e. during the rising phase of the initial $[Ca^{2+}]_i$ transient, normal monospermic fertilization takes place (Deguchi and Osanai, 1994a). In these oocytes, the duration of the initial transient is somewhat shortened, but the following $[Ca^{2+}]_i$ changes are little affected. Similar $[Ca^{2+}]_i$ changes after removal of external Ca^{2+} were detected in the control oocytes injected with 50 mg/ml de-N-sulfated heparin (n=5, Fig. 3A). In the oocytes injected with 20 mg/ml heparin, in contrast, the same treatment caused the elevating $[Ca^{2+}]_i$ to return to the resting level more quickly than in the presence of external Ca^{2+} (Fig. 3B, cf. Fig. 2B). Under these conditions, additional large $[Ca^{2+}]_i$ transients, which were sometimes detected in the presence of external Ca^{2+} (see Fig. 2C), did not take place (0/8).

We further tested whether combined application of heparin and D-600 could inhibit all sperm-induced $[Ca^{2+}]_i$ increases. In the oocytes injected with 20 mg/ml heparin, incubated with 100 μM D-600 for 5 minutes and then inseminated, only a single (4/8, Fig. 4A) or multiple (4/8, Fig. 4B) $[Ca^{2+}]_i$ transients of barely detectable size were observed. These results suggest that simultaneous inhibition of extracellular Ca^{2+} influx (by Ca^{2+} free-seawater or by D-600) and IICR (by heparin) can almost completely abolish sperm-induced $[Ca^{2+}]_i$ increases.

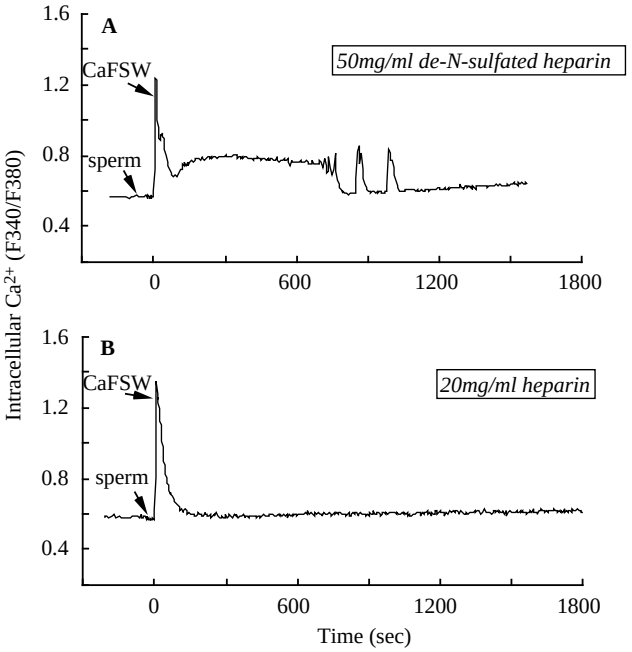

Fig. 3. Cooperative effects of heparin and removal of external Ca^{2+} on sperm-induced $[Ca^{2+}]_i$ changes. The oocyte was injected with Fura-2 together with 50 mg/ml de-N-sulfated heparin (A) or 20 mg/ml heparin (B), inseminated in NSW, and exposed to Ca^{2+}-free seawater (CaFSW) containing 10 mM EGTA at 6 seconds after fertilization. The estimated intracellular concentration of de-N-sulfated heparin or heparin was 740 (A) or 380 μg/ml (B). None of the oocytes showed polar body formation during the time of measurements.

Excess K^+-induced $[Ca^{2+}]_i$ increase absolutely depends on extracellular Ca^{2+} influx

To confirm whether D-600 can inhibit voltage-dependent Ca^{2+} influx, we examined the effect of D-600 on an excess K^+-induced $[Ca^{2+}]_i$ increase, which is thought to be caused by depolarization of the plasma membrane (Jaffe and Robinson, 1978) and the consequent opening of voltage-gated Ca^{2+} channels (Dubé and Guerrier, 1982; Jaffe, 1985). As shown in Fig. 5A, the addition of excess K^+ seawater to unfertilized oocytes, without insemination, caused an initial $[Ca^{2+}]_i$ transient followed by sustained $[Ca^{2+}]_i$ elevation (see also Deguchi and Osanai, 1994a). Injection of 20 mg/ml heparin did not inhibit such a $[Ca^{2+}]_i$ increase (n=8, Fig. 5B). In contrast, the presence of 100 μM D-600 almost completely blocked the excess K^+-induced $[Ca^{2+}]_i$ increase (n=6, Fig. 5C). Similar results were obtained when two other Ca^{2+} channel inhibitors, nifedipine (n=5) and nitrendipine (n=5), were applied at 100 μM, although their inhibitory effects were less strong than that of D-600 (see Fig. 6L,M,N). Even when 100 μM D-600 was added after normal stimulation with excess K^+, the elevated $[Ca^{2+}]_i$ rapidly returned nearly to the resting level (n=4, Fig. 5D). These results suggest that both the initial and the later $[Ca^{2+}]_i$ changes caused by excess K^+ are absolutely dependent on voltage-gated Ca^{2+} influx, and support the idea that the initial $[Ca^{2+}]_i$ transient at fertilization is due to extracellular Ca^{2+} influx.

