A caffeine- and ryanodine-sensitive intracellular Ca^{2+} store can act as a Ca^{2+} source and a Ca^{2+} sink in PC12 cells

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We have investigated the modulation of stimulus-induced changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by a caffeine- and ryanodine-sensitive Ca^{2+} store in PC12 cells. In populations of fura-2-loaded cells, caffeine caused a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ that was saturable, reversible and inhibited in a use-dependent fashion by ryanodine. Maximal Ca^{2+} release occurred with 40 mM caffeine, with an EC_{50} of 13 mM caffeine and a Hill coefficient (h) of 2.7, indicating that the release mechanism was co-operative. Pretreatment of intact cell populations with increasing concentrations of caffeine in nominally Ca^{2+} -free medium inhibited the subsequent Ca^{2+} response to a maximal concentration of ATP, in a dose-dependent manner. In permeabilized cells, a maximal concentration (40 μM) of InsP_3 still released Ca^{2+} in the presence of a supramaximal concentration (50 mM) of caffeine, whereas caffeine was unable to release Ca^{2+} after the InsP_3 -sensitive store had been completely emptied. These data suggest that PC12 cells contain a uniquely InsP_3 -sensitive Ca^{2+} store, and a store that is sensitive to both

InsP_3 and caffeine. Depletion of the caffeine-sensitive Ca^{2+} store by caffeine and ryanodine pretreatment in intact cells attenuated the Ca^{2+} response to ATP, but not to 55 mM K^+ , suggesting that the caffeine-sensitive Ca^{2+} store acts as a Ca^{2+} source after ATP stimulation, but not after depolarization with 55 mM K^+ . Pretreatment of intact cells with ATP and ryanodine resulted in a use-dependent block of both caffeine- and ATP-mediated Ca^{2+} release, confirming that ATP stimulation of PC12 cells brings about activation of ryanodine receptors. The rate of recovery, but not the magnitude or rate of onset, of the depolarization-induced $[\text{Ca}^{2+}]_i$ transient was modulated by the state of filling of the caffeine-sensitive Ca^{2+} store such that recovery was prolonged if the store was either full, or empty and unable to refill. We conclude that the caffeine- and ryanodine-sensitive Ca^{2+} store can act as a Ca^{2+} source and a Ca^{2+} sink in PC12 cells, and that its role may in part be governed by the nature of the stimulating agent.

INTRODUCTION

A change in the concentration of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) is important in the control of a diverse range of cellular processes. The main pathways through which external stimuli increase $[\text{Ca}^{2+}]_i$ include promotion of Ca^{2+} entry from the outside medium and release of Ca^{2+} from internal stores (Berridge, 1993a). In neuronal cells, inositol 1,4,5-trisphosphate (InsP_3)- (Higashida and Brown, 1986; Thayer et al., 1988b) and caffeine-sensitive (Kuba, 1980; Lipscombe et al., 1988; Thayer et al., 1988a; Marrion and Adams, 1992; Friel and Tsien, 1992a,b) Ca^{2+} stores have been distinguished. The importance of the caffeine-sensitive store is beginning to emerge, in part because caffeine-induced release of Ca^{2+} implicates the presence of Ca^{2+} -induced Ca^{2+} release (CICR), a Ca^{2+} -release mechanism involving Ca^{2+} -dependent gating of the ryanodine receptor (RyR) of the sarcoplasmic reticulum (Fabiato, 1983; Endo, 1985). In neuronal cells, interest in CICR has grown with the identification of neuronal forms of the RyR (Ellisman et al., 1990; McPherson et al., 1991), suggesting that CICR can cause regenerative increases in $[\text{Ca}^{2+}]_i$ (Kuba, 1980; Lipscombe et al., 1988; Henzi and MacDermott, 1992; Berridge, 1993a).

In spite of its intriguing properties, the role of the caffeine-sensitive store in non-muscle-cell Ca^{2+} homeostasis is not clear. For example, there is evidence suggesting the involvement of CICR in depolarization-induced $[\text{Ca}^{2+}]_i$ transients in some studies using bullfrog sympathetic neurons (Lipscombe et al., 1988; Friel and Tsien, 1992a,b; Hua et al., 1993), but not others

(Thayer et al., 1988a; Nohmi et al., 1992). In adrenal chromaffin cells, CICR was not implicated in the $[\text{Ca}^{2+}]_i$ transient induced by a single action potential (Neher and Augustine, 1992) but the state of filling of the caffeine-sensitive store clearly influenced the shape of the $[\text{Ca}^{2+}]_i$ transient induced by chemical depolarization (Cheek et al., 1990) and InsP_3 -mobilizing stimuli (Stauderman et al., 1991; Stauderman and Murawsky, 1991).

In neuronally derived rat pheochromocytoma (PC12) cells, caffeine-sensitive stores co-exist with InsP_3 -sensitive stores, although there is debate as to whether the InsP_3 receptor (InsP_3R) and RyR are uniquely present on distinct organelles (Reber et al., 1993) or co-exist on the same organelle (Zacchetti et al., 1991). In order to gain further insight into the role played by caffeine-sensitive stores in modulating intracellular Ca^{2+} signals, we have examined the ability of a variety of extracellular stimuli to bring about activation of the PC12-cell RyR, using fluorimetric techniques to monitor Ca^{2+} signals from both intact and permeabilized cell populations.

