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Activation of Ca^{2+}-Dependent Currents in Cultured Rat Dorsal Root Ganglion Neurones by a Sperm Factor and Cyclic ADP-Ribose

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The effects of intracellular application of two novel Ca^{2+} releasing agents have been studied in cultured rat dorsal root ganglion (DRG) neurones by monitoring Ca^{2+}-dependent currents as a physiological index of raised free cytosolic Ca^{2+} ($[Ca^{2+}]_i$). A protein based sperm factor (SF) extracted from mammalian sperm, has been found to trigger Ca^{2+} oscillations and to sensitize unfertilized mammalian eggs to calcium induced calcium release (CICR). In this study intracellular application of SF activated Ca^{2+}-dependent currents in approximately two-thirds of DRG neurones. The SF induced activity was abolished by heat treatment, attenuated by increasing the intracellular Ca^{2+} buffering capacity of the cells and persisted when extracellular Ca^{2+} was replaced by Ba^{2+}. In addition, activity could be triggered or potentiated by loading the cells with Ca^{2+} by activating a series of voltage-gated Ca^{2+} currents. Ca^{2+}-activated inward current activity was also generated by intracellular application of cyclic ADP-ribose (cADPR), a metabolite of NAD^+, which causes Ca^{2+} release in sea urchin eggs. This activity could also be enhanced by loading the cells with Ca^{2+}. The cADPR induced activity, but not the SF induced activity, was abolished by depleting the caffeine sensitive Ca^{2+} store. Ruthenium red markedly attenuated SF induced activity but had little action on cADPR induced activity or caffeine induced activity. Our results indicate that both SF and cADPR release intracellular Ca^{2+} pools in DRG neurones and that they appear to act on subtly distinct stores or distinct intracellular Ca^{2+} release mechanisms, possibly by modulating CICR.

INTRODUCTION

It is well established that changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) regulate many diverse cellular functions. In neurones these include neurite outgrowth, vesicular neurotransmitter release, activation of calcium dependent ion channels, gene expression, synaptic plasticity, and programmed cell death (Kennedy, 1989; Miller, 1991). To facilitate this, neurones possess complex calcium homeostatic mechanisms to control $[Ca^{2+}]_i$ (McBurney and Neering, 1987; Blaustein, 1988; Miller, 1991). Despite the importance and interest in this field many questions still remain unanswered about the modulation of $[Ca^{2+}]_i$. Considerable attention has focused on the release of Ca^{2+} from intracellular pools. Two types of intracellular Ca^{2+} release channel have so far been reported, namely the inositol 1,4,5 trisphosphate receptor (IP_3R)[1] and the ryanodine receptor (RyR) (Supattapone *et al.*, 1988; McPherson *et al.*, 1991; Ferris and Snyder, 1992). The receptors have both been shown to coexist in cerebellar purkinje neurons

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[1] Abbreviations used: cADPR, cyclic ADP-ribose; CICR, calcium induced calcium release; DRG, dorsal root ganglion; EGTA, 1,2-Di(2-aminoethoxy)ethane-*N,N,N',N'*-tetra-acetic acid; E_{rev}, reversal potential; IP_3, inositol 1,4,5 trisphosphate; IP_3R, inositol 1,4,5 trisphosphate receptor; RuR, ruthenium red; RyR, ryanodine receptor; SF, cytosolic sperm factor.

(Brorson *et al.*, 1991; Walton *et al.*, 1991), and molecular cloning has identified some sequences of striking similarity (Tsien and Tsien, 1990). The brain RyR is also sensitive to caffeine and calcium itself (Bezprozvanny *et al.*, 1991; McPherson *et al.*, 1991) and is probably a site of calcium induced calcium release (CICR) in neurones (Lipscombe *et al.*, 1988; Marrion and Adams, 1992). CICR has been implicated in many cellular functions including models of $[Ca^{2+}]_i$ oscillations, spreading waves of Ca^{2+} within cells, and the after-hyperpolarization in Guinea pig vagal neurones (Berridge and Galione, 1988; Berridge and Irvine, 1989; Dupont *et al.*, 1991; Sah and McLachlan, 1991). It has also been shown that the caffeine sensitive store can modulate stimulus evoked changes in $[Ca^{2+}]_i$ and presumably also Ca^{2+}-dependent processes (Friel and Tsien, 1992). It has been suggested that a means by which this could be controlled is modulation of the Ca^{2+} sensitivity of CICR. However to our knowledge no endogenous modulators of CICR have been reported in neurones.

Intracellular calcium stores are also present in oocytes and are involved in egg activation at fertilization. In all species studied the egg is activated by the sperm-causing transient increases in calcium (Whittingham, 1980; Jaffe, 1983; Whitaker and Steinhardt, 1985). In mammalian eggs the transients form a series of regular oscillations that persist for several hours, and there is strong evidence to suggest that release from intracellular stores is involved (Cuthbertson and Cobbold, 1985; Miyazaki 1988). One hypothesis on how the Ca^{2+} oscillations are triggered suggests that the sperm and egg first fuse allowing the transfer of a soluble cytosolic sperm factor into the egg that causes release of intracellular Ca^{2+} stores (Dale *et al.*, 1985; Dale, 1988; Stice and Robl, 1990; Swann, 1990; Swann and Whitaker, 1990). The actions of such a sperm factor (SF) have been described in hamster and mouse eggs and microinjecting SF into unfertilized eggs closely mimics the calcium transients and associated hyperpolarizing membrane responses seen at fertilization (Swann, 1990, 1992). The SF appears to be a protein (because it is sensitive to both heat treatment and trypsin) found in the high molecular weight extract of the cytosolic fraction from sperm. Moreover, its mode of action appears to be to sensitize the hamster eggs to CICR (Swann, 1990).

Another novel Ca^{2+} releasing agent has recently been reported in sea urchin eggs and egg homogenates (Lee *et al.*, 1989; Dargie *et al.*, 1990). Cyclic-ADP ribose (cADPR) is a metabolite of NAD^+, and the enzyme responsible for its synthesis appears to be present in rabbit liver, brain, heart, spleen, and kidney (Rusinko and Lee, 1989) with two isoforms of this enzyme (termed ADP ribosyl cyclase) having recently been purified (Hellmich and Strumwasser, 1991; Lee, 1991; Lee and Aarhus, 1991). Interestingly, in analogy with the SF, cADPR has been reported to act as a modulator of CICR in sea urchin eggs (Galione *et al.*, 1991; and for review see Galione, 1992). However, cADPR does not appear to cause release of Ca^{2+} in hamster eggs raising the possibility that they may be distinct agents and target distinct Ca^{2+} release mechanisms.

Given the apparent modulation by both these agents of CICR, we have investigated the action of intracellular application in cultured dorsal root ganglion (DRG) neurones. We have recorded calcium-dependent currents (predominantly nonselective cation but also chloride) previously reported in these cells (Currie and Scott, 1992) as indicators of raised $[Ca^{2+}]_i$. In addition to identifying Ca^{2+} releasing activity of the two agents in these neurones, we have attempted to pharmacologically distinguish the mechanism of action of SF and cADPR.

METHODS

Culture of Cells

One- to two-day old Wistar rats were decapitated and the dorsal root ganglia dissected out from the spinal column. The cells were then dissociated both enzymatically (collagenase, trypsin) and mechanically (trituration) before being plated on poly-L-ornithine/laminin coated coverslips. The cells were maintained in and fed every 7 d with Ham's F14 nutrient mixture (Imperial Laboratories, Andover, Hampshire UK) with 10% Horse Serum (GIBCO, Grand Island, NY) + NGF (10 μg/ml: Sigma, St. Louis, MO) + penicillin/streptomycin (Flow, McLean, VA) and kept at 37°C in humidified air with 5% CO_2. Electrophysiological recordings were made from cells maintained in culture for 1 d to 2 wk.

For some experiments cells up to 7 d in culture were freshly replated. This involved gently washing the cells off the coverslip with a small amount (≈0.5 ml) of culture medium (F14/horse serum) and then replating the cells onto new poly-L-ornithine coated coverslips (Dolphin and Menon-Johanssen, personal communication). The replated cells were attached to the new coverslip and were recorded from within 1–2 h after replating. Replated cells have the advantage of being spherical "neuroballs" with no processes. This leads to much improved voltage clamping and unequivocally isolates currents generated by channel openings on the cell body.