The effects of inhibitors on sperm- and excess K^+-induced initial $[Ca^{2+}]_i$ transients in Fura-2-injected oocytes are summarized in Fig. 6. Although the resting ratios (R_0) in individual oocytes were somewhat different, their average values in respective experiments were similar (approx. 0.5). We used normalized values of ratio against the resting ratio, $(R-R_0)/R_0$ (Stricker et al., 1994), to evaluate the amplitude of the initial transients, and carried out statistical comparisons by unpaired t-test data analysis. As for sperm-induced $[Ca^{2+}]_i$ changes, the initial transients for the control oocytes injected with Fura-2 alone and inseminated in NSW (Fig. 6A) were not significantly (P>0.1) different from those for the oocytes treated with 0.5%

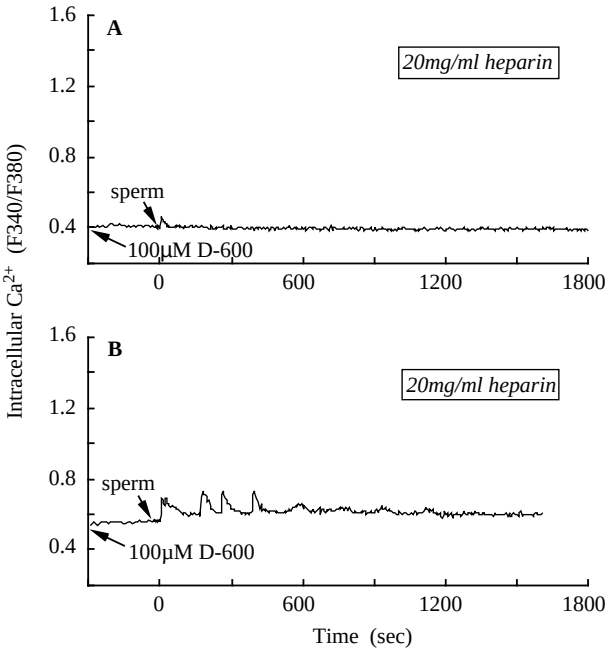

Fig. 4. Cooperative effects of heparin and D-600 on sperm-induced $[Ca^{2+}]_i$ changes. The oocyte was injected with Fura-2 together with 20 mg/ml heparin, incubated with 100 μM D-600 for 5 minutes, and then inseminated. Two typical $[Ca^{2+}]_i$ changes are shown in A and B. The estimated intracellular concentration of heparin was 210 (A) or 350 μg/ml (B). None of the oocytes showed polar body formation during the time of measurements.

DMSO:ethanol (Fig. 6B), 50 mg/ml de-N-sulfated heparin (Fig. 6E) or 8-50 mg/ml heparin (Fig. 6F,G,H). In contrast, application of 100 μM (Fig. 6C) or 200 μM D-600 (Fig. 6D) significantly (P<0.01 or P<0.001, compared with B) reduced the amplitude of the initial transients. Combined application of 100 μM D-600 and 20 mg/ml heparin (Fig. 6I) caused significantly lower transients than any sole application of 100 μM D-600 (Fig. 6C, P<0.01), 200 μM D-600 (Fig. 6D, P<0.001), 20 mg/ml heparin (Fig. 6G, P<0.001) or 50 mg/ml heparin (Fig. 6H, P<0.001).

As for excess K^+-induced $[Ca^{2+}]_i$ changes, the initial $[Ca^{2+}]_i$ transients for the control oocytes stimulated with excess K^+ without any inhibitor (Fig. 6J) were not significantly (P>0.1) different from those for the oocytes injected with 20 mg/ml heparin (Fig. 6K). In contrast, the oocytes treated with 100 μM D-600 (Fig. 6L), nifedipine (Fig. 6M) or nitrendipine (Fig. 6N) showed significantly (P<0.001) smaller transients than the control oocytes (Fig. 6J).

IP_3- or $GPIP_2$-induced $[Ca^{2+}]_i$ increases absolutely depend on Ca^{2+} release mediated by the IP_3 receptor

Using our $[Ca^{2+}]_i$ measurement system, direct injection of agents such as IP_3 into the experimental oocyte was unsuccessful because of technical limitations. Alternatively, to show the existence of IICR in *Mytilus* oocytes and to confirm the contribution of IICR to later $[Ca^{2+}]_i$ changes at fertilization, we induced a rise in intracellular IP_3 by photolysis of 'caged' derivatives of IP_3, the method which has recently been used with starfish (Stricker et al., 1994) and hamster oocytes (Shiraishi et al., 1995). The oocytes injected with 300 μM caged IP_3 and exposed to UV irradiation immediately showed a $[Ca^{2+}]_i$ transient (n=11). The long $[Ca^{2+}]_i$ recordings performed in 5 out of the 11 experiments revealed that the initial $[Ca^{2+}]_i$ transient was followed by no change in $[Ca^{2+}]_i$ (n=3, Fig. 7A) or by a short-lived elevation and repetitive small increases (n=2, Fig. 7B). Injection of 50 μM caged IP_3 still caused a UV-induced $[Ca^{2+}]_i$ transient (n=9, Fig. 7C), while