The results indicate that PC12 cells contain a Ca^{2+} store bearing only InsP_3R , and a caffeine-sensitive Ca^{2+} store bearing both InsP_3R and RyR. Cell stimulation with extracellular ATP results in Ca^{2+} release from the caffeine-sensitive store mediated by RyR activation. In contrast, depolarization with high K^+ does not activate RyR, though the state of filling of the caffeine-sensitive store was found to influence the rate of recovery of the K^+ -induced $[\text{Ca}^{2+}]_i$ transient. These results indicate that the caffeine-sensitive store can act both as a Ca^{2+} source and as a Ca^{2+} sink in PC12 cells, and that its role may, at least in part, be governed by the nature of the stimulating agent.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; Ca^{2+}_o , extracellular Ca^{2+} ; CICR, Ca^{2+} -induced Ca^{2+} release; RyR, ryanodine receptor; InsP_3R , InsP_3 receptor; KR medium, Krebs–Ringer medium.

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MATERIALS AND METHODS

Materials

Fura-2 AM (acetoxymethyl ester) and Fluo-3 (free acid) were from Molecular Probes (Eugene, OR, U.S.A.), ryanodine was from ICN Biomedicals (High Wycombe, Bucks., U.K.), and ATP, creatine kinase and phosphocreatine were from Boehringer (Lewes, Sussex, U.K.). Tissue-culture media were from Gibco (Paisley, Scotland, U.K.) and, unless otherwise stated, all other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Methods

Cell culture

PC12 cells were obtained from the European Cell Culture Laboratories (PHLS Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K.). Cells were grown on 10 cm plastic dishes coated with either collagen (Vitrogen; Imperial Laboratories, Andover, Hants., U.K.) or 1 mg/ml poly-L-ornithine, in 85% RPMI growth medium supplemented with L-glutamine, 10% horse serum, 5% fetal-calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone, and buffered with 2.2 g/l NaHCO₃. Cells were incubated in a humidified atmosphere (air/CO₂ 19:1) at 37 °C and medium was changed 3 times per week.

Fura-2 measurements of intact cell populations

Cells were harvested from tissue-culture dishes, when approaching confluence, by dissociation in trypsin/EDTA buffer. Cells were centrifuged (1000 g, 5 min) and resuspended at a density of 5×10^6 cells/ml in loading buffer containing RPMI medium + L-glutamine and 5% fetal-calf serum supplemented with 200 µM sulfinpyrazone and 3 µM fura-2 AM. Cells were loaded with fura-2 AM for 30 min at 37 °C with constant stirring, and then washed twice and resuspended at a density of 10×10^6 cells/ml in Krebs–Ringer medium (KR medium) containing 145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1.2 mM NaH₂PO₄, 10 mM glucose, 3 mM CaCl₂ and 20 mM Hepes (pH 7.4), again supplemented with 200 µM sulfinpyrazone.

Cell suspensions (300 µl) were transferred to a stirred thermostatically controlled cuvette at 37 °C. Fluorescence was continuously monitored with a Perkin–Elmer LS-5 luminescence spectrophotometer (alternate excitation wavelengths 340 nm and 380 nm), and the information was stored by using Perkin–Elmer Fura-2 analysis software on an IBM personal computer. Traces were calibrated in accordance with the following equations:

$$[\text{Ca}^{2+}]_i = k_d \times \frac{(F - F_{\min.})}{(F_{\max.} - F)} \quad (\text{A})$$

and

$$F_{\min.} = \frac{1}{3}(F_{\max.} - F_{\text{Mn}}) + F_{\text{Mn}} \quad (\text{B})$$

where F is fura-2 fluorescence from cells, $F_{\max.}$ is maximum fura-2 fluorescence (determined by addition of 50 µM digitonin to permeabilize the cells), $F_{\min.}$ is minimum fluorescence, F_{Mn} is fluorescence after quenching of fura-2 (determined by addition of 4 mM MnCl₂ to the cells after obtaining $F_{\max.}$), and k_d is equilibrium dissociation constant of fura-2–Ca²⁺. This was assumed to be 224 nM at 37 °C (Grynkiewicz et al., 1985).

Nominally Ca²⁺-free media [Ca²⁺_o (extracellular Ca²⁺)-free] was obtained by adding 4 mM EGTA to cells in KR medium. This was calculated to give a free [Ca²⁺] < 2 µM (Maxchelator; Dr. C. Patton, Stanford University). Concentration–response

data were fitted to a logistic equation by using GraphPAD Inplot (GraphPAD software, San Diego, CA, U.S.A.).

In all experiments, basal [Ca²⁺]_i was within the range 26–95 nM, as previously observed (Barry and Cheek, 1994).

Fluo-3 measurements of permeabilized cell populations

Cells were harvested as for the intact-cell population studies, washed three times and finally resuspended at a density of 5×10^6 cells/ml in the following medium: 120 mM KCl, 20 mM Hepes (pH 7.2), 1 mM MgCl₂, 1 mM ATP, 10 mM NaN₃ and 5 µM fluo-3 (free acid). Cell suspensions (300 µl) were transferred to a stirred thermostatically controlled cuvette at 37 °C, and Ca²⁺ uptake was started by adding 8 units of creatine kinase, 25 mM phosphocreatine, 5.8 mM pyrophosphate [to increase the Ca²⁺-accumulating capacity of non-mitochondrial pools (Palade, 1987)] and 50 µM digitonin to permeabilize the cells (Cheek et al., 1991). Fluorescence was continuously monitored with a Perkin–Elmer LS-5 spectrophotometer (excitation 503 nm, emission 530 nm) and results were collected with an accompanying IBM personal computer.

