RESEARCH COMMUNICATION

A cytosolic sperm factor triggers calcium oscillations in rat hepatocytes

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Previously it has been shown that injecting a cytosolic sperm protein factor into mammalian eggs induces sustained repetitive transients of cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$), or $[\text{Ca}^{2+}]_i$ oscillations [Swann (1990) *Development* **110**, 1295–1302]. These sperm-factor (SF)-induced $[\text{Ca}^{2+}]_i$ oscillations are similar to those seen at fertilization. Here we demonstrate that injecting the same cytosolic extracts of mammalian sperm into single rat hepatocytes induces a series of $[\text{Ca}^{2+}]_i$ oscillations, as measured by aequorin luminescence. SF injection into hepatocytes induced $[\text{Ca}^{2+}]_i$ oscillations that were of longer duration, lower frequency and greater amplitude than those seen with the $\text{Ins}(1,4,5)\text{P}_3$ -gen-

erating agonist phenylephrine. The SF-induced $[\text{Ca}^{2+}]_i$ responses appeared to be due to internal release of Ca^{2+} , since transients could occur in Ca^{2+} -free media. Addition of the phorbol ester phorbol 12,13-dibutyrate (PDBu) at low concentrations did not inhibit the SF-induced $[\text{Ca}^{2+}]_i$ oscillations; high concentrations of PDBu led to a sustained increase in $[\text{Ca}^{2+}]_i$ concentrations. These data demonstrate that sperm contain a protein factor capable of inducing a characteristic series of $[\text{Ca}^{2+}]_i$ oscillations in a somatic cell, the hepatocyte. Along with previous observations in dorsal root ganglion neurons, the data suggest a widespread efficacy of the factor in triggering Ca^{2+} oscillations.

INTRODUCTION

Oscillations, or repetitive transient rises, in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) have been demonstrated in many different cell types [1,2]. Such oscillations can be triggered by cell-specific stimuli such as neurotransmitters, hormones and growth factors [1,2]. $[\text{Ca}^{2+}]_i$ oscillations appear to play diverse roles in cellular signalling, affecting cell metabolism, secretion, cell-cycle progression and differentiation [1,2]. The existence of most $[\text{Ca}^{2+}]_i$ oscillations may be explained by complex mechanisms involving increased production of $\text{Ins}(1,4,5)\text{P}_3$ with co-activation of Ca^{2+} -release channels by InsP_3 and Ca^{2+} [2]. However, some of the features of cellular $[\text{Ca}^{2+}]_i$ oscillations are not yet fully understood. For example, in rat hepatocytes the time course of individual $[\text{Ca}^{2+}]_i$ spikes is a characteristic of the stimulating agonist [3]. These agonist-induced $[\text{Ca}^{2+}]_i$ oscillations in hepatocytes also show characteristic differences in their sensitivity to the protein kinase C-activating phorbol esters [4] and to the Ca^{2+} -channel modulator ryanodine [5]. Differences in the amplitude and duration of $[\text{Ca}^{2+}]_i$ transients with different agonists are also seen in various secretory cell types and oocytes [6,7]. It is possible that factors other than InsP_3 are involved in modulating $[\text{Ca}^{2+}]_i$ oscillations and waves in non-excitable cells.

One of the first and most sustained physiological $[\text{Ca}^{2+}]_i$ oscillators to be described was that occurring after fertilization *in vitro* in mammalian eggs [8–10]. It is not known how the sperm triggers these Ca^{2+} oscillations at fertilization, but one hypothesis suggests that the sperm introduces a Ca^{2+} -releasing factor into the egg after gamete membrane fusion [11,12]. This is supported by the demonstration that the injection of cytosolic sperm extracts can trigger sustained $[\text{Ca}^{2+}]_i$ oscillations in mouse, hamster and human eggs [12–14]. The oscillations induced by the sperm factor (SF) resemble the low-frequency large-amplitude $[\text{Ca}^{2+}]_i$ transients that are characteristic of fertilization [12,13]. The SF has

been shown to be protein-based and appears to be a novel agent that may be capable of triggering $[\text{Ca}^{2+}]_i$ changes in other cell types. Injecting the SF into cultured rat dorsal root ganglion neurons has been shown to activate Ca^{2+} -dependent membrane currents [15]. In the present study, we demonstrate directly that the SF can induce $[\text{Ca}^{2+}]_i$ oscillations in single aequorin-injected rat hepatocytes. We show that the SF induces a characteristic pattern of $[\text{Ca}^{2+}]_i$ oscillations in these cells.