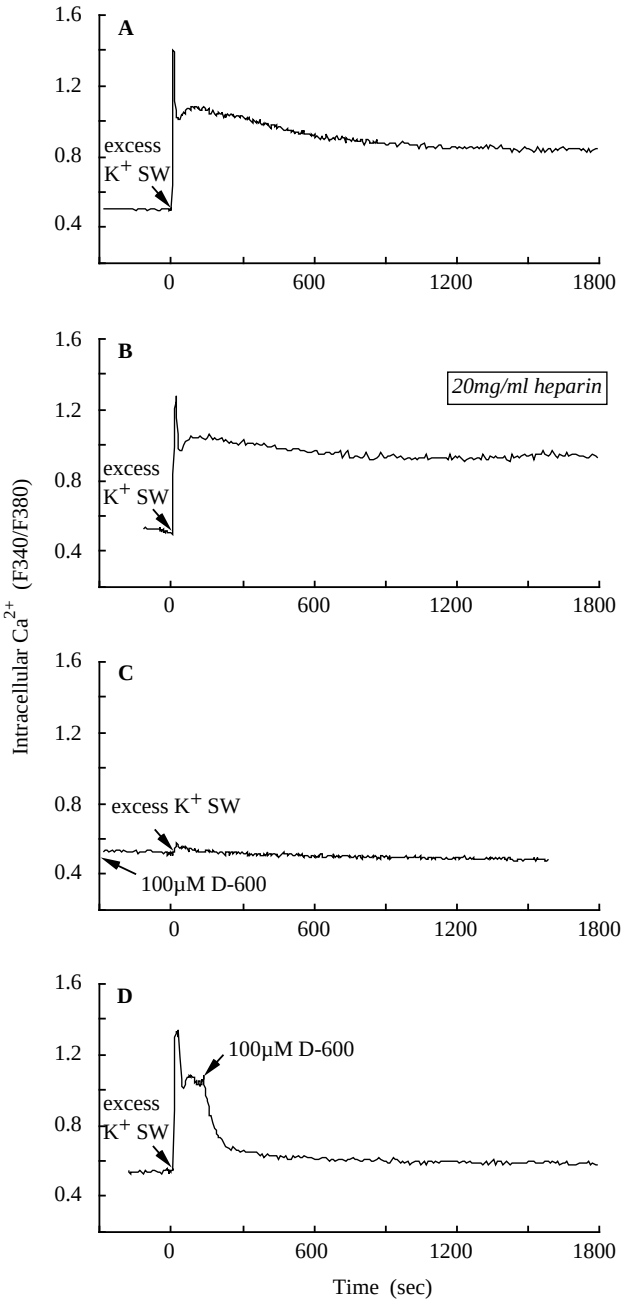

Fig. 5. Respective effects of D-600 and heparin on excess K^+-induced $[Ca^{2+}]_i$ changes. Oocytes were injected with Fura-2 alone (A,C,D) or together with 20 mg/ml heparin (B) and exposed to excess K^+ seawater. D-600 at 100 μM was added at 5 minutes before (C) or 2 minutes after (D) the stimulation with excess K^+. The estimated intracellular concentration of heparin was 330 μg/ml (B). Only control oocytes (A) underwent polar body formation.

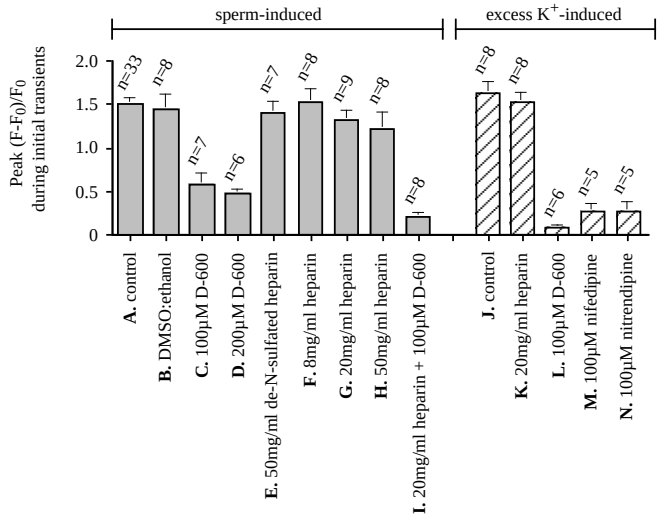

Fig. 6. Effects of inhibitors on sperm- and excess K^+-induced initial $[Ca^{2+}]_i$ transients in Fura-2-injected oocytes. The error bars represent s.e.m. of the number of oocytes examined (*n* is given for each value).

12 μM caged IP_3 no longer produced the response (*n*=7, Fig. 7D).

Neither Ca^{2+}-free seawater containing 10 mM EGTA (*n*=7, Fig. 7E) nor normal seawater containing 100 μM D-600 (*n*=7, Fig. 7F) inhibited a UV-induced $[Ca^{2+}]_i$ transient in oocytes injected with 300 μM caged IP_3. On the other hand, co-injection of 20 mg/ml heparin completely blocked the response (*n*=8, Fig. 7G). Lower concentration of heparin (8 mg/ml) also gave a strong inhibitory effect (*n*=8, see Fig. 9F), but 20 mg/ml de-N-sulfated heparin did not (*n*=7, Fig. 7H).

To induce a larger $[Ca^{2+}]_i$ increase, we tested the effect of a caged compound of $GPIP_2$, a poorly metabolized analog of IP_3 (Bird et al., 1992). Oocytes injected with 300 μM caged $GPIP_2$ and exposed to UV irradiation showed a much larger $[Ca^{2+}]_i$ increase than those injected with the same concentration of caged IP_3 (*n*=7, Fig. 8A, cf. Fig. 7A,B). When these oocytes were exposed to second and third UV irradiations following the first irradiation, they showed second and third $[Ca^{2+}]_i$ increases, which prolonged the total duration of $[Ca^{2+}]_i$ elevation (Fig. 8B). Such UV-induced $[Ca^{2+}]_i$ increases were suppressed, in a dose-dependent manner, by co-injection of heparin (Fig. 8C,D).