To determine any effect on the extent of InsP₃-mediated Ca²⁺ release by 50 mM caffeine (Figure 3, inset), traces were calibrated by using eqn. A, above. $F_{\max.}$ was obtained by addition of 1 mM CaCl₂ to the cuvette and $F_{\min.}$ by subsequently adding 10 mM K-EGTA (pH 7.2). A k_d of 864 nM was used (Merritt et al., 1990). The remaining traces are uncalibrated because the calculated [Ca²⁺] values are arbitrary; they vary depending on the method of permeabilization used [compare Cheek et al. (1991), Stauderman et al. (1991) and Robinson and Burgoyne (1991), where three different permeabilization protocols were used with the same cell type]. This may have reflected differences in endoplasmic-reticulum luminal continuity between these preparations (Renard-Rooney et al., 1993). As a consequence, reliable quantitative data are obtainable only from intact cells.

Determination of inositol phosphates

PC12 cells were incubated for 24 h in inositol-free RPMI supplemented with 5% dialysed mixed serum, 2.2 g/l NaHCO₃ and 1 µCi/ml myo-[³H]inositol (86 Ci/mmol; Amersham International). Cells were removed from their dishes by gentle agitation, centrifuged (1000 g, 5 min) and resuspended in KR medium at 37 °C. After 30 min, 30 mM LiCl was added to inhibit inositol monophosphatases, and 10 min later the stimulus was applied. After a further 10 min incubation period, the reaction was terminated by rapidly heating the cells to ~90 °C for 1 min. Cell debris was pelleted by centrifugation (2000 g, 10 min) and the resulting supernatants were applied to anion-exchange columns (AG1X8, formate form, 200–400 mesh; Bio-Rad). [³H]Inositol and [³H]glycerophosphoinositol were eluted with 8 ml of 60 mM ammonium formate/5 mM Na₂B₄O₇, InsP₁ was eluted with 8 ml of 0.2 M ammonium formate/0.1 M formic acid, and InsP₂ with 8 ml of 0.5 M ammonium formate/0.1 M formic acid. InsP₃ and InsP₄ were eluted together with 8 ml of 1.25 M ammonium formate/0.1 M formic acid. The activity in each fraction was expressed as a fraction of the total label incorporated into the cells.

Use of caffeine and ryanodine

We used caffeine and theophylline instead of ryanodine to activate the putative CICR channel in these cells because ryanodine binds very slowly to the inactive channel ($t_1 = 24.8$ min; Pessah et al., 1986). Caffeine and theophylline are poorly soluble in water, so we prepared stocks of 400–500 mM in

KR medium (containing no added Ca^{2+} or EGTA) by shaking a solution warmed to $\sim 60^\circ\text{C}$, and when the methylxanthine had dissolved the solution was maintained at 37°C .

The effect of ryanodine and caffeine in inhibiting the putative CICR channel was achieved by incubating fura-2 loaded cell populations with $10\ \mu\text{M}$ ryanodine (stock $100\ \text{mM}$ in methanol) for 10 min, and then adding a maximal caffeine concentration ($40\ \text{mM}$) for a further 5 min. Cells were then pelleted in a microfuge, resuspended in Ca^{2+} -containing KR medium without caffeine or ryanodine, and depolarized with $55\ \text{mM}$ K^+ to allow refilling of the caffeine-sensitive store. Cells were stimulated with either $40\ \text{mM}$ caffeine (Figures 4a and 5a) or $100\ \mu\text{M}$ ATP (Figures 4b and 5b) after the medium had been made nominally Ca^{2+} -free by addition of $4\ \text{mM}$ EGTA (see above). Depolarization with $55\ \text{mM}$ K^+ did not deplete the caffeine-sensitive store, and therefore did not alter the effects of ryanodine pretreatment (see the Results section, Figure 6). The PC12 cell clone used in this study absolutely required this depolarizing pulse for store refilling. Poorly refilling caffeine-sensitive Ca^{2+} stores have been observed in some PC12 cell clones (Reber and Reuter, 1991; Reber et al., 1993), but not others (Zacchetti et al., 1991).

RESULTS

Caffeine and theophylline mobilize Ca^{2+} from intracellular stores in PC12 cells

Caffeine acts at the RyR, where it appears to shift the Ca^{2+} -sensitivity of the channel to lower concentrations (Nagasaki and

Kasai, 1984; Endo, 1985), thereby increasing the probability of channel opening (Rousseau et al., 1988; Iino, 1989). The concentration-dependence of the caffeine-induced $[\text{Ca}^{2+}]_i$ rise in PC12 cells is shown in Figure 1(a). Populations of fura-2-loaded cells were challenged with caffeine over the range $5\text{--}50\ \text{mM}$ in the presence or absence of Ca^{2+}_o . In both cases, Ca^{2+} release was found to be saturable, with maximal release occurring at around $40\ \text{mM}$ caffeine. The EC_{50} values were similar under both conditions: $13\ \text{mM}$ caffeine in the presence and $17\ \text{mM}$ caffeine in the absence of Ca^{2+}_o . These data indicate that caffeine is a relatively low-affinity Ca^{2+} -mobilizing agonist in these cells. The Hill coefficients (h) for release were found to be 2.7 in the presence and 3.0 in the absence of Ca^{2+}_o , suggesting that Ca^{2+} release from the caffeine-sensitive stores is co-operative.