MATERIALS AND METHODS

Hepatocytes were isolated from fed male Wistar rats (180–250 g) by collagenase (Boehringer) digestion and microinjected with the Ca^{2+} -sensitive photoprotein aequorin as described previously [3,16]. A second microinjection with the SF was then performed, with the double-injected hepatocyte being positioned under the photomultiplier as described previously (see also [17]). The experimental medium was Williams Medium E (WME; Gibco) gassed with CO_2/air (1:19), to which all compounds were added. Essentially Ca^{2+} -free WME was produced by addition of 2 mM EGTA (Sigma) to the WME, which contains 1.8 mM Ca^{2+} . Phenylephrine, ATP and phorbol 12,13-dibutyrate (PDBu) were all from Sigma, with the agonists being made up in WME freshly each day, whereas the phorbol ester and chelerythrine (LC Services Corp.) were stored as stock solutions in DMSO (Sigma) at -20°C . Vasopressin (Sigma) was made up as a stock solution buffered to pH 4.0 and stored at 4°C .

The cytosolic hamster sperm extract (SF) was the semi-purified '1M Blue' fraction as previously described [13], and it was stored in batches at -70°C . Before use, and on a weekly basis, a batch of the SF was thawed and kept under paraffin oil (light; BDH) at 4°C . The SF was diluted (1:2 or 1:4), on a daily basis, into aequorin dialysis buffer (KCl, 150 mM; Pipes buffer, 1 mM;

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; SF, sperm factor; PDBu, phorbol 12,13-dibutyrate.

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EGTA, 25 μM ; EDTA, 100 μM , pH 7.2), and kept under paraffin oil before microinjection [17]. To inactivate the SF, dialysed extracts were heated at 70 °C for 15 min [12]. Both aequorin and the SF were routinely sequentially injected into the single hepatocytes at volumes that represented between 1 % and 2 % of the total cell volume. All data collection and analysis were performed as described previously [3,16].

RESULTS AND DISCUSSION

Figure 1(A) illustrates the $[\text{Ca}^{2+}]_i$ oscillations that originated from a single rat hepatocyte after microinjection of a 1:4 dilution of the SF stock. Figure 1(B) illustrates the SF-induced $[\text{Ca}^{2+}]_i$ oscillations (1:2 dilution) in a different cell, where the response can be compared with oscillations induced by prior application of 10 μM phenylephrine. The SF-induced oscillations consisted of two types of $[\text{Ca}^{2+}]_i$ transients. Early in the response, between one and 19 regular $[\text{Ca}^{2+}]_i$ transients occurred that lasted for a total of 30–150 s, about half of which displayed further secondary $[\text{Ca}^{2+}]_i$ oscillations during the falling phase (Figure 2A). Later in the response, up to eight $[\text{Ca}^{2+}]_i$ transients occurred that were more variable in $[\text{Ca}^{2+}]_i$ peak height, and which never displayed secondary oscillations. These two types of transient occurred with a variable period that was different in different cells, showing a range from 3 to 15 min ($n = 10$ cells). There was usually a longer break between the occurrence of the first and second sets of the different types of transients. Some cells only produced 1–7 early-type $[\text{Ca}^{2+}]_i$ oscillations before a loss of control of the basal $[\text{Ca}^{2+}]_i$ level that often led to a full discharge of the injected

Figure 1 (A) $[\text{Ca}^{2+}]_i$ oscillations induced by injection of SF into a single hepatocyte; (B) phenylephrine-induced (10 μM) $[\text{Ca}^{2+}]_i$ oscillations, followed by SF injection and SF-induced $[\text{Ca}^{2+}]_i$ oscillations

In (A), injection of SF was made at the time indicated by the arrow. In (B) phenylephrine (PE) was applied for the time shown by the bar. Time constants in all Figures were 10 s for resting $[\text{Ca}^{2+}]_i$ levels and 1 s for $[\text{Ca}^{2+}]_i$ spikes.

