Ca\(^{2+}\) oscillations can be induced in mammalian eggs and somatic cells by microinjection of a cytosolic sperm protein factor. The nature of the sperm factor-induced Ca\(^{2+}\) signaling was investigated by adding sperm protein extracts to homogenates of sea urchin eggs, which contain multiple classes of Ca\(^{2+}\) release mechanisms. We show that the sperm factor mobilizes Ca\(^{2+}\) from non-mitochondrial Ca\(^{2+}\) stores in egg homogenates after a distinct latency. This latency is abolished by preincubation of sperm extracts with egg cytosol. The preincubation step is highly temperature-dependent and generates a high molecular weight, protein-based Ca\(^{2+}\) releasing agent that can also mobilize Ca\(^{2+}\) from purified egg microsomes. This Ca\(^{2+}\) release appears to be mediated via both inositol 1,4,5-trisphosphate and ryanodine receptors, since homologous desensitization of these two release mechanisms by their respective agonists inhibits further release by the sperm factor. However, sperm factor-induced Ca\(^{2+}\) release by these channels is independent of inositol 1,4,5-trisphosphate or cADPR since antagonists of either of these two messengers did not block the Ca\(^{2+}\) release effected by the sperm factor. The sperm protein factor may cause Ca\(^{2+}\) release via an enzymatic step that generates a protein-based Ca\(^{2+}\)-releasing agent.

At fertilization in mammals and some marine invertebrates, the sperm activates the egg by inducing a series of Ca\(^{2+}\) oscillations involving release from intracellular stores (1, 2). In eggs, the propagation of Ca\(^{2+}\) waves is thought to be mediated by Ca\(^{2+}\)-induced Ca\(^{2+}\) release operating via inositol 1,4,5-trisphosphate (InsP\(_3\))-sensitive Ca\(^{2+}\) channels (1, 3, 4), possibly in concert with ryanodine receptors (RyR) (3–5). The sequence of events leading to the initiation of Ca\(^{2+}\) oscillations is unclear (6). One hypothesis for signaling at fertilization proposes that sperm-egg fusion allows the diffusion of a soluble cytosolic sperm factor into the egg cytoplasm which leads to the activation of Ca\(^{2+}\) release (7, 8). This is consistent with the finding that sperm-egg membrane fusion occurs before Ca\(^{2+}\) release in the eggs of sea urchins and mice (9, 10). Further...

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The abbreviations used are: InsP\(_3\), inositol 1,4,5-trisphosphate; RyR, ryanodine receptor; cADPR, cyclic adenosine diphosphate ribose.

A Cytosolic Sperm Protein Factor Mobilizes Ca\(^{2+}\) from Intracellular Stores by Activating Multiple Ca\(^{2+}\) Release Mechanisms Independently of Low Molecular Weight Messengers*

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Perkin-Elmer LS-50B fluorimeter. Additions were made in 5-μl volumes, except where stated otherwise, and all chemicals were added in intracellular medium containing 10 mM EGTA and continuously stirred. Basal concentrations of Ca²⁺ were typically between 100 and 150 nM. Sequestered Ca²⁺ was determined by monitoring decrease in fluo-3 fluorescence during microsomal loading and by measuring Ca²⁺ release in response to ionomycin (5 μM) and was constant between experiments. For each experiment, standard Ca²⁺ calibration was performed by adding known amounts of Ca²⁺ (range of 0–8 nmol) to homogenates and monitoring fluorescence changes. Xenopus egg homogenates were prepared and used by a modified protocol of the above as described previously (4). Microsomes were purified from 5% egg homogenates by the Percoll density centrifugation method described previously (20). Briefly, a fractionating buffer was prepared by diluting Percoll stock to 1:2 ratio, respectively, at 24 °C, except where stated otherwise. Aliquots of the resulting mixtures were added by pipette to sea urchin egg homogenates or microsomal fractions to examine their Ca²⁺ releasing activities. In some cases, the resulting mixtures were further fractionated using a series of 3-, 10-, and 100-kDa cut-off filters (Amicon), in which the mixture was placed on top of the filtration columns and microcentrifuged for 10–30 min at 4 °C. Both resulting filtrate and retentate fractions from each of these columns were tested for their Ca²⁺ releasing activities. Sperm factor extracts or fractions derived from incubation and filtration protocols were treated with by dilution into buffer containing trypsin (100 μg/ml) for 30 min at room temperature, before reconcentration back to the original volume (7). Aliquots of the resulting mixture were then tested for activity in the egg homogenates and microsomal fractions. 8-NH₂-cADPR and fluo-3 were from Molecular Probes; fluo-3 was from Calbiochem (Molecular Probes); all other chemicals were from Sigma. Chemicon cut-off filters were obtained from Amicon, Beverly, MA.