Preparation of Cytosolic Sperm Factor

The full preparation has previously been described (Swann, 1990). Briefly this entailed collection of hamster or boar sperm that was lysed in a KCl/*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer with 100 μM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N,N*′-tetraacetic acid (EGTA) by freeze/thaw cycles or by sonication. The homogenates were then spun at 100 000 × *g* for 1 h at 4°C and the supernatant isolated as the cytosolic fraction. Size fractionation was achieved by concentration of extracts on centricon-100 ultrafiltration membranes for boar samples and C-10 membranes for hamster samples followed by dilution into an intracellular-like medium and reconcentration. Stocks were frozen (−18°C) until use and then diluted in patch recording medium to yield 0.1–5.0 mg/ml total protein concentration. The Ca^{2+} concentration in the stock before dilution (10–100 times) was typically 100–200 nM (Swann, 1990). No detergents were used in the extraction of SF, and it was fully soluble in distilled water and 1M KCl, strongly suggesting it is a soluble cytosolic factor. Cytosolic brain extracts (BE) were made from rat brain in the same way as sperm extracts and were of similar protein concentrations. The SF was introduced into the cells by inclusion in the patch pipette solution used in the whole-cell patch-clamp technique.

Preparation of Cyclic ADP-Ribose

Cyclic ADP-ribose (cADPR), a generous gift from Dr. H. C. Lee, was prepared by incubating NAD^+ with the purified enzyme, ADP-ribosyl

cyclase (Lee and Aarhus, 1991). Its authenticity was confirmed by high performance liquid chromatography (h.p.l.c.) profile (Walseth *et al.*, 1991) and by its ability to release Ca^{2+} from sea urchin egg microsomes that had previously not been desensitized to cADPR (Galione *et al.*, 1991).

Electrophysiology

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used to record from DRG neurones at room temperature (~23°C). Low resistance patch pipettes (3–7 MΩ) were used and the cells voltage clamped with an Axoclamp-2A amplifier operated at a sampling rate of 25–35 kHz in the discontinuous single electrode voltage-clamp mode. Sodium currents were blocked with tetrodotoxin (TTX), and potassium currents were blocked with extracellular tetraethylammonium (TEA) and intracellular Cs^+, leaving intact the voltage-activated Ca^{2+} currents and Ca^{2+}-activated chloride and Ca^{2+}-activated nonselective cation currents (Currie and Scott, 1992). The recording bath medium contained (in mM) 130 choline chloride; 3.0 KCl; 0.6 $MgCl_2$; 1.0 $NaHCO_3$; 10 HEPES; 4.0 glucose; 25 TEA; 0.0025 TTX; and 2.0 or 4.0 $CaCl_2$. The pH and OsM were adjusted to 7.4 and 320 mOsm with NaOH and sucrose, respectively. Tris (hydroxymethyl) aminomethane (Tris) sucrose (T/S) based recording medium used in some experiments contained in (mM) 145 Tris-Cl; 4.0 $CaCl_2$; 0.3 $MgCl_2$; 2.5 glucose; 5.0 HEPES; 0.00125 TTX. The pH of this solution was adjusted to 7.4 with HCl. Patch pipettes were filled with a solution containing (in mM) either 140 CsCl or CsAcetate; 2.0 $MgCl_2$; 2.0 ATP-Na; 10 HEPES; 0.1 $CaCl_2$; and 1.1 or 10 EGTA. The pH was adjusted to 7.2 with Tris and OsM to 310 mOsm with sucrose. For extracellular application SF was diluted in the choline chloride-based recording medium. In some experiments ruthenium red (RuR) (BDH limited) was also included in the patch solution at a concentration of 100 μM–1 mM. For experiments with SF the RuR-containing patch solution was used to make the appropriate dilution of SF.

Inclusion of the highest concentrations (2–5 mg/ml) of SF in the patch solution had no action on pH and increased the osM slightly to 320 mOsm. Perfusion of drugs and modified media was performed by low pressure ejection (~7 kPa) from micropipettes ~10 μM tip diam) placed ~100 μM from the cell. The data was stored by using both a digital audio tape recorder and a Gould 2200S (Gould, Cleveland, OH) twin-channel pen recorder.

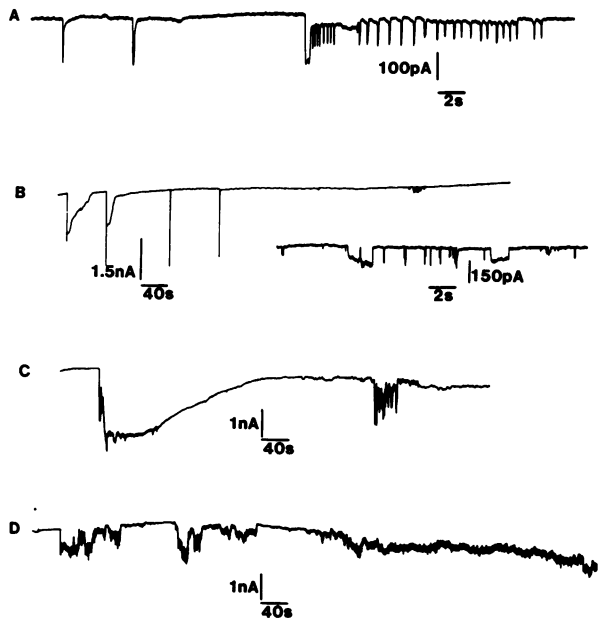

Figure 1. Inward currents activated in response to intracellular SF. Examples of the different types of activity elicited by intracellular application of SF (0.2 mg/ml). Records are extracts of traces from 4 different cells all loaded with CsCl based patch solution. (A) Short duration spike like inward current activity, showing a burst of regular oscillations. (B) More prolonged transient inward currents and also large spike like activity. The insert shows the small burst of low amplitude activity towards the end of the recording at higher gain. (C) A much more sustained response (~5–6 min) followed by a burst of noisier more transient type activity. (D) More sustained activity followed by an increase in noise and sustained increase in holding current.

RESULTS

In 61% (52 of 85 cells) of DRG neurones loaded with CsCl patch solution, intracellular application of SF (0.1–5.0 mg/ml total protein concentration) elicited inward current activity after a delay of between 10–25 min. The inward currents induced by the SF were of variable nature and amplitude (Figure 1) consisting of several distinct types of activity including regular oscillations. The amplitude of the responses ranged from 100 pA to 3.0 nA and the currents could be subdivided into several distinct qualitative groups. These different types of activity include short duration (<1–2 s) spike-like inward currents (Figure 1A), more prolonged transients lasting for 10–20 s or for ≤1–2 min (Figure 1B), more sustained responses lasting for >5–10 min (Figure 1C), and finally stepwise sustained increases with no recovery (Fig. 1D). Often two or more types of activity would be present in the same cell and superimpose on one another limiting our study to a qualitative analysis.

These effects were not caused by a nonspecific effect of loading the cells with protein. No activity was seen in 12 cells that were clamped at −90 mV with 2.0 mg/ml of serum albumin fraction V present in the patch pipette solution for up to 30 min after entering the whole-cell configuration. Only 4 of these 12 cells showed any activity between 30–60 min, and this activity consisted of a few very small isolated events (<200 pA). Likewise with a crude cytosolic brain extract, applied at similar protein concentrations to SF, only one of seven cells showed any inward current activity within 30 min. SF was also applied extracellularly by low pressure ejection and again was found to produce no activity (n = 5).