Figure 2 (A) (i), (ii), (iv): Expanded time courses of phenylephrine (PE)- and SF-induced $[\text{Ca}^{2+}]_i$ oscillations from data shown in Figure 1(B); (iii) early SF-induced $[\text{Ca}^{2+}]_i$ oscillations from another cell, demonstrating secondary $[\text{Ca}^{2+}]_i$ oscillations on top of the main transient; (B) addition of phenylephrine (10 μM) during the course of SF-induced responses

aequorin ($n = 10$; results not shown), whereas others only displayed the early-type $[\text{Ca}^{2+}]_i$ transients ($n = 5$). As previously found in hamster eggs [12], injecting heat-inactivated SF did not induce any $[\text{Ca}^{2+}]_i$ oscillations ($n = 4$; results not shown).

In cells that were pre-stimulated with 10 μM phenylephrine, the SF-induced $[\text{Ca}^{2+}]_i$ oscillations had peak heights slightly larger than those induced by phenylephrine ($n = 5$; 1090.9 ± 14.8 nM and 1181.7 ± 27.9 nM for phenylephrine and the SF respectively). However, due to the way aequorin signals are calibrated and to the loss of a portion of the total aequorin during the second microinjection, the estimates for the $[\text{Ca}^{2+}]_i$ peak heights for the phenylephrine-induced $[\text{Ca}^{2+}]_i$ oscillations are probably an overestimate. The early $[\text{Ca}^{2+}]_i$ oscillations induced by the SF are probably of even greater amplitude compared with those induced by phenylephrine.

When a maximal concentration of phenylephrine was added to cells already undergoing SF-induced $[\text{Ca}^{2+}]_i$ oscillations, a train of high-frequency $[\text{Ca}^{2+}]_i$ oscillations was induced that resembles that seen with phenylephrine application alone. This suggests that phenylephrine over-rides the SF-induced $[\text{Ca}^{2+}]_i$ oscillations (Figure 2B). Upon removal of the phenylephrine, the $[\text{Ca}^{2+}]_i$ oscillations reverted to the characteristics associated with those of the SF (Figure 2B). Phenylephrine was effective in causing Ca^{2+} oscillations early in the course of the SF response, but neither phenylephrine (10 μM ; $n = 15$) nor vasopressin (3 nM; $n = 7$) was able to induce any $[\text{Ca}^{2+}]_i$ oscillatory behaviour if applied later in the course of the SF-induced $[\text{Ca}^{2+}]_i$ oscillations. This effect may have been due to proteins other than the SF in our extracts, since application of 10 μM phenylephrine was

Figure 3 Effects of Ca^{2+} -free medium on SF-induced $[\text{Ca}^{2+}]_i$ oscillations

After SF had been injected, medium containing EGTA was perfused during the time indicated by the bar.

unable to induce $[\text{Ca}^{2+}]_i$ oscillations in some cells (5/8) injected with heat-treated extracts.