RESULTS AND DISCUSSION
Diluted sea urchin egg homogenates (2.5% v/v) containing the fluorescent Ca²⁺ indicator, fluo-3, and mitochondrial inhibitors were allowed to sequester Ca²⁺ in the presence of an ATP-regenerating system over 3 h at 17 °C. After a stable baseline of fluorescence had been attained, aliquots of soluble cytoplasmic (>30 kDa) extracts from boar sperm were added to the cuvette. Fig. 1A (i) shows that a delayed and transient increase in the fluorescence signal occurred after sperm extract addition, indicating that the sperm extracts contained a Ca²⁺-mobilizing activity. The latencies of Ca²⁺ release were at least 100 s, and after this release the Ca²⁺ was resequestered (Fig. 1A, i). A protein component rather than a small molecular

![Image of Fig. 1](https://www.jbc.org/content/119/2/28902/F1)

Fig. 1. Sperm factor mobilizes Ca²⁺ from sea urchin and Xenopus egg homogenates. A, i, the effect of the sperm factor (10 μl) on Ca²⁺ release from sea urchin homogenates (2.5%) at 17 °C measured by changes in fluo-3 fluorescence. Sperm factor solution was added at the time indicated by the arrow. A, ii, the effect of trypsin-pretreated sperm factor on Ca²⁺ release under the same conditions as in i. B, sperm factor mobilizes Ca²⁺ from Xenopus egg homogenates. The effect of sperm factor (10 μl) on Ca²⁺ release from Xenopus egg homogenates (10%) at 22 °C measured by changes in fluo-3 fluorescence. Sperm factor solution was added at the time indicated by the arrow. The horizontal bars indicate 5 min. C, effect of two subsequent additions of sperm factor on Ca²⁺ release from 2.5% sea urchin egg homogenates. After the first challenge with sperm factor (15 μl) followed by resequestration of Ca²⁺ a second addition of sperm factor (15 μl) failed to induce a second Ca²⁺ release. D, typical responses illustrating the dose-response relationships between varying the volume of sperm factor added to four separate cuvettes on fluo-3 fluorescence traces. Increasing volumes of sperm factor resulted in shortened latencies to the initiation of Ca²⁺ release and also to a higher maximal Ca²⁺ release. E, graphical summary of the effect of varying the volumes of sperm factor additions on the latencies (diamonds) and magnitude of Ca²⁺ release (squares). The amount of Ca²⁺ released was calibrated from fluorescent traces by adding known amounts of Ca²⁺ to a new cuvette containing 2.5% sea urchin egg homogenate from the same batch of extract. The horizontal bars indicate 5 min.
A sperm factor was likely to be responsible for this effect since sperm extracts preincubated with trypsin at room temperature abolished the sperm extract-induced Ca\textsuperscript{2+} release. 10 μl of sperm factor was incubated for 30 min with 20 μl of egg supernatant, and then the mixture was added to 2.5% egg homogenates. Up to 50 μl of supernatant alone kept at room temperature for 30 min had no discernible effect on Ca\textsuperscript{2+} release (data not shown). C, 10 μl of sperm factor was incubated for 30 min with 20 μl of egg microsomes and then the total mixture was added to 2.5% egg homogenates. A prominent latency to Ca\textsuperscript{2+} release was observed similar to Fig. 1A without any preincubation or additions of egg fractions. D, time dependence of sperm factor incubation on the amount of Ca\textsuperscript{2+} released (squares) by sperm factor:supernatant mixtures in a 1:2 ratio, respectively, for a total addition of 30 μl. Incubations were performed at room temperature. The effect of lowering the temperature to 4 °C is shown for a single time point (circle) at 20 min. E, size fractionation of sperm factor superantant incubates on their Ca\textsuperscript{2+} releasing properties. Sperm factor (60 μl) was incubated with supernatant (120 μl) as in C and then separated using 3-kDa or 100-kDa cut-off filters. 30 μl of either filtrate (F) or retentate (R) were then tested for Ca\textsuperscript{2+} release activities. The most active fractions were the 3-kDa and 100-kDa retentates. However, when aliquots of both these fractions were incubated with trypsin (T), their Ca\textsuperscript{2+} mobilizing activities were lost. F, the effect of sperm factor on Ca\textsuperscript{2+} release from Percoll-purified microsomes. Sperm factor alone was unable to release Ca\textsuperscript{2+} from 5% microsomal suspensions. However, sperm factor preincubated with supernatant as in B elicited a large release. Supernatant cytosolic fractions (20 μl) had no effect on Ca\textsuperscript{2+} release per se. The horizontal bars indicate 5 min.