Oocytes injected with 500 μM caged ATP (*n*=6, data not shown) or Calcium Green alone (*n*=14, see Fig. 9I) did not show a UV-induced $[Ca^{2+}]_i$ increase, thus the $[Ca^{2+}]_i$ increase in caged IP_3- or caged $GPIP_2$-injected oocytes derives from the function of IP_3 or $GPIP_2$ photoreleased from caged IP_3 or caged $GPIP_2$, and not from any nonspecific action of UV irradiation on the caged compound itself or Calcium Green itself.

There was a possibility that Calcium Green might lose its sensitivity to $[Ca^{2+}]_i$ changes when injected simultaneously with heparin and caged IP_3 or with heparin and caged $GPIP_2$, since such oocytes showed a weak response to UV irradiation (Figs 7G, 8D). To rule out this possibility, we checked whether these oocytes still showed an initial $[Ca^{2+}]_i$ transient at fertilization. Insemination of control oocytes injected with Calcium Green alone generated an intact pattern of $[Ca^{2+}]_i$ changes (*n*=8, data not shown). The initial $[Ca^{2+}]_i$ transient was not inhibited by co-injection of 300 μM caged IP_3 and 20 mg/ml heparin (*n*=6, see Fig. 9N) or by 300 μM caged $GPIP_2$ and 20 mg/ml heparin (*n*=4, see Fig. 9O), although the later $[Ca^{2+}]_i$ changes were inhibited. The results described above show that the UV-induced $[Ca^{2+}]_i$ increase in caged IP_3- or caged $GPIP_2$-injected oocytes, namely, IICR, can be specifically inhibited by heparin, and confirm the contribution of IICR to heparin-sensitive, later $[Ca^{2+}]_i$ increases at fertilization of *Mytilus* oocytes.

The data on UV- and sperm-induced initial $[Ca^{2+}]_i$ transients and the effects of their inhibitors in Calcium Green-injected oocytes are summarized in Fig. 9. When oocytes were exposed to three successive UV irradiations (see Fig. 8B,C,D), only the peak value after the first irradiation was used for the analysis. In case of no obvious peak after UV irradiation (see Fig. 7G), the maximal value during the first 1-minute period after UV irradiation was defined as the peak value. UV-induced $[Ca^{2+}]_i$ transients for 300 μM caged IP_3 (Fig. 9A) were significantly (P<0.001) reduced by co-injection of 8 mg/ml (Fig. 9F) or 20 mg/ml heparin (Fig. 9G), but not (P>0.1) by the absence of external Ca^{2+} (Fig. 9D), application of 100 μM D-600 (Fig. 9E), or co-injection of 20 mg/ml de-N-sulfated heparin (Fig. 9H). UV-induced transients for 300 μM caged $GPIP_2$ (Fig. 9J) were also significantly (P<0.001) lowered by co-injection of 8 mg/ml (Fig. 9K) or 20 mg/ml heparin (Fig. 9L). In contrast, the initial transients after insemination (Fig. 9M) were not significantly (P>0.1) affected by simultaneous injection of 20 mg/ml heparin and 300 μM caged IP_3 (Fig. 9N) or caged $GPIP_2$ (Fig. 9O).

Both extracellular Ca^{2+} influx and Ca^{2+} release from IP_3-sensitive stores can induce polar body formation

Polar body formation is the first visible morphological change after meiosis reinitiation from metaphase I in bivalve oocytes and is triggered by stimulation with excess K^+ as well as by fertilization in *Mytilus* oocytes (Dufresne-Dubé et al., 1983; Deguchi and Osanai, 1994a). In this study, a long-term $[Ca^{2+}]_i$ elevation caused by excess K^+ resulted in 75% (6/8) polar body formation in the Fura-2-injected oocytes (Fig. 5A). Polar body formation was also induced by a long-term $[Ca^{2+}]_i$ increase caused by UV irradiations in the oocytes injected with 300 μM caged $GPIP_2$: 29% (2/7) by one UV irradiation and 71% (5/7) by three UV irradiations (Fig. 8B). In contrast, no polar body formation was triggered by a short-lived $[Ca^{2+}]_i$ increase after one UV irradiation in the oocytes injected with the same concentration of caged IP_3 (0/11, Fig. 7A,B).

Application of 100 μM D-600 completely blocked the excess K^+-induced $[Ca^{2+}]_i$ increase and polar body formation (0/6, Fig. 5C). The same or higher (200 μM) concentration of D-600 inhibited only an initial $[Ca^{2+}]_i$ transient at fertilization and permitted these oocytes to complete meiosis normally and develop to early trochophores after being withdrawn from measurement chambers and cultured in NSW (6/7 with 100 μM D-600, Fig. 1B; 6/6 with 200 μM D-600), as observed in the control oocytes (8/8, Fig. 1A).