The removal of extracellular Ca^{2+} from the medium by addition of medium with 2 mM EGTA resulted in a progressive inhibition of early SF-induced $[\text{Ca}^{2+}]_i$ oscillations (Figure 3). During the run-down of $[\text{Ca}^{2+}]_i$ oscillations, the frequency and the duration of the oscillations decreased (4/4 cells). This demonstrates that each $[\text{Ca}^{2+}]_i$ increase does not rely on direct influx of Ca^{2+} , although some Ca^{2+} influx may be required for maintaining a series of oscillations. When extracellular Ca^{2+} was returned to the SF-injected cells that had been perfused in Ca^{2+} -free media, the cells showed either a single $[\text{Ca}^{2+}]_i$ transient (2/4 cells), or else $[\text{Ca}^{2+}]_i$ oscillations characteristic of the later-type transients (2/4 cells; Figure 3). A number of specific agents, such as phorbol esters, cyclic AMP analogues and ryanodine, have been reported to have effects on $[\text{Ca}^{2+}]_i$ oscillations in hepatocytes ([4,18,5] respectively). Addition of 10 nM of the phorbol ester PDBu during the early $[\text{Ca}^{2+}]_i$ oscillations induced by the SF had little or no effect on the SF-induced $[\text{Ca}^{2+}]_i$ oscillations ($n = 3$), suggesting that protein kinase C does not play a central role in the generation of these oscillations (Figure 4A). This effect contrasts with the findings that PDBu concentrations of 5–10 nM abolish most other agonist-stimulated $[\text{Ca}^{2+}]_i$ oscillations in these cells [4,19]. However, it resembles the effects of PDBu on ATP-induced $[\text{Ca}^{2+}]_i$ oscillations of long duration that have been shown to be refractory to PDBu even at greatly elevated concentrations (100 nM [20]). The addition of higher concentrations of PDBu (100 nM) during the early SF-induced $[\text{Ca}^{2+}]_i$ oscillations resulted in a continued elevation of $[\text{Ca}^{2+}]_i$ (4/4 cells; Figure 4B), which is unlike the response to PDBu during ATP stimulation [20]. Application of other agents, such as the cell-permeant cyclic AMP analogue dibutyryl cyclic AMP, or low concentrations of ryanodine, had no effect on the SF-induced oscillations (results not shown).

The present study provides the first direct demonstration that the cytosolic SF can induce $[\text{Ca}^{2+}]_i$ oscillations in a somatic cell, the rat hepatocyte. This finding is consistent with previous observations that the SF triggers Ca^{2+} -dependent membrane currents in rat dorsal root ganglion neurons [15]. In hepatocytes we can compare the SF-induced $[\text{Ca}^{2+}]_i$ oscillations with those of a wide variety of receptor-linked agonists that act through the phosphoinositide signalling pathway. It is clear that the SF-induced $[\text{Ca}^{2+}]_i$ transients are of longer duration, greater amplitude and lower frequency, and are more resistant to the effects of phorbol esters, than the $[\text{Ca}^{2+}]_i$ oscillations seen with phenyl-

Figure 4 (A) Effect of a low concentration (10 nM) of PDBu on SF-induced $[\text{Ca}^{2+}]_i$ oscillations; (B) effect of a higher concentration (100 nM) of PDBu on SF-induced $[\text{Ca}^{2+}]_i$ oscillations

eprine [3]. The ability of the SF to induce distinctive early- and late-type $[\text{Ca}^{2+}]_i$ transients distinguishes it from phenylephrine- or vasopressin-induced $[\text{Ca}^{2+}]_i$ oscillations [3,16]. The overall pattern of SF-induced $[\text{Ca}^{2+}]_i$ oscillations is closest to the pattern of response of a subpopulation (28 %) of ATP-stimulated hepatocytes [21], or to cells stimulated with bovine growth factor [22]. However, the sustained elevations of $[\text{Ca}^{2+}]_i$ in response to high concentrations of phorbol esters in SF-injected cells still distinguish the response from that seen in ATP-stimulated cells [21].