**Fig. 2. Requirement for cytosolic factors for sperm factor-induced Ca\textsuperscript{2+} release.** A, sperm factor-induced Ca\textsuperscript{2+} release from a concentrated (50%) sea urchin egg homogenate. Sperm factor (10 μl) caused an immediate rise in fluorescence with no discernible latency. B, preincubation of the sperm factor with supernatant from Percoll-fractionated sea urchin egg homogenate abolishes the latency and increases Ca\textsuperscript{2+} release. 10 μl of sperm factor was incubated for 30 min with 20 μl of egg supernatant, and then the mixture was added to 2.5% egg homogenates. Up to 50 μl of supernatant alone kept at room temperature for 30 min had no discernible effect on Ca\textsuperscript{2+} release (data not shown).
Preincubation of sperm extract with egg cytosol (homogenate supernatant) before addition to 2.5% homogenates substantially reduced the latency and greatly increased the magnitude of response to sperm extract (Fig. 2B). The enhancement of the sperm factors’ Ca\(^{2+}\) mobilizing actions was time-dependent and significantly reduced by incubations of sperm extracts and egg cytosol at 4 °C (Fig. 2D). The Q\(_{10}\) of the reaction was investigated by incubating 10 μl of sperm factor with 20 μl of egg supernatant for 15 min at 17 °C or at 7 °C. The mixture was then added to sea urchin egg homogenates (2.5% v/v) and Ca\(^{2+}\) release monitored at 17 °C. The latency to release was 319 ± 28 s (n = 3; S.E.) and 690 ± 49 s (n = 3; S.E.) at 17 °C and 7 °C, respectively. These data suggest a Q\(_{10}\) of 2.2 for the latency and imply that an enzymatic reaction is rate-limiting between the sperm factor and soluble egg components (22). The Q\(_{10}\) is similar to that of 2.3 for the latent period at fertilization in sea urchin eggs (23, 24).
fact that these preparations responded to InsP_3 and cADPR (data not shown). However, when boar sperm extract was preincubated with egg cytosolic fractions for 30 min (as above) and then added to microsomes immediate Ca^{2+} release occurred, while egg cytosolic fractions were not able themselves to cause Ca^{2+} release (Fig. 2F). These data suggested that the preincubation of boar sperm extract with an egg cytosolic protein leads to the formation of an active principle that can directly trigger Ca^{2+} release via channels in the endoplasmic reticulum or microsomal fractions. The active principle in egg cytosol was in excess of 100 kDa since rapid Ca^{2+} releasing activity generated by incubation of sperm extract with supernatant fractions was apparent only with retentate from 100 kDa cut-off filters rather than the filtrate (Fig. 2E). This argued against the idea that the sperm factor exerts its effects by generating small molecular intracellular messengers such as InsP_3 or cADPR. Furthermore, it is unlikely that sperm extract interacts with large molecules to produce these low molecular weight intermediates which could trigger Ca^{2+} release since size filtration (100-kDa cut-off filter) of the sperm extract preincubated with egg cytosol for up to 60 min resulted in the active fraction being retained by the filter (>100 kDa) and did not appear in the filtrate (<100 kDa) (Fig. 2E). That an additional protein is required for sperm extract action is supported by the finding that transient treatment of egg cytosol with agarose-trypsin beads abolished the enhancing effect of egg cytosol on sperm factor-induced Ca^{2+} release (Fig. 2E). A role for protein kinases in the preincubation reaction did not seem likely, because the preincubation reaction still gave rise to a rapid Ca^{2+}-release agent when it was carried out in the presence of a 10 μM concentration of the potent protein kinase inhibitor staurosporine, or the absence of any added ATP (data not shown).