A low concentration (8 mg/ml) of heparin blocked UV-induced polar body formation in caged $GPIP_2$-injected oocytes (0/8, Fig. 8C), while the oocytes injected with this concentration of heparin underwent the normal meiotic process after fertilization and developed to early trochophores in all cases (8/8). In contrast, higher concentrations (20 or 50

mg/ml) of heparin or de-N-sulfated heparin, independent of their effects on $[Ca^{2+}]_i$ changes, never permitted the injected oocytes to undergo polar body formation within at least 30 minutes after insemination (Fig. 2A,B,C), stimulation with excess K^+ (Fig. 5B), or UV irradiations in caged $GPIP_2$-injected oocytes (Fig. 8D).

DISCUSSION

Our results strongly suggest that an initial $[Ca^{2+}]_i$ transient at fertilization derives mainly from extracellular Ca^{2+} entry. This is based on the following observations: (1) D-600 suppressed the initial $[Ca^{2+}]_i$ transient without much affecting the following $[Ca^{2+}]_i$ increases; (2) D-600 actually blocked an excess K^+-induced $[Ca^{2+}]_i$ increase; and (3) D-600 did not affect IICR caused by photolysis of caged IP_3.

In contrast, later phases of $[Ca^{2+}]_i$ increases at fertilization appear to derive mainly from IICR, since (1) heparin inhibited the later $[Ca^{2+}]_i$ increases without much affecting the initial transient; (2) heparin actually blocked IICR in a dose-dependent manner; and (3) heparin did not prevent an excess K^+-induced $[Ca^{2+}]_i$ increase.

Extracellular Ca^{2+} influx through voltage-dependent channels

Fertilized oocytes of prophase I type protostomes, such as the bivalves Spisula and Barnea and the echiuroid worm *Urechis*, show a long-term (5-10 minutes) depolarization of the plasma membrane (Jaffe et al., 1979; Finkel and Wolf, 1980) and resultant Ca^{2+} uptake (Johnston and Paul, 1977; Dubé and Guerrier, 1982). The Ca^{2+} uptake at fertilization of Barnea oocytes is blocked by the addition of 100 μM D-600 (Dubé and Guerrier, 1982), and $[Ca^{2+}]_i$ increases at fertilization of the same type of bivalve *Mactra* are abolished by removal of external Ca^{2+} (see Introduction) or by application of 100 μM D-600 (R. Deguchi, unpublished data). These results suggest that, unlike in deuterostomes (see Introduction), extracellular Ca^{2+} is the main source of sperm-induced $[Ca^{2+}]_i$ increases in protostomes, especially in prophase I type protostomes (see Jaffe, 1985).

Excess K^+ seawater induces a long-term $[Ca^{2+}]_i$ elevation in *Mytilus* oocytes, which is inhibited by the absence of external Ca^{2+} (Deguchi and Osanai, 1994a) or by addition of the voltage-gated Ca^{2+} influx blockers D-600 (Fig. 5C,D), nifedipine and nitrendipine. These results suggest that long-term voltage-gated Ca^{2+} influx, as detected at fertilization of