The amplitude of the $[\text{Ca}^{2+}]_i$ rise evoked by caffeine in the absence of Ca^{2+}_o was decreased compared with the response in the presence of Ca^{2+}_o (Figure 1a). The difference in amplitude was not due to depletion of the intracellular Ca^{2+} stores caused by the $30\text{--}60\ \text{s}$ incubation in EGTA-containing medium, as the stores still contained $74 \pm 7\%$ ($n = 3$) of their Ca^{2+} after a 3 min incubation in EGTA-containing medium, suggesting that Ca^{2+} leaks only very slowly from caffeine-sensitive Ca^{2+} stores.

In skeletal-muscle sarcoplasmic-reticulum vesicles, theophylline (1,3-dimethylxanthine) was slightly more potent than caffeine (1,3,7-trimethylxanthine) at eliciting Ca^{2+} efflux (Rousseau et al., 1988). The concentration-response of a PC12 cell population responding with a rise in $[\text{Ca}^{2+}]_i$ to theophylline in Ca^{2+} -free medium is presented in Figure 1(b). The EC_{50} for the theophylline-induced $[\text{Ca}^{2+}]_i$ rise was $12.5\ \text{mM}$, and maximal Ca^{2+} release occurred at about $40\ \text{mM}$, indicating that theophylline and caffeine had a similar potency under these conditions. When fura-2-loaded PC12 populations were challenged firstly with $40\ \text{mM}$ caffeine in Ca^{2+} -free medium, and then subsequently with $40\ \text{mM}$ theophylline, the theophylline-evoked rise in $[\text{Ca}^{2+}]_i$ was completely abolished (*, Figure 1b). Similarly, theophylline pretreatment abolished the response to $40\ \text{mM}$ caffeine in a concentration-dependent manner (results not shown). Therefore, akin to skeletal muscle (Rousseau et al., 1988), caffeine and theophylline activated the same intracellular Ca^{2+} -release channel in PC12 cells.

Do the caffeine- and InsP_3 -sensitive and Ca^{2+} stores overlap?

To investigate the interaction between InsP_3 - and caffeine-sensitive Ca^{2+} mobilization, experiments were performed in Ca^{2+} -free medium ($1\ \text{mM}$ excess EGTA) to eliminate any contribution from stimulus-induced Ca^{2+} influx. Fura-2-loaded PC12 cells were challenged sequentially with two stimuli: firstly caffeine in increasing concentrations, followed (at the end of the $[\text{Ca}^{2+}]_i$ transient) by a maximal concentration ($100\ \mu\text{M}$) of the InsP_3 -mobilizing agonist ATP (Figure 2). The larger the primary, the smaller was the secondary response (Figure 2a). This inverse relationship between the amount of Ca^{2+} mobilized by a sub-maximal caffeine concentration and the amount mobilized by a subsequent addition of a maximal ATP concentration is clearly shown in Figure 2(b). Caffeine has been shown to inhibit InsP_3 production in some cell types (Stauderman et al., 1991; Zacchetti et al., 1991; Toescu et al., 1992). In order to investigate whether the caffeine-induced attenuation of ATP-induced Ca^{2+} mobilization was due to an inhibitory effect on inositol phosphate accumulation, anion-exchange chromatography was used to quantify the extent of ATP-induced phosphoinositide hydrolysis in the presence or absence of caffeine. The effect of a 10 min incubation with $40\ \text{mM}$ caffeine alone, or in concert with $100\ \mu\text{M}$ ATP, on the accumulation of inositol phosphates in Ca^{2+} -free

Figure 1 Caffeine- and theophylline-stimulated $[\text{Ca}^{2+}]_i$ rise in populations of PC12 cells

(a) Populations of fura-2-loaded PC12 cells were stimulated with the caffeine concentrations shown, either in KR medium (●; $[\text{Ca}^{2+}] = 3\ \text{mM}$) or after $30\text{--}60\ \text{s}$ in EGTA-containing KR medium (■; $[\text{Ca}^{2+}] < 2\ \mu\text{M}$). (b) Populations of fura-2-loaded PC12 cells were stimulated with the theophylline concentrations shown after $30\text{--}60\ \text{s}$ in EGTA-containing KR medium (●; $[\text{Ca}^{2+}] < 2\ \mu\text{M}$). The asterisk (*) indicates peak $[\text{Ca}^{2+}]_i$ in response to $40\ \text{mM}$ theophylline after stimulation with $40\ \text{mM}$ caffeine. The data represent the peak $[\text{Ca}^{2+}]_i$ rise obtained after addition of the methylxanthine. The plots show means $\pm$ S.E.M., $n \geq 3$.

Figure 2 Concentration-dependent inhibition by caffeine of the ATP-induced rise in $[Ca^{2+}]_i$ in PC12 cells

Populations of fura-2-loaded PC12 cells were stimulated with increasing concentrations of caffeine, and then within 90 s with 100 μ M ATP, in EGTA-containing KR medium. (a) Traces show the $[Ca^{2+}]_i$ changes in three populations of cells, responding to 5, 10 or 40 mM caffeine, followed by 100 μ M ATP. (b) Plot of the complete experiment from which the traces in (a) were taken. Symbols: ●, peak $[Ca^{2+}]_i$ rise above the basal level, stimulated by addition of the caffeine concentration shown on the abscissa; ■, peak $[Ca^{2+}]_i$ stimulated by a subsequent application of 100 μ M ATP. The data are means $\pm$ S.E.M., $n = 3$.