Heat Treatment Attenuates SF Induced Activity

Heat treated SF (either 60°C for 1 h or boiling in a water bath for 15 min) was applied to 10 cells to see if the SF activity was heat sensitive in neurones and as a further control against effects of adding a high molecular weight substance to the cells. Batches of the SF were split in two: one-half was heat treated while the other one-half was untreated and used to test for SF mediated responses. In four of five cells to which heat treated 0.2 mg/ml SF was added, there was no activity elicited for >1 h (Figure 2B). The higher concentration of sperm factor induced activity that was attenuated by heat

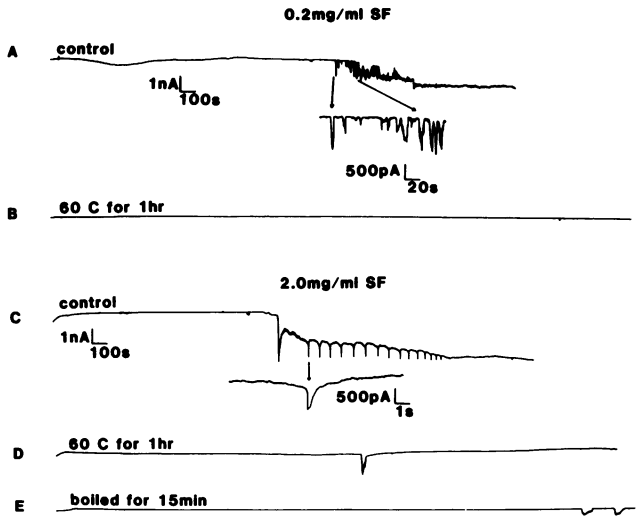

Figure 2. Heat treatment attenuates the action of SF. Heat treating the lower concentration of SF used (0.2 mg/ml protein) at 60°C for 1 h abolished activity in 4 of 5 cells (A–B). The top (A) shows a control cell with the initial transient inward current activity expanded in the insert. The bottom (B) shows a cell with heat treated SF present in the patch pipette. Heat treatment of the higher concentration (2.0 mg/ml protein) SF (C–E). The top (C) shows a control cell with one of the oscillating inward currents expanded in the insert. (D) and (E) show cells with SF applied following heat treatment at 60°C for 1 h and boiling for 15 min, respectively. The traces show attenuation of activity with the two small events shown in (E) occurring 58–59 min into the recording. All traces are continuous records from cells at holding potentials of −90 mV, records begin from the point of going into the whole-cell configuration.

treating at 60°C for 1 h (3 cells) (Figure 2D) while boiling for 15 min virtually abolished activity (2 cells) with only small residual current being activated after an extremely long delay (>40 min) (Figure 2E) when compared with cells loaded with untreated SF.

The SF Induced Currents are Ca^{2+} Dependent

Raising the intracellular calcium buffering capacity of the cells by increasing the EGTA concentration of the patch solution from 1.1 or 2 mM to 10 or 20 mM attenuated SF induced activity (Figure 3). The calculated free Ca^{2+} in patch pipette solutions containing 1.1, 2.0, 10, and 20 mM EGTA were 1.6×10^{-8} M, 8×10^{-9} M, 1.6×10^{-9} M, and 8×10^{-10} M, respectively. With raised EGTA concentration, the overall amount of activity seen in cells was reduced, and the activity that remained was shorter lasting and of smaller amplitude than that typically seen in control cells (n = 12 cells). The free Ca^{2+} concentration of CsCl based patch solution was increased by raising the [Ca^{2+}] from 0.1 to 1.1 mM (with 1.1 mM EGTA present). This gave an estimated free calcium concentration in the patch solution of 4.59×10^{-6} M. In six of eight cells loaded with this patch solution (with no SF present), inward current activity was generated that showed similar components to that elicited by application of SF (Figure 4A). The "normal" patch solution (in which the SF was applied) had an estimated free calcium concentration of 16 nM and did not give rise to any activity of Ca^{2+}-dependent currents (Figure 4B). Ba^{2+} does not directly support the Ca^{2+}-dependent Cl^- and nonselective cation currents in DRG neurones (Currie and Scott, 1992). However substituting extracellular Ca^{2+} with Ba^{2+} did not abolish the SF induced activity. In three of five cells with Ba^{2+} in the extracellular medium and loaded with CsCl based patch solution SF induced inward current activity was generated. These data add further support to the contention that SF is activating Ca^{2+}-dependent currents by releasing calcium from intracellular stores.

Ionic Nature of the SF-Induced Inward Currents

In cells loaded with CsAcetate based patch solution to prevent any inward chloride current component of the activity, intracellular application of SF again elicited inward currents in 67% (14 of 21 cells) of DRG neurones. The same components as described for CsCl loaded cells were present but more sustained, and stepwise increases in current tended to predominate (see Figure 5). This has also been found with Ca^{2+}-dependent currents activated by applications of caffeine (1–10 mM) to DRG neurones and may represent some interaction of the intracellular anion with Ca^{2+} release or Ca^{2+} homeostasis (Currie and Scott, 1992).

In cells loaded with CsAcetate based patch solution, the reversal potential of the SF induced sustained inward cation currents was estimated by extrapolating the current/voltage relationship of the difference current (as shown in Figure 5). A series of 30-ms duration volt-

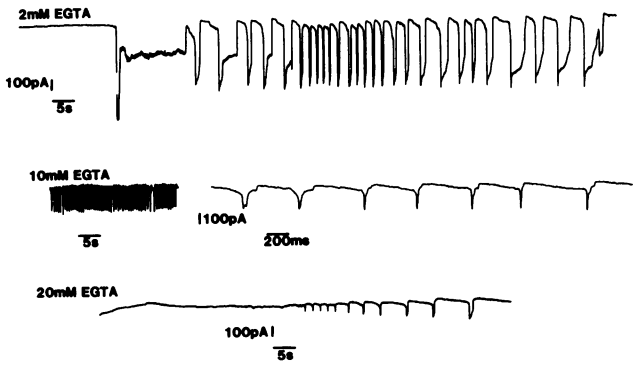

Figure 3. SF induced inward currents can be shown to be Ca^{2+} dependent by attentuation with EGTA. The traces show extracts of records from 3 cells in which oscillating inward currents were activated by application of 0.2 mg ml^{-1} SF with either 2, 10, or 20 mM EGTA present in the patch solution. Increasing the EGTA concentration reduced the overall amount of activity seen in cells and reduced the amplitude. In the middle trace there is a burst of high frequency oscillations that have been expanded, but note the small amplitude. Likewise with 20 mM EGTA only very small events remain.

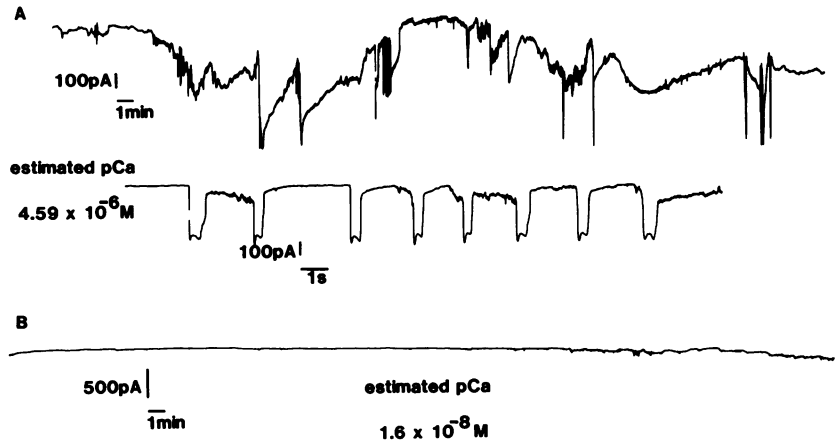

Figure 4. Activation of calcium-dependent currents by increasing the free calcium concentration ($[Ca^{2+}]_i$) of the patch pipette solution. Both cells were voltage clamped at −90 mV with patch pipettes containing CsCl based patch solution with the $[Ca^{2+}]_i$ adjusted as described in RESULTS. (A) Record of a cell clamped by using patch solution with 4.59 μM $[Ca^{2+}]_i$. Inward current activity was generated almost immediately. The insert shows oscillatory currents on an expanded time scale. (B) A control cell loaded with a patch solution containing 16 nM $[Ca^{2+}]_i$. No inward current activity was generated for ≤1 h.

age-step commands were activated from a holding potential of −90 mV to command potentials of between −180 and −40 mV before and during the SF evoked currents. These currents were then subtracted and the current/voltage relationship of the difference current was plotted and extrapolated to estimate the reversal potential of the current (steps to more depolarized potentials than −40 mV could not be done because they would activate voltage-gated calcium currents). In eight cells the difference current I/V relationship was linear and was extrapolated to estimate E_{rev} (Figure 5). The mean E_{rev} for the SF current in eight cells was +2 ± 7 (SE) mV.