We examined which Ca^{2+} release channels mediated sperm extract-induced Ca^{2+} release. Ca^{2+} release in sea urchin egg homogenates by InsP_3 and cADPR can be fully inactivated by prior stimulation with a maximal concentration of each respective agonist (25). Fig. 3A shows the effect of prior desensitization to InsP_3 on the cytosol-preactivated, sperm factor-induced Ca^{2+} release, and Fig. 3B shows the same for cADPR-inactivated homogenates. Sperm extract-induced Ca^{2+} release still occurred in homogenates refractory to either of these agents. However, if both InsP_3 and cADPR mechanisms are inactivated in the same homogenate, the sperm factor-induced Ca^{2+} release was abolished (Fig. 3C). In addition, prior exposure of the homogenates to cytosol-preactivated sperm factor desensitized the microsomes to both InsP_3 and cADPR (Fig. 3D). Previous studies indicate that InsP_3 and cADPR are located on substantial overlapping Ca^{2+} pools and that lack of response to one agent probably reflects a desensitization of the Ca^{2+} release mechanism rather than pool depletion (19, 26). Additionally, a distinct Ca^{2+} pool that is sensitive to nictonic acid adenine dinucleotide phosphate does not increase in size after mobilization of Ca^{2+} by the other two agents (25, 26). It is thus thought that after desensitization of cADPR- and InsP_3-sensitive Ca^{2+} release mechanisms, the pool expressing these channels is still able to sequester Ca^{2+}. These data therefore suggest that sperm extract can trigger Ca^{2+} release by activating both InsP_3-Rs and RyRs. The effects of sperm factor resemble those of the sulphydryl reagent thimerosal, which can stimulate both InsP_3-Rs and RyRs (27, 28) and shares the ability to mimic fertilization and trigger sustained Ca^{2+} oscillations in a variety of mammalian eggs (8).

To confirm that the sperm factor induces Ca^{2+} release by activating multiple Ca^{2+} release mechanisms present on internal stores, we tested the effects of the sperm factor on homogenates pretreated with thapsigargin (10 μM). Thapsigargin is a potent microsomal Ca^{2+}-ATPase inhibitor and functionally removes Ca^{2+} stores and renders sea urchin egg microsomes insensitive to InsP_3 and cADPR (25, 26). Fig. 4A shows that the sperm factor-induced Ca^{2+} release in egg homogenates was abolished by pretreatment with thapsigargin. However, a combination of heparin plus 8-NH_2-cADPR, which competitively blocks both InsP_3-Rs and RyRs to activation by InsP_3 and cADPR, respectively (3), did not affect egg cytosol-preincubated, sperm factor-induced Ca^{2+} release (Fig. 4B).

Our data support the view that the sperm extract modulates Ca^{2+} release via InsP_3-Rs and RyRs by a novel mechanism that is independent of low molecular weight messengers such as InsP_3 or cADPR. These features of the sperm factor do not correlate with the 23-kDa tr-kit sperm protein, which has been suggested to generate InsP_2-induced Ca^{2+} release in mouse eggs (29). The active component causing Ca^{2+} oscillations in the sperm extract fraction used is correlated with a high molecular mass complex composed of 33-kDa subunits which has been named oscillin and has 53% amino acid sequence homology with an Escherichia coli enzyme glucosamine-6-phosphate isomerase (18). It is not clear how this class of enzyme might be linked to Ca^{2+} release channel activation. However, the features of the sperm factor-induced Ca^{2+} mobilization in egg homogenates suggest a novel signaling mechanism and further studies should reveal any possible connection with this class of enzymes. In view of the ability of the sperm factor to trigger distinct Ca^{2+} oscillations in somatic cells (30, 31), it is possible that it may also reveal a novel component of cellular Ca^{2+} oscillators.

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