Table 1 Total inositol phosphates accumulated after stimulation of PC12 cells with ATP or caffeine

Populations of PC12 cells were incubated in $[^3H]$ inositol for 24 h before stimulation with 100 μ M ATP, 40 mM caffeine, or ATP after a 10 min caffeine pretreatment. After 10 min the total amount of $[^3H]$ inositol phosphates ($[^3H]InsP_s$) accumulated were quantified by anion-exchange chromatography (see the Materials and methods section). The results show the 3H radioactivity of total inositol phosphates as a percentage of the cellular 3H radioactivity, as means $\pm$ S.E.M. ($n = 3$).

Stimulus	$[^3H]InsP_s$ (%)
Basal	0.020 ± 0.02
100 μ M ATP	0.126 ± 0.03
40 mM Caffeine	0.050 ± 0.03
100 μ M ATP + 40 mM caffeine	0.124 ± 0.03

medium is presented in Table 1: 40 mM caffeine generated little or no inositol phosphates, and left the ATP-induced response unchanged.

Figure 3 Caffeine and $InsP_3$ release Ca^{2+} from digitonin-permeabilized PC12 cells

Populations of PC12 cells were permeabilized with digitonin in the presence of an ATP-regenerating system and the fluorescent Ca^{2+} indicator, fluo-3. Traces (a) and (b) illustrate that depletion of the caffeine-sensitive Ca^{2+} store with a supra-maximal caffeine concentration (50 mM) does not inhibit a subsequent response to 40 μ M $InsP_3$ (a maximal concentration). Conversely, trace (c) illustrates that depletion of the $InsP_3$ -sensitive Ca^{2+} store abolishes a subsequent response to 40 mM caffeine (a maximal concentration). These data are typical of 5 independent experiments. The inset shows the concentration-response of $InsP_3$ -mediated Ca^{2+} release (a), and the lack of effect of 50 mM caffeine on this relationship (b). Data in the inset show peak rises in $[Ca^{2+}]_i$ (mean $\pm$ S.E.M., $n = 3$).

To investigate further the nature of the intracellular Ca^{2+} stores, agonist-induced events at the plasma membrane were bypassed, and direct access to the cell interior was achieved by permeabilizing PC12-cell populations with digitonin. The assay medium was supplemented with 5.8 mM sodium pyrophosphate to increase the Ca^{2+} -accumulating capacity of the non-mitochondrial intracellular Ca^{2+} stores that contained a specific anion transporter for pyrophosphate (Palade, 1987; Cheek et al., 1991). A maximal concentration of $InsP_3$ (40 μ M; Figure 3 inset) induced an increase in $[Ca^{2+}]_i$, even after prior depletion of the caffeine-sensitive Ca^{2+} store with a supra-maximal concentration of caffeine (50 mM) (Figures 3a and 3b). Conversely, depletion of the $InsP_3$ -sensitive store with a maximal concentration of $InsP_3$ abolished the $[Ca^{2+}]_i$ rise evoked by a maximal caffeine concentration (40 mM) (Figure 3c). A potential problem when using caffeine in these experiments is that this compound has been shown to inhibit non-specifically the Ca^{2+} release triggered by

sub-maximal concentrations of InsP_3 (Brown et al., 1992). However, preincubation of permeabilized PC12 cells with 50 mM caffeine had no effect on the amount of Ca^{2+} released over a range of InsP_3 concentrations (Figure 3 inset).

These experiments, using intact and permeabilized cells, suggested that PC12 cells contain a uniquely InsP_3 -sensitive Ca^{2+} store, and a Ca^{2+} store that is sensitive to both InsP_3 and caffeine.

Extracellular ATP releases Ca^{2+} from the caffeine-sensitive Ca^{2+} store

It was important to confirm that the caffeine-sensitive Ca^{2+} store could be released by InsP_3 -mobilizing stimuli such as ATP. Intact cells were pretreated with 10 μM ryanodine and 40 mM caffeine, to deplete the caffeine-sensitive Ca^{2+} store, and then challenged with 100 μM ATP, in Ca^{2+} -free medium. Confirmation that ryanodine and caffeine pretreatment depleted the caffeine-sensitive store in a use-dependent manner is shown in Figure 4(a). Pretreatment of a cell population with ryanodine (10 μM) together with caffeine (40 mM) virtually abolished the subsequent response to 40 mM caffeine (Figure 4a, iv). Ryanodine alone gave a slight decrease in the Ca^{2+} response to 40 mM caffeine (Figure 4a, ii), whereas caffeine pretreatment alone gave a $50.2 \pm 2.5\%$ ($n = 6$) decrease in the subsequent response (Figure 4a, iii). These data indicate that only a combination of ryanodine plus caffeine was maximally effective at inhibiting caffeine-sensitive Ca^{2+} release.