In three of these eight cells T/S perfusion during the response reversibly attenuated activity. The mean reduction in the three cells, with two applications to each cell, was 43 ± 8%. Both the estimated E_{rev} and the sensitivity of the SF induced current to T/S medium suggest responses were nonselective cation currents (I_{CAN}) and agree with data previously found with caffeine application (Currie and Scott, 1992).

Activity can be Triggered and/or Potentiated in SF Loaded Cells by Activating Voltage-Gated Calcium Currents

In 15 cells in which there had been little or no activity 15–25 min after application of SF via the patch pipette, activity could be started or potentiated by applying a series of depolarizing voltage steps from −90 to 0 mV for 100 ms at 3 to 5-s intervals. During the 100 ms voltage-step command to 0 mV voltage-gated calcium channels are opened allowing influx of Ca^{2+} into the cells. Thus performing a series of these steps will load the cell with Ca^{2+}. In some cells as few as five steps could trigger inward current activity (Figure 5) but the same protocol applied to control cells with only CsCl or CsAcetate patch (no SF present) did not elicit activity over 45 min recording (n = 10) (Figure 5).

$I_{1,4,5}P_3$ does not Mimic SF Induced Inward Current Activity

Ten to one hundred of μM IP_3 were included in the patch pipette solution (CsCl) in 13 cells to determine if it would elicit similar types of activity to that generated by SF. In only 2 cells were inward currents activated with a short delay (<5 min) with some small events occurring in two further cells only after considerable delay > 30 min and following in both cases series (5 to 20) of inward voltage-gated calcium currents to load the cells with Ca^{2+}. In the remaining 9 cells no inward current activity was seen. Inclusion of 10–80 μM Inositol 1,4,5-trisphosphorothioate (IPS_3), a nonhydrolysable analogue of IP_3, did not elicit any consistant inward current activity. Only 3 out of 8 cells showed any inward current activity up to 30 min after entering the whole-cell patch configuration, and this activity was limited to single events except in one case in which several small amplitude (−50 pA) events occurred.

Cyclic ADP Ribose (cADPR) Activated Inward Current Activity

Intracellular application of 10^{-8} − 10^{-6} M cyclic ADP ribose (cADPR) also elicited inward current activity in 23 of 32 cells (Figure 6 A and B). Again the nature of the response was varied and in some cells resembled that seen with either SF or raising the pCa of the patch solution including oscillatory currents (Figure 6A). The delay to onset of activity was usually within 10 min with some cells showing activity within 2–3 min. Six cells were also seen to rapidly swell and burst 5–10 min after cADPR application. Activity of the cADPR was abolished by repetitive freeze/thawing (Figure 6C). Another similarity between the cADPR and SF was the ability of a Ca^{2+} load generated by a series of voltage-gated Ca^{2+} channel currents to trigger or potentiate activity (n = 5) (Figure 6D).

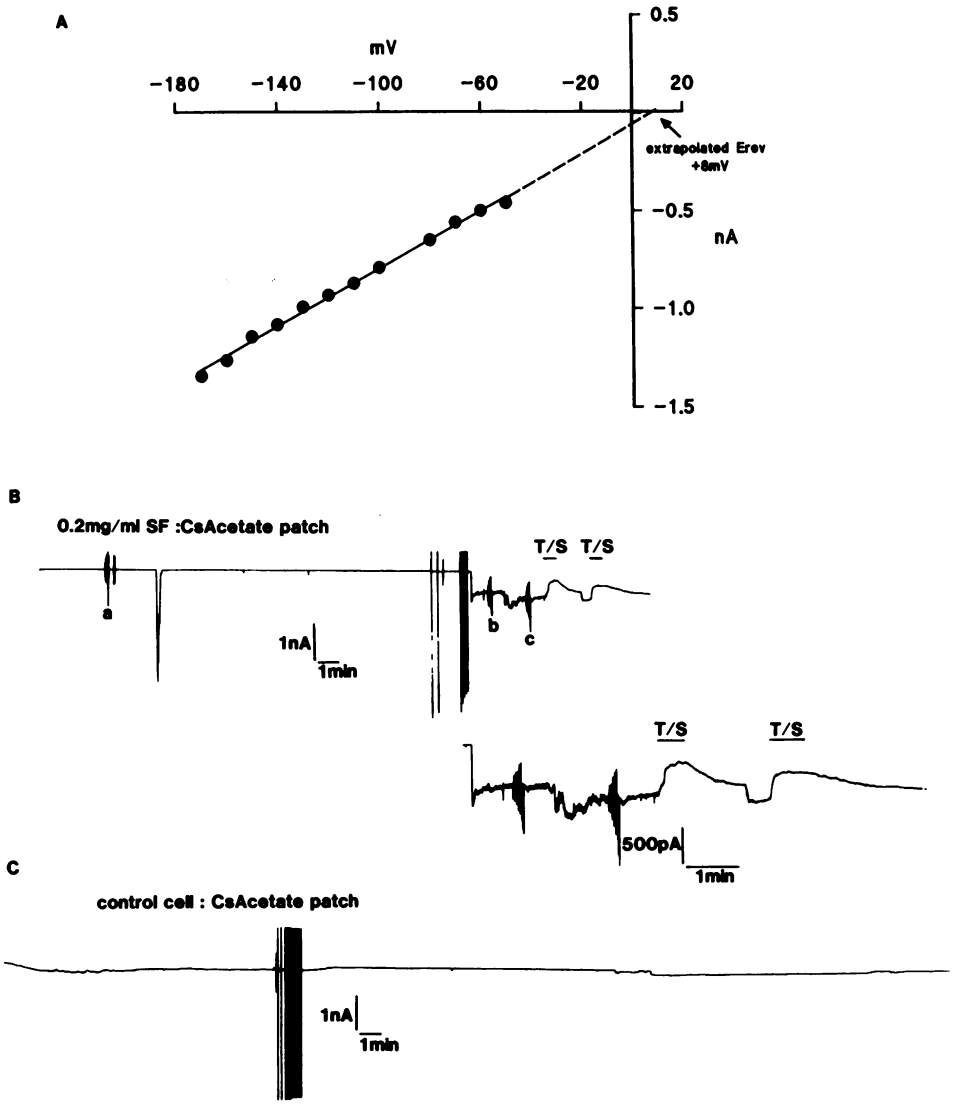

Figure 5. (A) Current voltage relationship for the difference current calculated from the cell shown in (B). The extrapolated E_{rev} was calculated from the data by using linear regression analysis (the correlation coefficient is 0.998). (B) Complete record of a cell loaded with CsAc based patch solution and 0.2 mg/ml SF. A large transient inward current was followed by a period of inactivity. Upon applying a series of voltage step commands (5 steps, −90 to 0 mV, 5 s apart, 100 ms duration) to activate voltage-gated Ca^{2+} currents more sustained inward current activity was generated. The estimated reversal potential was calculated by performing a series of 30 ms voltage step commands to between −180 and −40 mV from a holding potential of −90 mV before the current was activated (a) and during the response (b) and (c). The baseline current/voltage relationship was subtracted from the current/voltage relationship activated during the response and the resultant current/voltage relationship of the difference current plotted and extrapolated (A). T/S indicates perfusion of T/S based recording medium and shows reversible attenuation of the current during perfusion. The insert trace shows the prolonged response, current/voltage relationships, and T/S attenuation on an expanded scale. (C) A control cell loaded with CsAc in which a series of 12 voltage-step commands from −90 to 0 mV (100 ms duration) to activate voltage-gated Ca^{2+} current, which did not elicit any inward current activity.