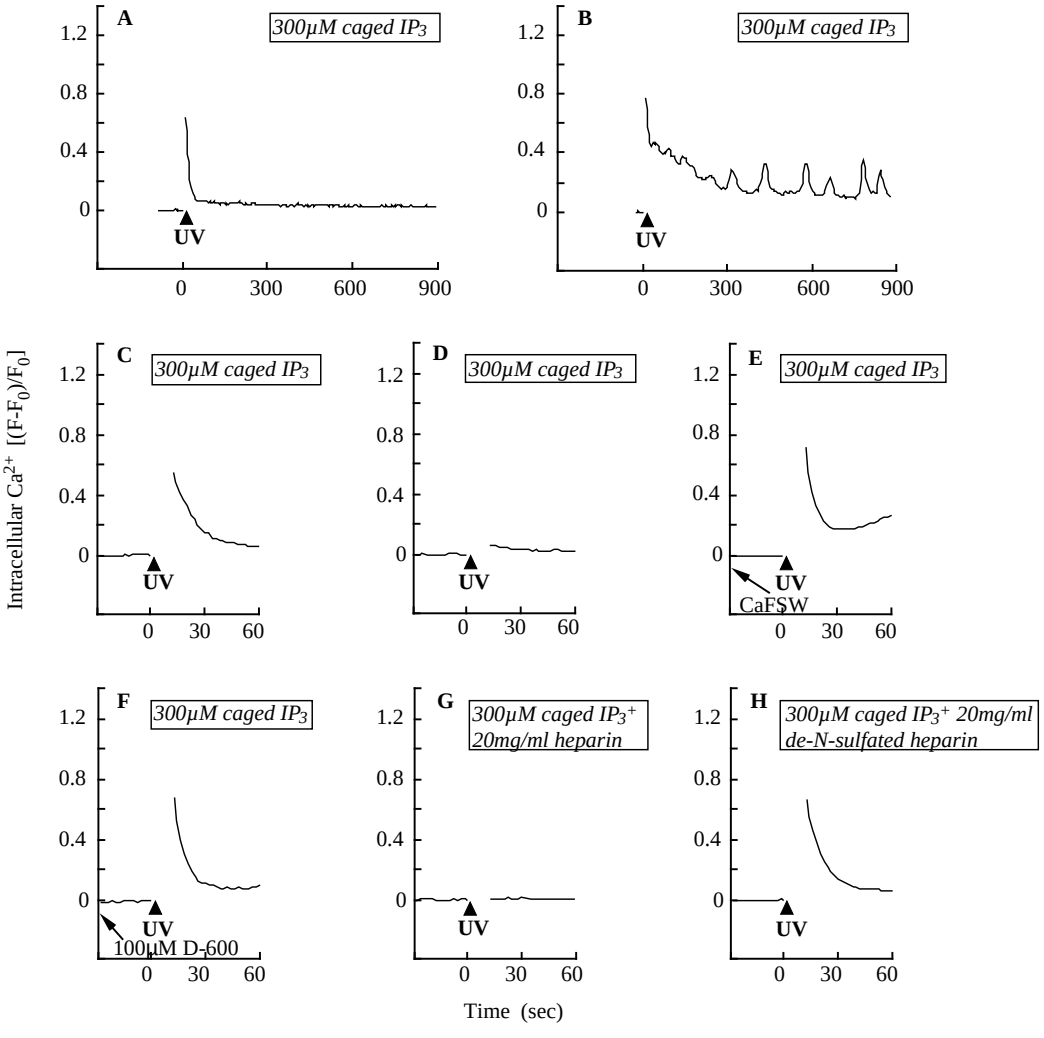

Fig. 7. IP_3-induced $[Ca^{2+}]_i$ changes caused by photolysis of caged IP_3. Oocytes were injected with Calcium Green together with 300 μM caged IP_3 (A,B,E,F), 50 μM caged IP_3 (C), 12 μM caged IP_3 (D), 300 μM caged IP_3 and 20 mg/ml heparin (G), or 300 μM caged IP_3 and 20 mg/ml de-N-sulfated heparin (H), and irradiated with UV light for 5 seconds. Ca^{2+}-free seawater containing 10 mM EGTA (E) or normal seawater containing 100 μM D-600 (F) was added at 5 minutes before the UV irradiation. None of the eight oocytes showed polar body formation when observed after incubation for at least 30 minutes.

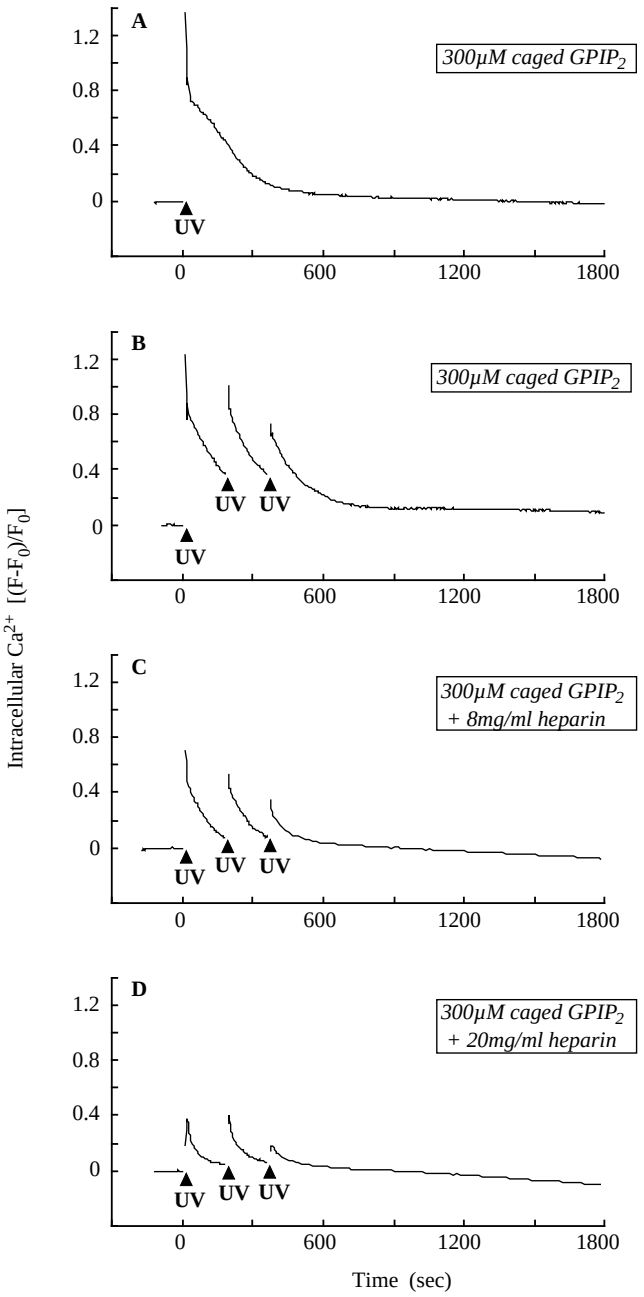

Fig. 8. $GPIP_2$-induced $[Ca^{2+}]_i$ changes caused by photolysis of caged $GPIP_2$. Oocytes were injected with Calcium Green together with 300 μM caged $GPIP_2$ (A,B), 300 μM caged $GPIP_2$ and 8 mg/ml heparin (C), or 300 μM caged $GPIP_2$ and 20 mg/ml heparin (D), and irradiated with UV light for 5 seconds. The second and third UV irradiations were performed 3 and 6 minutes after the first irradiation (B,C,D). Only oocytes in B showed polar body formation.

prophase I type protostomes, also occurs in oocytes of the metaphase I type protostome *Mytilus* during the excess K^+-induced, continuous membrane depolarization.