Pretreatment of cells with ryanodine and caffeine in this way inhibited the subsequent Ca^{2+} response induced by 100 μM ATP by $79.0 \pm 8.2\%$ ($n = 6$) (Figure 4b, iv), as compared with control cells pretreated with caffeine alone (Figure 4b, iii). These data suggested firstly that most ($\sim 80\%$) of the Ca^{2+} mobilized in response to ATP originates from a caffeine-sensitive Ca^{2+} store, and secondly that the remaining 20% or so of ATP-releasable Ca^{2+} is contained in a uniquely InsP_3 -sensitive store, which is in agreement with data obtained with intact and permeabilized cells (Figures 2 and 3). We have previously shown that the pyrimidine nucleotide UTP activates the same P_2 purinergic receptor as ATP in these cells (Barry and Cheek, 1994). Caffeine and ryanodine pretreatment of PC12 cells inhibited the subsequent Ca^{2+} response induced by 1 mM UTP by $66.0 \pm 8.0\%$ ($n = 2$), as compared with control cells pretreated with caffeine alone, suggesting that UTP also releases Ca^{2+} from the caffeine-sensitive store.

ATP releases Ca^{2+} from the caffeine-sensitive Ca^{2+} store by activating RyR

ATP-induced Ca^{2+} mobilization involves Ca^{2+} release from a uniquely InsP_3 -sensitive store, and Ca^{2+} release from a store that is sensitive to both caffeine and ryanodine (Figure 4). However, because the caffeine- and ryanodine-sensitive store is also InsP_3 -sensitive (Figures 2 and 3), it was important to confirm that ATP released Ca^{2+} from the caffeine-sensitive store by activating RyR. Cell populations were pretreated with 10 μM ryanodine as above (Figure 4), but this time in combination with 100 μM ATP rather than with 40 mM caffeine. It was considered that if ATP activates the RyR, then pretreatment of cells with ATP in the presence of ryanodine should induce a use-dependent block of caffeine-stimulated Ca^{2+} release (see Figure 4a, iv, ATP instead of caffeine). Ryanodine plus ATP pretreatment inhibited the subsequent Ca^{2+} response to 40 mM caffeine by $92.9 \pm 4.1\%$ ($n = 6$) (Figure 5a, iii), compared with control cells treated with ATP alone (Figure 5a, ii). This suggested that ATP stimulation of

Figure 4 Ryanodine inhibits the caffeine- and ATP-induced $[\text{Ca}^{2+}]_i$ rise in a use-dependent manner

The Figures show the Ca^{2+} responses of four populations fura-2-loaded PC12 cells to either (a) a maximal caffeine concentration (40 mM, arrows) or (b) a maximal ATP concentration (100 μM , arrows), after various pretreatments. (i) Control response of cells preincubated for 15 min in KR medium. (ii) Cells were pretreated for 15 min in KR medium containing 10 μM ryanodine, before centrifugation, refilling (see the Materials and methods section) and resuspension in control buffer. (iii) Cells were pretreated for 15 min in KR medium containing 40 mM caffeine, before centrifugation, refilling and resuspension in control buffer. (iv) Cells were incubated in KR medium containing 10 μM ryanodine for 10 min, after which 40 mM caffeine was added and the cells were incubated for a further 5 min. The cells were then centrifuged, refilled and resuspended in KR medium. In all experiments, stimuli were added 1 min after the medium had been made nominally Ca^{2+} -free by addition of EGTA (see the Materials and methods section). These data are typical of 6 independent experiments for both (a) and (b) (see the text).

Figure 5 ATP releases Ca^{2+} from the caffeine-sensitive store by activating RyR

The Figures show the Ca^{2+} responses of three populations of fura-2-loaded PC12 cells to either (a) a maximal caffeine concentration (40 mM, arrows) or (b) a maximal ATP concentration (100 μM , arrows), after various pretreatments. (i) Control response of cells preincubated for 15 min in KR medium. (ii) Cells were pretreated for 15 min in KR medium containing 100 μM ATP, before centrifugation, refilling (see the Materials and methods section) and resuspension in control buffer. (iii) Cells were incubated in KR medium containing 10 μM ryanodine for 10 min, after which 100 μM ATP was added and the cells were incubated for a further 5 min. The cells were then centrifuged, refilled and resuspended in KR medium. In all experiments, stimuli were added 1 min after the medium had been made nominally Ca^{2+} -free by addition of EGTA (see the Materials and methods section). These data are typical of 6 independent experiments for both (a) and (b) (see the text).

Figure 6 Effect of the filling state of the caffeine-sensitive Ca^{2+} store on intracellular Ca^{2+} homeostasis in PC12 cells

Populations of fura-2-loaded PC12 cells were pre-stimulated with either (a) 40 mM caffeine followed by 55 mM K^+ , to ensure that the caffeine-sensitive Ca^{2+} store was replete, or (b) 10 μ M ryanodine and 40 mM caffeine, to deplete and maintain empty the caffeine-sensitive Ca^{2+} store, before stimulation with 55 mM K^+ (arrows). (i) Control cells; (ii) pretreated cells. The rates of the depolarization-induced rise in $[Ca^{2+}]_i$ in pretreated cells are shown in (c). Both the magnitude and the rate of rise of the Ca^{2+} signal in Ca^{2+} -deplete (i) and Ca^{2+} -replete (ii) cells were indistinguishable. These data are typical of 3 independent experiments for each of (a), (b) and (c).