Some of the characteristics of the SF-induced $[\text{Ca}^{2+}]_i$ oscillations in hepatocytes are similar to those seen in mammalian eggs. In both cell types the individual $[\text{Ca}^{2+}]_i$ transients last about 1 min [6,23]. Secondary oscillations are also a feature of SF-induced and fertilization-induced $[\text{Ca}^{2+}]_i$ transients in mouse eggs [10,13], as well as the early SF-induced responses in hepatocytes. Another similarity with hepatocytes is that the SF-induced $[\text{Ca}^{2+}]_i$ oscillations in hamster eggs are relatively refractory to inhibition by phorbol esters [12]. In mammalian eggs it was suggested that the SF does not mediate its effects through increased InsP_3 production [13]. It is possible that a direct effect on $[\text{Ca}^{2+}]_i$ release channels is involved, since SF extracts can trigger Ca^{2+} release from sea-urchin egg homogenates (A. Galione and K. Swann, unpublished work). The SF appears to mediate its effects by sensitizing the process of Ca^{2+} -induced Ca^{2+} release [12,13,15]. This does not appear to involve cyclic ADP-ribose (cADPR), since injecting cADPR is ineffective at triggering $[\text{Ca}^{2+}]_i$ increases in rodent eggs [24]; K. Swann, unpublished work), and in dorsal root ganglion neurons cADPR and SF act on distinct Ca^{2+} -release stores [15]. The recent identification of the SF as a 33 kDa protein should allow for a precise definition of its molecular mechanisms of action in the near future [25].

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REFERENCES

- 1 Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- 2 Tsien, R. W. and Tsien, R. Y. (1990) *Annu. Rev. Cell Biol.* **6**, 715–760
- 3 Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1987) *Cell Calcium* **8**, 79–100
- 4 Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1987) *Biochem. J.* **246**, 519–523
- 5 Sanchez-Bueno, A. and Cobbold, P. H. (1993) *Biochem. J.* **291**, 169–172
- 6 Swann, K. and Ozil, J. P. (1994) *Int. Rev. Cytol.* **152**, 183–222
- 7 Hellman, B., Gylfe, E., Grapengiesser, E., Lund, P.-E. and Berts, A. (1992) *Biochim. Biophys. Acta* **1113**, 295–305
- 8 Cuthbertson, K. S. R., Whittingham, D. G. and Cobbold, P. H. (1981) *Nature (London)* **294**, 754–757
- 9 Miyazaki, S. and Igusa, Y. (1981) *Nature (London)* **290**, 706–707
- 10 Cuthbertson, K. S. R. and Cobbold, P. H. (1985) *Nature (London)* **316**, 541–542
- 11 Dale, B., De Felice, L. J. and Ehrenstein, G. (1985) *Experientia* **41**, 1068–1070
- 12 Swann, K. (1990) *Development* **110**, 1295–1302
- 13 Swann, K. (1994) *Cell Calcium* **15**, 331–339
- 14 Homa, S. T. and Swann, K. (1994) *Hum. Reprod.* **9**, 2356–2361
- 15 Currie, K. P. M., Swann, K., Galione, A. and Scott, R. H. (1992) *Mol. Biol. Cell* **3**, 1415–1425
- 16 Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1986) *Nature (London)* **319**, 600–602
- 17 Cobbold, P. H. and Lee, J. A. C. (1991) in *Cellular Calcium: A Practical Approach* (McCormack, J. G. and Cobbold, P. H., eds.), pp. 55–81, Oxford University Press
- 18 Woods, N. M., Dixon, C. J., Cuthbertson, K. S. R. and Cobbold, P. H. (1990) *Cell Calcium* **11**, 353–360
- 19 Sanchez-Bueno, A., Marrero, I. and Cobbold, P. H. (1993) *Biochem. J.* **291**, 163–168
- 20 Dixon, C. J., Cobbold, P. H. and Green, A. K. (1995) *Biochem. J.* **309**, 145–149
- 21 Dixon, C. J., Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1990) *Biochem. J.* **269**, 499–502
- 22 Marrero, I., Green, A. K., Cobbold, P. H. and Dixon, C. J. (1996) *Biochem. J.* **313**, 525–528
- 23 Miyazaki, S., Shirakawa, H., Nakada, K. and Honda, Y. (1993) *Dev. Biol.* **158**, 62–78
- 24 Kline, J. T. and Kline, D. (1994) *Biol. Reprod.*, **50**, 193–203
- 25 Parrington, J., Swann, K., Shevchenko, V., Sesay, A. K. and Lai, F. A. (1996) *Nature (London)*, in the press

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