Pharmacological Distinction Between cADPR and SF Induced Activity

Because both agents have been reported to act by a CICR mechanism in other cell types, we investigated the effects of depleting the caffeine sensitive Ca^{2+} pool on both SF and cADPR induced activity in DRG neurones. To do this cells were preincubated for two periods of seven and one-half min with 1 mM extracellular caffeine in Ba^{2+}-containing bath solution. The cells were then washed twice to remove the caffeine. In addition, extracellular Ca^{2+} was replaced by extracellular Ba^{2+} for the duration of the experiment to prevent refilling of the caffeine sensitive Ca^{2+} store from the extracellular environment. In five cells, which had undergone this pretreatment, pressure ejection of 1–10 mM caffeine ≤30 min after the start of whole-cell recording did not elicit any inward currents suggesting that no Ca^{2+} was available for release. cADPR or SF was applied as usual by inclusion in the patch pipette solution and patch clamping the pretreated cells.

In nine cells to which cADPR was applied after caffeine pretreatment, there was no inward current activity > 25–30 min. In six of these cells it was also shown that a series of 20 depolarizing voltage-step commands of 100-ms duration activated every 3–5 s to load the cells with Ba^{2+} (through voltage-gated Ca^{2+} channels) did not trigger activity (Figure 7). In four of these nine cells, after 25–30 min had elapsed with no activity, Ca^{2+} (2 mM) containing recording medium was perfused onto the cells. Three of four of these cells subsequently showed inward current activity either spontaneously (Figure 7A) or after a series of depolarizing steps to load the cells with Ca^{2+} (Figure 7B).

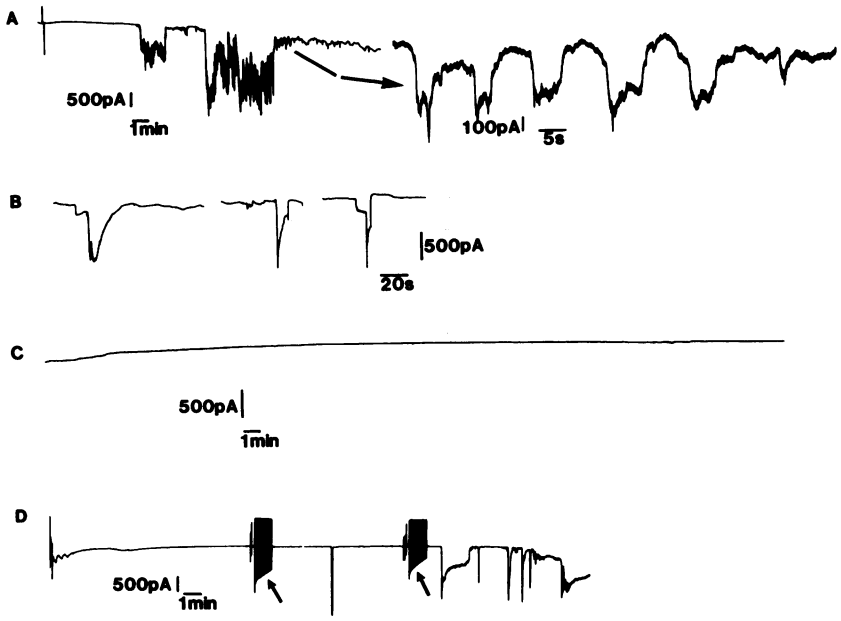

Figure 6. Intracellular cADPR induces oscillating inward currents in some cells. All cells were voltage clamped at −90 mV. (A) A complete trace of a cell loaded with CsCl based patch solution containing 10 nM cADPR (left). The area arrowed is expanded on the right to show the currents more clearly (note the change in scale bars). (B) Examples of inward currents obtained from another cell with 10 nM intracellular cADPR. (C) Trace shows that after 4 freeze/thaw cycles the activity of the cADPR (10 nM) was abolished. (D) Complete record of a cell containing 1 μM cADPR. The trace shows some slight activity within 1–2 min and the triggering of activity by a series of 20 voltage-gated calcium currents activated at 0 mV every 3 s (←).

The same pretreatment to cells loaded with SF did not abolish activity with 6 of 10 cells still showing some inward current activity after caffeine pretreatment (Figure 7C).

RuR has been reported to block CICR in a variety of preparations, so we investigated its actions on activity induced by SF, cADPR, and caffeine. RuR was included in the patch pipette solution along with the SF at a concentration of 100 μM. In a total of eight cells, three showed inward current activity, which was qualitatively less and of smaller amplitude than that seen with control cells run on the same day with the same batch of SF (Figure 8A). This attenuation of activity was not due to any cell membrane channel blocking action of RuR because both voltage-gated Ca^{2+} currents and Ca^{2+}-activated tail currents were shown to persist in cells loaded with RuR (Figure 8B).

It has also been shown that in DRG neurones that possess a Ca^{2+}-activated chloride tail current ($I_{Cl(Ca)}$), following a voltage-gated Ca^{2+} current that application of caffeine (10 mM) leads to a rapid onset transient inward current due to activation of $I_{Cl(Ca)}$ (Currie and Scott, 1992). RuR, either 100 μM (n = 3) or 1 mM (n = 3), did not block this caffeine activated $I_{Cl(Ca)}$ in this study (Figure 8C). This is further confirmation that RuR does not block the Ca^{2+}-activated currents used to monitor $[Ca^{2+}]_i$.

RuR (100 μM) was also found to have little or no action on cADPR (1 μM) induced activity with six of eight cells showing typical responses indistinguishable from responses seen in cells loaded with cADPR alone (Figure 8D).

DISCUSSION

Under the recording conditions used in this study, we have previously identified two types of Ca^{2+}-dependent currents—a Ca^{2+}-activated chloride current ($I_{Cl(Ca)}$) and a Ca^{2+}-activated nonselective cation current (I_{CAN}) and have shown that both can be activated by release of Ca^{2+} from intracellular stores (Currie and Scott, 1992). In this study we used these currents as a physiological index of raised $[Ca^{2+}]_i$ to investigate the actions of two novel Ca^{2+} releasing agents, namely a sperm factor (SF) and cyclic ADP ribose (cADPR).

SF activates calcium-dependent currents in cultured DRG neurones and appears to do so by releasing calcium from intracellular stores as the responses are attenuated by increasing the EGTA concentration and persist when barium is substituted for calcium in the extracellular medium. Further evidence for this comes from the finding that activity with similar components to that induced by SF can be generated by increasing the free cytosolic calcium concentration of the DRG neurones.

The sometimes considerable delay before activity in cells loaded with SF is probably in large part due to the mode of application. Inclusion in the patch pipette solution will result in an initial bulk flow followed by diffusion into the cell, once the membrane patch at the tip of the pipette is ruptured. A delay due to the mechanism of action of SF may also contribute, but the short delay (<1 min) seen upon microinjection into eggs (Swann, 1990) suggests this may not be a major factor. However, if SF acts as a CICR enhancing protein, Ca^{2+} levels in the cell may still need to rise before substantial Ca^{2+} is released to activate Ca^{2+}-dependent currents. Differences in the buffering capacity of patch clamped DRG neurones supported by EGTA in the patch solution and injected eggs may also account for differences. However, differences in Ca^{2+} stores and mechanisms of action may be important. For example, the DRG neurones unlike mammalian eggs are sensitive to caffeine.