PC12 cells resulted in activation of RyR. Similarly, ryanodine plus ATP pretreatment decreased the subsequent Ca^{2+} response induced by 100 μ M ATP to $27.7 \pm 12.4\%$ ($n = 6$) (Figure 5b, iii) of that of control cells pretreated with ATP alone (Figure 5b, ii). This is consistent with the finding that $\sim 20\%$ of ATP-releasable Ca^{2+} is contained in a uniquely $InsP_3$ -sensitive store (Figure 4b).

Depolarization with 55 mM K^+ does not activate RyR

Interestingly, although stimulation of PC12 cells with ATP resulted in the activation of the RyR, depolarization with high (55 mM) K^+ appeared not to have the same effect. Pretreatment of cells with ryanodine plus caffeine to deplete, and prevent the refilling of, the caffeine-sensitive store did not attenuate the magnitude of the Ca^{2+} response evoked by depolarization with 55 mM K^+ (Figure 6b). In the converse experiment, ensuring that the caffeine-sensitive Ca^{2+} store was replete before depolarization

also failed to modulate the Ca^{2+} response evoked by 55 mM K^+ (Figure 6a). In this experiment, cells were pretreated with 40 mM caffeine for 5 min and then depolarized with 55 mM K^+ , to ensure the emptying and refilling of the caffeine-sensitive store. Cells were depolarized with 55 mM K^+ (Figure 6a, ii), and the magnitude of the $[Ca^{2+}]_i$ rise was not significantly different from that observed in control cells (Figure 6a, i). In addition to modulating the magnitude of the $[Ca^{2+}]_i$ rise, RyR activation has also been reported to increase the rate of rise of the depolarization-induced Ca^{2+} signal (Reber and Reuter, 1991; Friel and Tsien, 1992a). Comparing the rates of rise of the 55 mM K^+ -induced $[Ca^{2+}]_i$ signal in Ca^{2+} -deplete (Figure 6c, i) and Ca^{2+} -replete (Figure 6c, ii) PC12 cells indicated that these rates were indistinguishable, irrespective of the state of filling of the caffeine-sensitive Ca^{2+} store. All these data suggest that RyR activation does not contribute to the $[Ca^{2+}]_i$ response induced by continual depolarization with 55 mM K^+ in PC12 cells. This is puzzling, because both K^+ and ATP elicit similar global elevations in $[Ca^{2+}]_i$ [55 mM K^+ , 113 ± 9 nM ($n = 14$); 100 μ M ATP, 74 ± 10 nM ($n = 5$)]. However, the finding that 55 mM K^+ did not activate the RyR was important for the experimental protocol devised to refill the caffeine-sensitive Ca^{2+} store after ryanodine pretreatment (e.g. Figure 4a; see the Materials and methods section).

The caffeine-sensitive Ca^{2+} store acts as a Ca^{2+} sink, but not a Ca^{2+} source, after depolarization with 55 mM K^+

The rate of recovery of the $[Ca^{2+}]_i$ transient observed in control cells after depolarization with 55 mM K^+ varied; some responses were more transient than others (Figure 7c). These variations may have been due to a difference in the state of filling of the caffeine-sensitive Ca^{2+} store; the more replete the caffeine-sensitive Ca^{2+} store, the less able it would be to sequester intracellular Ca^{2+} , and hasten the return of $[Ca^{2+}]_i$ to pre-stimulus levels. This is illustrated in Figure 6(a). The Ca^{2+} signal in response to 55 mM K^+ remained at an elevated plateau in a cell population pretreated with caffeine and K^+ to fill the caffeine-sensitive store (Figure 6a, ii), in contrast with the relatively rapid return to baseline levels in control cells (Figure 6a, i). In the converse experiment, cells were pretreated with ryanodine and caffeine to deplete the caffeine-sensitive Ca^{2+} store and to ensure that the store could not refill (e.g. Figure 4a). Cytosolic Ca^{2+} remained elevated in pretreated cells (Figure 6b, ii), but not in control cells (Figure 6b, i), presumably because ryanodine had prevented the caffeine-sensitive store from sequestering cytoplasmic Ca^{2+} , by locking open the RyR.

Further evidence consistent with the view that the caffeine-sensitive Ca^{2+} store can act as a Ca^{2+} sink after depolarization with 55 mM K^+ is presented in Figure 7; 55 mM K^+ induced an elevation in $[Ca^{2+}]_i$ that was much more transient after pretreatment with 40 mM caffeine than before caffeine pretreatment (Figure 7b), suggesting that emptying of the caffeine-sensitive Ca^{2+} store enhances Ca^{2+} re-uptake, and therefore the return to pre-stimulus $[Ca^{2+}]_i$. When applied after 55 mM K^+ , caffeine induced a transient increase in $[Ca^{2+}]_i$ followed by a rapid decrease in $[Ca^{2+}]_i$ to near resting levels (Figure 7a). The increase in $[Ca^{2+}]_i$ induced by caffeine in the midst of a K^+ -induced Ca^{2+} signal again indicates that the increase in $[Ca^{2+}]_i$ produced by 55 mM K^+ did not deplete caffeine-sensitive Ca^{2+} stores by activating RyR. The decrease in Ca^{2+} -dependent fluorescence induced by caffeine was probably due, at least in part, to enhanced Ca^{2+} uptake into empty caffeine-sensitive Ca^{2+} stores (see the Discussion section), although the contribution of a direct inhibitory effect of caffeine on voltage-operated Ca^{2+} channels