The shape and height of initial $[Ca^{2+}]_i$ transient at fertilization are similar to those induced by excess K^+ in *Mytilus* oocytes (Deguchi and Osanai, 1994a), and the initial transient at fertilization was also greatly suppressed by D-600 (Fig. 1B), nifedipine and nitrendipine. These results suggest that the initial $[Ca^{2+}]_i$ transient at fertilization is mainly mediated by voltage-dependent Ca^{2+} channels, although possible contributions by other Ca^{2+} channels (e.g. ligand-gated channels) cannot be excluded.

Recently, Togo et al. (1995) found a transient (approx. 30 second) membrane depolarization shortly after insemination in *Mytilus* oocytes. Our previous study showed that an initial transient at fertilization of *Mytilus* oocytes was a uniform $[Ca^{2+}]_i$ increase, rather than a wave-like increase as detected at fertilization of deuterostomes (Deguchi and Osanai, 1994a). In this study, only an initial transient during sperm-induced $[Ca^{2+}]_i$ increases was sensitive to D-600 (Fig. 1B). It is possible that Ca^{2+} entry from the whole surface occurs initially at natural fertilization, but this influx does not persist for long, owing to the short-lived membrane depolarization, as compared with that induced by excess K^+ or that occurring at fertilization of prophase I type protostomes.

Intracellular Ca^{2+} release via an IP_3-dependent pathway

IICR is considered a common system in deuterostome oocytes or eggs (see Miyazaki et al., 1993). In protostomes, in contrast, direct evidence for IICR has been reported only for shrimp oocytes, in which injection of IP_3 causes a wave-like $[Ca^{2+}]_i$ increase (Lindsay and Clark, 1994). Our present results obtained from experiments with caged compounds (Figs 7, 8) are the first direct demonstration of the existence of IICR in bivalve oocytes. The increased $[Ca^{2+}]_i$ after photolysis of 300 μM caged IP_3 in *Mytilus* oocytes did not persist long (Fig. 7A,B), presumably because the released IP_3 was rapidly metabolized. In contrast, the same concentration of caged $GPIP_2$, which releases the poorly metabolized IP_3 analog $GPIP_2$, caused a much larger and longer $[Ca^{2+}]_i$ increase (Fig. 8A). Similar relationships between caged IP_3 and caged $GPIP_2$ have been reported in mouse lacrimal acinar cells (Bird et al., 1992).

Heparin blocks IICR in oocytes or eggs of various deuterostomes (Rakow and Shen, 1990; DeLisle and Welsh, 1992; Fissore and Robl, 1993; Stricker et al., 1994; Yue et al., 1995), although its effect is less potent than that of the monoclonal antibody 18A10 against the IP_3 receptor in hamster oocytes (Miyazaki et al., 1993). In *Mytilus* oocytes, heparin worked as an effective IICR antagonist (Figs 7G, 8D), supporting the conclusion that heparin-sensitive, later phases of $[Ca^{2+}]_i$ increases at fertilization are mainly caused by IICR. Its inhibitory effects seem unlikely to result from nonspecific toxicity of polyanionic polysaccharides, considering the effects of de-N-sulfated heparin (Figs 2A, 7H).

CICR mediated by the ryanodine receptor is another important intracellular Ca^{2+} release system, and its existence has been reported in sea urchin eggs (Dargie et al., 1990; Galione et al., 1991, 1993; Lee et al., 1993), starfish oocytes (Stricker et al., 1994) and bovine oocytes (Yue et al., 1995). In *Mytilus* oocytes, caffeine, a potent activator of CICR (Galione et al., 1991; Yue et al., 1995), produced no $[Ca^{2+}]_i$ increase even if applied at 50 mM (data not shown). Furthermore, injection of 5 or 10 mM ruthenium red, a potent inhibitor of CICR (Galione et al., 1991; Yue et al., 1995), did not inhibit sperm-induced $[Ca^{2+}]_i$ changes, and combined injection of 10 mM ruthenium red and 50 mg/ml heparin gave the same effect

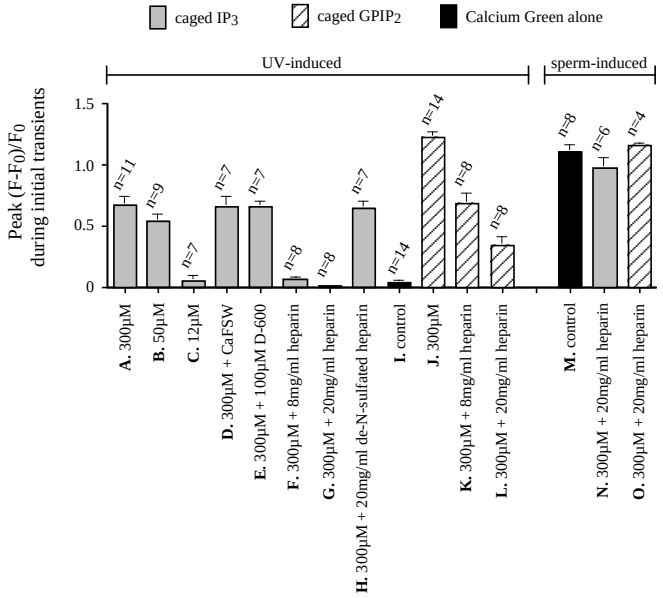

Fig. 9. Amplitude of UV- and sperm-induced initial $[Ca^{2+}]_i$ transients and the effects of inhibitors on them in Calcium Green-injected oocytes. The error bars represent s.e.m. of the number of oocytes examined (*n* is given for each value).

as 50 mg/ml heparin alone (data not shown). These data do not support the existence of CICR, or at least any contribution by it to the sperm-induced $[Ca^{2+}]_i$ increases in this species.