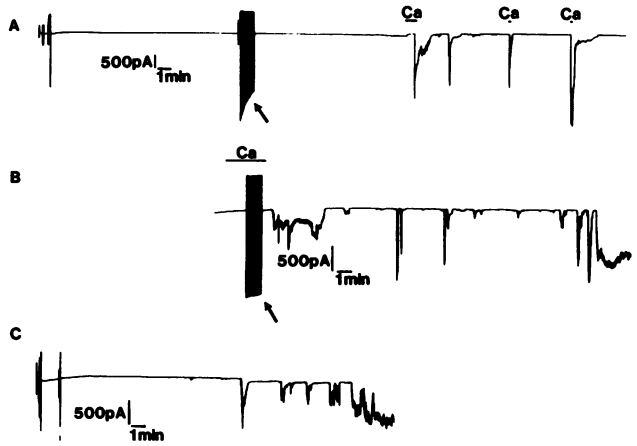

Figure 7. The effects of caffeine pretreatment on activity induced by cADPR or SF. All cells were voltage clamped at −90 mV and calcium channel currents were activated by 100-ms voltage step commands to 0 mV (←). The cells were preincubated with caffeine (1 mM) and maintained in extracellular medium containing Ba^{2+} rather than Ca^{2+} to deplete the caffeine sensitive Ca^{2+} store and prevent its refilling from the extracellular environment. (A) A cell with intracellular cADPR (1 μM) present. No inward current activity is generated over the first 25 min, even after a series of 20 depolarizing voltage steps activated at 0.33 Hz causing Ba^{2+} influx through voltage-gated Ca^{2+} channels. On perfusion of recording medium containing 2 mM Ca^{2+} (indicated by the ■) inward current activity was generated. (B) A second cell with cADPR present. Note that the record shown starts 22 min into the cell. Up to this point there had been no inward current activity, and again a series of Ba^{2+} currents had no effect. Ca^{2+} was perfused onto the cell (■), and during this perfusion a series of 20 depolarizing steps to 0 mV at 0.33 Hz was activated to load the cell with Ca^{2+}. After a short delay inward current activity was generated. (C) A cell with intracellular sperm factor present. Despite the pretreatment SF still induced inward current activity in this cell, which was maintained in extracellular Ba^{2+} for the whole experiment.

The variable nature of the currents activated by SF could arise due to many factors including variability in the SF/Ca^{2+} store interaction, the state of the Ca^{2+} store before SF application (i.e., depleted, full etc.), variability between cells with respect to other components of the Ca^{2+} homeostatic machinery of the cell, different topographic relationships between the stores and Ca^{2+}-activated channels, differences in the actual Ca^{2+}-activated channels between cells, and variability between batches of SF. In addition to these factors we feel that access problems may play a role not only in the delay but also possibly in determining the type of response to SF. This notion is supported by the finding that cells clamped with patch pipettes of higher series resistances (>7 MΩ) were less likely to show activity triggered by SF or cADPR. Attenuation of activity by heat treatment mirrors findings in eggs (Swann, 1990) and is also a further control against any possibility of Ca^{2+} contamination of the SF medium.

We have also investigated the ionic nature of the SF induced activity. By using CsAcetate based patch solution we can remove any Cl^- component of the SF response and show that I_{CAN} is activated by SF by estimating E_{rev} and by attenuation of the current by perfusion of T/S recording medium. It is likely that with CsCl based patch solution $I_{Cl(Ca)}$ will also contribute to the SF responses, but we have not been able as yet to

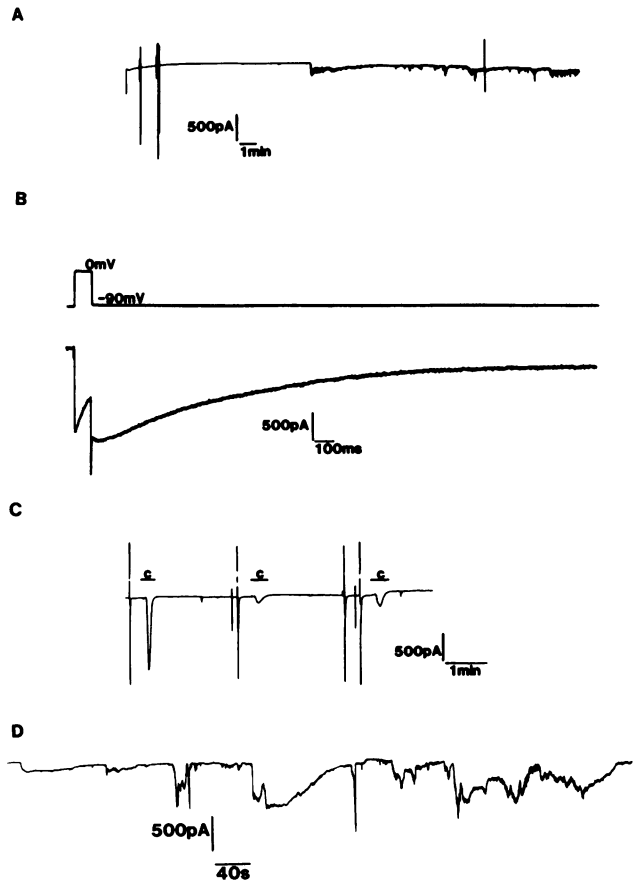

Figure 8. Ruthenium red (RuR) actions on SF, cADPR and caffeine induced inward current activity and voltage-gated Ca^{2+} currents and associated Ca^{2+}-activated tail currents. All cells were voltage clamped at −90 mV. (A) A cell loaded with SF and 100 μM RuR. After a typical delay SF induced activity started but is composed of only small amplitude noisy events suggesting attenuation by RuR. (B) A voltage-gated Ca^{2+} current and associated inward Ca^{2+}-activated Cl^- tail current recorded from a cell with 100 μM intracellular RuR in CsCl based patch solution. The top of the two traces shows the 100-ms duration voltage step command from −90 to 0 mV used to activate the Ca^{2+} current. The current shown was activated 10 min after entering the whole-cell configuration of the patch clamp technique, allowing ample time for RuR to diffuse into the cell. The trace shows that neither the Ca^{2+} current or the tail current is blocked by RuR. (C) An extract of a trace from a cell loaded with CsCl based patch solution containing 100 μM RuR. Caffeine (10 mM) perfusion, indicated by the horizontal bars labeled c, activates Ca^{2+}-dependent chloride currents due to release of Ca^{2+} from intracellular stores. This action is not blocked by RuR. Note that the 2nd and 3rd responses to caffeine are always smaller than the 1st response even in control cells. (D) An extract from a trace of a cell loaded with CsCl based patch solution containing 1 μM cADPR + 100 μM RuR. The RuR did not block the cADPR induced activity.

identify which components of the responses are due to which current. It would appear though that I_{CAN} may predominate, at least during noisy bursts of activity, because preliminary studies showed that the chloride channel blocker 5-nitro 2-(3-phenylpropylamino) benzoic acid (NPPB) (Wangemann *et al.*, 1986; Currie and Scott, 1992) produced little or no attenuation of this activity. It may be the case that $I_{Cl(Ca)}$ is associated with the more transient initial responses but this remains to be determined. The potentiation/triggering of SF induced activity by loading the cells with Ca^{2+} by activating a series of voltage-gated Ca^{2+} currents supports the notion that it may act by sensitizing the CICR mechanism. However, it may also be that the Ca^{2+} load simply acts to refill the Ca^{2+} pool on which SF is acting.

Cyclic ADP ribose has also been reported to act by a CICR mechanism (Galione *et al.*, 1991; Galione, 1992) and has been reported to release Ca^{2+} from microsomes from a pituitary cell line (Koshiyama *et al.*, 1991). In this study we have found that like SF, cADPR also activated Ca^{2+}-dependent inward current activity, in some cases with concentrations as low as 10 nM. Again as with SF, the activity can be triggered or potentiated by a series of Ca^{2+} currents. Given the proposed modulation by both these agents of CICR, we attempted to identify which stores and by which mechanisms the agents may be acting in neurones.