Relationships between Ca^{2+} influx and Ca^{2+} release from IP_3-sensitive stores

Injection of Ca^{2+} buffers or heparin into frog oocytes blocks a $[Ca^{2+}]_i$ increase at fertilization, but the treatment does not inhibit an increase in IP_3 mass at fertilization (Stith et al., 1994). Injection of Ca^{2+} chelators into sea urchin eggs blocks a $[Ca^{2+}]_i$ increase without affecting an initial increase in inward current at fertilization (Swann et al., 1992), and injection of heparin is also ineffective in inhibiting the initial current increase (Mohri et al., 1995). Extracellular Ca^{2+} entry during the current increase (Chambers and de Armendi, 1979) can be visualized as a barely detectable $[Ca^{2+}]_i$ increase in the whole cortical region, and nifedipine abolishes the cortical $[Ca^{2+}]_i$ increase without affecting the subsequent main $[Ca^{2+}]_i$ increase (Shen and Buck, 1993). These facts are consistent with our present findings that suppression of initial Ca^{2+} influx by D-600 did not inhibit the following IICR-mediated $[Ca^{2+}]_i$ increases in *Mytilus* oocytes (Fig. 1B) and that inhibition of IICR by heparin did not affect the initial Ca^{2+} influx (Fig. 2B). It is, therefore, possible that at fertilization of these species, external Ca^{2+} influx and IICR are basically regulated by independent mechanisms. However, our results also suggest some mutual relationships between the two mechanisms in fertilized *Mytilus* oocytes (see below).

Application of 100 μM D-600, which almost completely blocked an excess K^+-induced Ca^{2+} increase (Fig. 6L), did not completely block an initial $[Ca^{2+}]_i$ transient at fertilization (Fig. 6C). In addition, a higher concentration (200 μM) of D-600 was less effective than combined application of 100 μM D-600 and 20 mg/ml heparin in inhibiting the initial transient (Fig. 6D,I). It is, therefore, possible that when the sperm-induced Ca^{2+} influx through the plasma membrane is prevented, IICR compensates the loss of the influx to some extent and still produces a small $[Ca^{2+}]_i$ transient.

In oocytes injected with high concentrations of heparin, an initial $[Ca^{2+}]_i$ transient at fertilization was not followed by a rapid return to the resting $[Ca^{2+}]_i$ level, but was accompanied by a shoulder of the $[Ca^{2+}]_i$ elevation (Fig. 2B,C). In half of these oocytes, one or two additional large $[Ca^{2+}]_i$ transients took place after the initial increase (Fig. 2C). Although injection of high concentrations of heparin sometimes causes multiple sperm entry in frog oocytes (Nuccitelli et al., 1993) and sea urchin eggs (Mohri et al., 1995), the additional large transients in heparin-injected *Mytilus* oocytes seem unlikely to result from polyspermy, considering that they occurred even after injection of 8 mg/ml heparin, which always allowed the injected oocytes to undergo normal cleavage. Since these additional transients and shoulders were completely abolished by removal of external Ca^{2+} (Fig. 3B), they may be produced by external Ca^{2+} entry itself. Alternatively, it is possible that intracellular Ca^{2+} stores are overloaded with Ca^{2+} by continued Ca^{2+} influx and then the stored Ca^{2+} is released. In either case, extracellular Ca^{2+} entry may supplement the loss of IICR to some extent during the sperm-induced $[Ca^{2+}]_i$ increases. The mechanisms underlying these mutual relationships between Ca^{2+} influx and IICR at fertilization are unknown and should be clarified in future studies.

Contribution of Ca^{2+} influx and IP_3-induced Ca^{2+} release to meiosis reinitiation

External Ca^{2+}-dependent, excess K^+-induced meiosis reinitiation has been detected in both prophase I type and metaphase I type protostomes (see Jaffe, 1985; Gould and Stephano, 1989). IP_3-induced meiosis reinitiation has been observed in the prophase I type bivalve *Spisula*, although $[Ca^{2+}]_i$ changes during the process have not investigated (Bloom et al., 1988). Our present results show that a long-term $[Ca^{2+}]_i$ increase, which can be induced not only by stimulation with excess K^+ (Fig. 5A) but also by UV irradiations in oocytes injected with caged $GPIP_2$ (Fig. 8B), triggers meiosis reinitiation from metaphase I in *Mytilus* oocytes without insemination.

Sperm-induced meiosis reinitiation in *Mytilus* oocytes was prevented by injection of high concentrations of heparin, but not by application of high concentrations of D-600. These results imply that heparin-sensitive, D-600-insensitive, later $[Ca^{2+}]_i$ increases are sufficient to trigger meiosis reinitiation in fertilized oocytes. In contrast, it is unclear whether a D-600-sensitive, heparin-insensitive initial $[Ca^{2+}]_i$ increase is sufficient for meiosis reinitiation, since high concentrations of heparin or de-N-sulfated heparin seem to have nonspecific toxic effects on meiotic division itself, independent of their effects on $[Ca^{2+}]_i$ changes. Nevertheless, we can conclude that the two types of Ca^{2+} mobilizing systems, extracellular Ca^{2+} influx and IICR, are commonly present in *Mytilus* oocytes, and that both of them can generate a sufficient $[Ca^{2+}]_i$ increase to trigger meiosis reinitiation from metaphase I when continuously activated.

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