The caffeine sensitive Ca^{2+} pool is commonly accepted as being a probable site of action for CICR in neurones (Kuba, 1980; Lipscombe *et al.*, 1988). So far two types of intracellular Ca^{2+}-release channels have been reported—the IP_3R and the RyR (Suppattapone *et al.*, 1988; McPherson *et al.*, 1991; Ferris and Snyder, 1992), the latter of which is sensitive to Ca^{2+} itself and to caffeine (Bezprozvanny *et al.*, 1991; McPherson *et al.*, 1991). By pretreating the cells with caffeine to dump this Ca^{2+} pool and by maintaining the cells in extracellular Ba^{2+} to prevent its refilling, we investigated if SF and cADPR were acting on this Ca^{2+} pool. It was found that cADPR does seem to be acting on the caffeine sensitive Ca^{2+} store since activity was abolished by this treatment but responses could subsequently be initiated by replacing extracellular Ca^{2+}. It is suggested that replacing the extracellular Ca^{2+} allows refilling of the depleted store and subsequent release by the already present cADPR. Interestingly though, the SF response was not abolished in this manner by the same pretreatment. Although difficult to quantify, the caffeine treatment may have attenuated the SF response to some degree. This would not be surprising because an agent which may not act directly on the caffeine sensitive pool could still be influenced by its depletion due to 1) mobilization of Ca^{2+} between pools or 2) interaction between different pools in generating repetitive activity, such as has been suggested in models of intracellular Ca^{2+} oscillations (Berridge, 1991; Dupont *et al.*, 1991). Nevertheless, it appears that SF and cADPR do not act on the same mechanism or site within DRG neurones. Interestingly, mammalian eggs, in which SF activity was first identified, are not sensitive to caffeine (Swann, 1990; Miyazaki, 1991) or cADPR whereas sea urchin eggs, in which cADPR was first identified, are sensitive to caffeine (Galione *et al.*, 1991). In this respect it is also of interest to note that a caffeine insensitive CICR mechanism has been reported in cultured *Xenopus* spinal neurones (Holliday *et al.*, 1991).

Given the failure of caffeine to block the SF response, we decided to investigate the actions of RuR that has been reported to block CICR in some systems e.g., rat lacrimal gland acinar cells (Marty and Tan, 1989). Somewhat surprisingly we found that 100 μM RuR or 1 mM RuR failed to block the caffeine responses seen in these cells. These responses are due to caffeine releasing intracellular Ca^{2+}, which then activates the Ca^{2+}-dependent chloride channels (Currie and Scott, 1992). Although unexpected, because caffeine is reported to sensitize CICR and RuR to block CICR, a similar lack of block of caffeine mediated increases in $[Ca^{2+}]_i$ has been previously reported in neurones (Marrion and Adams, 1992). This finding also shows that RuR does not block the plasma membrane Ca^{2+}-activated channels, and this is further demonstrated by the observation that the calcium current and associated calcium activated tail currents in these cells were not blocked by RuR presence. Additionally, RuR (100 μM) does not affect cADPR induced activity, indicating again a close similarity between caffeine and cADPR induced Ca^{2+} release. In contrast we found that RuR does attenuate SF induced activity within these cells.

These findings suggest that there may exist two distinct (but quite possibly interacting) Ca^{2+} release mechanisms, one modulated by cADPR and caffeine, and one by SF. This certainly requires further study and the consideration of other mechanisms such as IP_3 mediated Ca^{2+} release or a possible involvement of the IP_3R, which may act as a CICR site under certain conditions (Lechleiter and Clapham, 1992). With this in mind, we investigated the action of 10-100 μM IP_3 applied via the patch pipette. The very poor responsiveness of these cells to IP_3 was slightly surprising given reports of IP_3-sensitive pools in DRG neurones (Thayer *et al.*, 1988). The unlikely possibility that this was due to rapid hydrolysis of the IP_3 is ruled out by the use of IPS_3. The IP_3 used was active as it was found to produce intracellular Ca^{2+} oscillations in mouse eggs as previously reported (Swann, 1992). It may therefore be that these particular neurones are relatively insensitive to IP_3, and there have been some other studies in which poor responsiveness to IP_3 has been reported (Morris *et al.*, 1987; Penner *et al.*, 1989). It may also be the case that the IP_3 stimulated rise in Ca^{2+} in the majority of these cells was too small to be reliably detected in this study. Indeed Thayer *et al.*, (1988) reported only a modest rise in $[Ca^{2+}]_i$ of 108 $\pm$ 37 nM in response to bradykinin

application to activate the IP_3 pathway. A modest rise in $[Ca^{2+}]_i$ and the possibility that the Ca^{2+} stores may be topographically remote from the Ca^{2+}-activated channels used to monitor $[Ca^{2+}]_i$ in this study could also explain the low proportion of cells responding to IP_3. Nevertheless, the data do suggest that the idea that SF is generating IP_3 or sensitizing the IP_3R to endogenous low levels of IP_3 is unlikely. A direct activation of G-proteins by SF also seems unlikely since intracellular application of GTP or its relatively nonhydrolysable analogues fails to elicit such inward current activity in DRG neurones (Scott *et al.*, 1991). Similarly we believe it unlikely that SF is in some way causing Ca^{2+} influx from the extracellular environment because 1) extracellular application has no effect, 2) activity persists when extracellular Ca^{2+} is replaced by Ba^{2+}, and 3) the potentiation of activity by a series of Ca^{2+} currents supports a role for intracellular stores/CICR. It is interesting to note at this point that there is now some evidence pointing to the existence of subtypes of the RyR (Lai *et al.*, 1988; Otsu *et al.*, 1990; Shoshan-Barmatz *et al.*, 1991), which may vary slightly in their CICR mechanism and possible modulatory sites. Similarly there is also evidence for subtypes of the IP_3R (Nakagawa *et al.*, 1991). Because both the RyR and IP_3R can act as CICR channels, (Bezprozvanny *et al.*, 1991; Lechleiter and Clapham, 1992) it is possible that SF is releasing Ca^{2+} by an interaction with one or more subtypes of either category of Ca^{2+} release channel.

In conclusion it seems that both SF and cADPR activate Ca^{2+}-dependent currents in cultured DRG neurones by releasing intracellular Ca^{2+} stores possibly by modulation of CICR. The parallels between caffeine and cADPR induced activity are quite striking and it seems that they may act on the same or a closely related site. In contrast it would seem that SF may act on another or perhaps multiple sites of action. The involvement of CICR in an increasing number of cellular processes and possible pathophysiological processes (see INTRODUCTION) makes the potential modulation of this phenomenon all the more exciting for our understanding of the physiological control and release of intracellular Ca^{2+} levels.

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REFERENCES

Berridge, M.J. (1991). Cytoplasmic calcium oscillations: a two pool model. Cell Calcium *12*, 63–72.

Berridge, M.J., and Galione, A. (1988). Cytosolic calcium oscillators. FASEB J. *2*, 3074–3082.

Berridge, M.J., and Irvine, R.F. (1989). Inositol phosphates and cell signalling. Nature *341*, 197–205.

Bezprozvanny, I., Watras, J. and Ehrlich, B.E. (1991). Bell-shaped calcium-response curves of $Ins(1,4,5)P_3$ and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature *351*, 751–754.

Blaustein, P. (1988). Calcium transport and buffering in neurons. TINS *11*, 438–443.

Brorson, J.R., Bleakman, D., Gibbons, S.J. and Miller, R.J. (1991). The properties of intracellular calcium stores in cultured rat cerebellar neurons. J. Neurosci. *11*, 4024–4043.

Currie, K.P.M. and Scott, R.H. (1992). Calcium-activated currents in cultured neurones from rat dorsal root ganglia. Br. J. Pharmacol. *106*, 593–602.

Cuthbertson, K.S.R. and Cobbold, P.H. (1985). Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. Nature *316*, 541–542.

Dale, B. (1988). Primary and secondary messengers in the activation of ascidian eggs. Exp. Cell Res. *207*, 205–211.

Dale, B., De Felice, L.J., and Ehrenstein, G. (1985). Injection of a soluble sperm extract into sea urchin eggs triggers the cortical reaction. Experimentia *41*, 1068–1070.

Dargie, P.J., Agre, M.C., and Lee, H.C. (1990). Comparison of Ca^{2+} mobilizing activities of cyclic ADP-ribose and inositol trisphosphate. Cell Regul. *1*, 279–290.

Dupont, G., Berridge, M.J., and Goldbetter, A. (1991). Signal-induced Ca^{2+} oscillations: properties of a model based on Ca^{2+}-induced Ca^{2+} release. Cell Calcium *12*, 73–85.

Ferris, C.D. and Snyder, S.H. (1992). Inositol 1,4,5 trisphosphate-activated calcium channels. Annu. Rev. Physiol. *54*, 469–488.

Friel, D.D., and Tsien, R.W. (1992). A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurones modulates effects of Ca^{2+} entry on $[Ca^{2+}]_i$. J. Physiol. *450*, 217–246.

Galione, A. (1992). Ca^{2+}-induced Ca^{2+} release and its modulation by cyclic ADP-ribose. TIPS *13*, 304–306.

Galione, A., Lee, H.C. and Busa, W.B. (1991). Ca^{2+}-induced Ca^{2+} release in sea urchin egg homogenates and its modulation by cyclic ADP-ribose. Science *253*, 1143–1146.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane. Pflügers Arch. *391*, 85–100.

Hellmich, M.R., and Strumwasser, F. (1991). Purification and characterization of a molluscan egg-specific NADase, a second messenger enzyme. Cell Reg. *2*, 193–202.

Holliday, J., Adams, R.J., Sejnowski, T.J., and Spitzer, N.Z. (1991). Calcium induced release of calcium regulates differentiation of cultured spinal neurones. Neuron *7*, 787–796.

Jaffe, L.F. (1983). Sources of calcium in egg activation; a review and hypothesis. Dev. Biol. *99*, 265–276.

Kennedy, M.B. (1989). Regulation of neuronal function by calcium. TINS, *12*, 417–420.

Koshiyama, H., Lee, H.C., and Tashjian, A.H. (1991). Novel mechanism of intracellular calcium release in pituitary cells. J. Biol. Chem. *266*, 16985–16988.

Kuba, K. (1980). Release of calcium ions linked to the activation of potassium conductance in a caffeine treated sympathetic neurone. J. Physiol. *298*, 251–269.

Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q-Y. and Meissner, G. (1988). Purification and reconstitution of the calcium release channel from skeletal muscle. Nature *331*, 315–319.

Lechleiter, J.D., and Clapham, D.E. (1992). Molecular mechanisms of intracellular calcium excitability in X. *laevis* oocytes. Cell *69*, 283–294.

Lee, H.C. (1991). Specific binding site of cyclic ADP-ribose to calcium storing microsomes from sea urchin eggs. J. Biol. Chem. *266*, 2276–2281.

Lee, H.C., and Aarhus, R. (1991). ADP-ribosyl cyclase: an enzyme that cyclizes NAD^+ into a calcium-mobilizing metabolite. Cell Regul. *2*, 203–209.

Lee, H.C., Walseth, T.F., Bratt, G.T., Hayes, R.N., and Clapper, D.L. (1989). Structural determination of a cyclic metabolite of NAD^+ with intracellular Ca^{2+} mobilizing activity. J. Biol. Chem. *264*, 1608–1615.

Lipscombe, D., Madison, D.V., Poenie, M., Reuter, H., Tsien, R.W., and Tsien, R.Y. (1988). Imaging of cytosolic Ca^{2+} transients arising from Ca^{2+} stores and Ca^{2+} channels in sympathetic neurons. Neuron *1*, 355–365.

Marrion, N.V., and Adams, P.R. (1992). Release of intracellular calcium and modulation of membrane currents by caffeine in Bull-frog sympathetic neurones. J. Physiol. *445*, 515–535.

Marty, A., and Tan, Y.P. (1989). The initiation of calcium release following muscarinic stimulation in rat lacrimal glands. J. Physiol. *419*, 665–687.

McBurney, R.N., and Neering, I.R. (1987). Neuronal calcium homeostasis. TINS *10*, 164–169.

McPherson, P.S., Kim, Y-K., Valdivia, H., Knudson, C.M., Takekura, H., Franzini-Armstrong, C., Coronado, R., and Campbell, K.P. (1991). The brain ryanodine receptor: a caffeine-sensitive calcium release channel. Neuron *7*, 17–25.

Miller, R.J. (1991). The control of neuronal Ca^{2+} homeostasis. Prog. Neurobiol. *37*, 255–285.

Miyazaki, S. (1988). Fertilization potential and calcium transients in mammalian eggs. Dev. Growth & Differ. *30*, 603–610.

Miyazaki, S. (1991). Repetitive calcium transients in hamster oocytes. Cell Calcium *12*, 205–216.

Morris, A.P., Gallacher, D.V., Irvine, R.F., and Peterson, O.H. (1987). Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. Nature *330*, 653–655.

Nakagawa, T., Okano, H., Furuichi, T., Aruga, J., and Mikoshiba, K. (1991). The subtypes of the mouse inositol 1,4,5-trisphosphate receptor are expressed in a tissue-specific and developmentally specific manner. Proc. Natl. Acad. Sci. USA *88*, 6244–6248.

Otsu, K., Willard, H.F., Khanna, V.K., Zorzatto, F., and MacLennan, D.H. (1990). Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J. Biol. Chem. *265*, 13472–13483.

Penner, R., Neher, E., Takeshima, H., Nishimura, S. and Numa, S. (1989). Functional expression of the calcium release channel from skeletal muscle ryanodine receptor cDNA. FEBS Lett. *259*, 217–221.

Rusinko, N., and Lee, H.C. (1989). Widespread occurrence in animal tissues of an enzyme catalyzing the conversion of NAD^+ into a cyclic metabolite with intracellular Ca^{2+} mobilizing activity. J. Biol. Chem. *264*, 11725–11731.

Sah, P., and McLachlan, E.M. (1991). Ca^{2+}-activated K^+ currents underlying the after hyperpolarization in Guinea Pig vagal neurons: a role for Ca^{2+}-activated Ca^{2+} release. Neuron. *7*, 257–264.

Scott, R.H., Pearson, H.A., and Dolphin, A.C. (1991). Aspects of vertebrate neuronal voltage-activated calcium currents and their regulation. Prog. Neurobiol. *36*, 485–520.

Shoshan-Barmatz, V., Pressley, T.A., Higham, S., and Kraus-Friedmann, N. (1991). Characterisation of high affinity ryanodine binding sites of ray liver endoplasmic reticulum. Biochem. J. *276*, 41–46.

Stice, S.L., and Robl, J.M. (1990). Activation of mammalian oocytes by a factor obtained from rabbit sperm. Mol. Reprod. Dev. *25*, 272–280.

Supattapone, S., Worley, P.F., Baraban, J.M., and Snyder, S.H. (1988). Solubilization, purification and characterisation of an inositol trisphosphate receptor. J. Biol. Chem. *263*, 1530–1534.

Swann, K. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development *110*, 1295–1302.

Swann, K. (1992). Different triggers for calcium oscillations in mouse eggs involve a ryanodine sensitive calcium store. Biochem. J. *287*, 79–84.

Swann, K., and Whitaker, M.J. (1990). Second messengers at fertilization in sea urchin eggs. J. Reprod. Fertil. Suppl. *42*, 141–153.

Thayer, S.A., Perney, T.M., and Miller, R.J. (1988). Regulation of calcium homeostasis in sensory neurons by bradykinin. J. Neurosci. *8*, 4089–4097.

Tsien, R.W., and Tsien, R.Y. (1990). Calcium channels, stores, and oscillations. Annu. Rev. Cell Biol. *6*, 715–760.

Walseth, T.F., Aarhus, R., Zeleznikar, J., and Lee, H.C. (1991). Determination of endogenous levels of cyclic ADP-ribose in rat tissues. Biochim. Biophys. Acta *1094*, 113–122.

Walton, P.D., Airey, J.A., Sutko, J.L., Beck, C.F., Mignery, G.A., Südhof, T.C., Deerinck, T.J., and Ellisman, M.H. (1991). Ryanodine and inositol trisphosphate receptors coexist in avian cerebellar purkinje neurones. J. Cell Biol. *113*, 1145–1157.

Wangemann, P., Wither, M., Distefano, A., Englert, H.C., Lang, H.J., Schlatter, E., and Greger, R. (1986). Cl^--channel blockers in the thick ascending limb of the loop of Henle. Structure activity relationship? Pflügers Arch. *407*, S128–S141.

Whitaker, M.J., and Steinhardt, R.A. (1985). Ionic signalling in sea urchin egg fertilization. In: Biology of Fertilization, ed. C. Metz, and A. Monray, New York: Academic Press, 167–221.

Whittingham, D.G. (1980). Parthenogenesis in mammals. Oxford Reviews of Reproductive Biology *2*, 205–231.