Figure 7 Effect of caffeine on the depolarization-induced $[\text{Ca}^{2+}]_i$ signal in PC12 cells

Populations of fura-2-loaded PC12 cells were stimulated with either (a) 55 mM K^+ followed by 40 mM caffeine, or (b) 40 mM caffeine followed by 55 mM K^+ . Caffeine did not have adverse effects on fura-2 fluorescence in these experiments, as judged by the single-wavelength (340 nm and 380 nm) recordings shown in the insets. (c) Three populations of control fura-2-loaded PC12 cells responding with a $[\text{Ca}^{2+}]_i$ transient to depolarization with 55 mM K^+ (arrow). These data are typical of at least 4 independent experiments for each of (a), (b) and (c).

cannot be excluded (Hughes et al., 1990). One reported non-specific effect of caffeine that does not contribute to the decrease in fluorescence is its potential ability to interfere with fura-2 fluorescence causing the ratio signal to quench artifactually (Xu and Forsberg, 1993). The insets, showing the fluorescence signal at the individual excitation wavelengths, clearly show that caffeine had no adverse effects on fura-2 fluorescence in these experiments (Figure 7).

DISCUSSION

In addition to containing an InsP_3 -sensitive intracellular Ca^{2+} store, many neuronal cells also contain a caffeine- and ryanodine-sensitive Ca^{2+} store. The role that this store plays in cytoplasmic Ca^{2+} homeostasis is not clear, because some studies indicate that the store can act as a Ca^{2+} source after cell stimulation (Lipscombe et al., 1988; Holliday et al., 1991; Friel and Tsien, 1992a; Simpson et al., 1993; Hua et al., 1993), whereas others

suggest it does not contribute signal Ca^{2+} to the stimulus-induced $[\text{Ca}^{2+}]_i$ transient (Thayer et al., 1988a; Nohmi et al., 1992; Neher and Augustine, 1992). In this study we show that undifferentiated PC12 cells possess a caffeine- and ryanodine-sensitive Ca^{2+} store that releases Ca^{2+} through the RyR after stimulation with ATP, but not after depolarization with high (55 mM) K^+ . Instead, after depolarization, the caffeine-sensitive store acts as a Ca^{2+} sink to promote the re-uptake of cytoplasmic Ca^{2+} .

PC12 cells possess a caffeine- and ryanodine-sensitive Ca^{2+} store

In the present study, caffeine induced a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ that was saturable (Figure 1), reversible (Figure 2), independent of Ca^{2+}_o (Figure 2) and inhibited by ryanodine in a use-dependent manner (Figure 4a). The caffeine-induced rise in $[\text{Ca}^{2+}]_i$ was independent of inositol phosphate turnover (Table 1), and unrelated to the inhibitory effects of caffeine on cyclic nucleotide phosphodiesterases, because preincubation of PC12-cell populations with 1 μM forskolin, which directly activates adenylate cyclase, or 100 μM dibutyryl cyclic AMP, had no effect on control or caffeine-induced Ca^{2+} mobilization (results not shown). All these data indicate that caffeine acts at the RyR to mobilize intracellular Ca^{2+} in these experiments. The steep caffeine concentration-response curve ($h > 1$) indicates that the caffeine-induced Ca^{2+} release is co-operative. The opening of the muscle and brain RyR is known to have a biphasic dependency on $[\text{Ca}^{2+}]_i$: in the low- μM range (0.1–1 μM) the probability of channel opening is increased, whereas higher $[\text{Ca}^{2+}]_i$ (0.1–1 mM) inhibits channel opening (Rousseau et al., 1988; Bezprozvanny et al., 1991; reviewed by Henzi and MacDermott, 1992). The positive co-operativity associated with caffeine-induced Ca^{2+} release in PC12 cells could therefore result, at least in part, from mobilized Ca^{2+} feeding back on the channel to promote Ca^{2+} release, i.e. CICR.

The nature of ATP-sensitive and caffeine-sensitive Ca^{2+} stores

Pretreatment of intact cells with caffeine decreased the amount of Ca^{2+} released by a subsequent application of 100 μM ATP, in a concentration-dependent manner (Figure 2). Caffeine has been shown to inhibit InsP_3 production (Zacchetti et al., 1991; Toescu et al., 1992) and InsP_3 -mediated Ca^{2+} release (Brown et al., 1992) in some cell types, but the decrease in ATP-induced Ca^{2+} mobilization after prior stimulation with caffeine observed in the present study did not reflect either an inhibition of ATP-induced phosphoinositide hydrolysis or inhibition of Ca^{2+} release, because a maximal concentration of caffeine had no effect on the inositol phosphates accumulated in response to 100 μM ATP (Table 1) or the amount of Ca^{2+} released by InsP_3 (Figure 3). A similar result, a concentration-dependent inhibition of the subsequent Ca^{2+} response to an InsP_3 -mobilizing stimulus after caffeine stimulation, was observed when using another PC12 clone, where it was proposed that both the InsP_3R and the RyR were co-localized on the same intracellular compartment (Zacchetti et al., 1991). However, there are several reasons for supposing that the PC12 clone under current investigation does not contain one single InsP_3 - and caffeine-sensitive Ca^{2+} store. Firstly, even after depleting the caffeine-sensitive store with a supramaximal concentration of caffeine, ATP was still able to induce an elevation in $[\text{Ca}^{2+}]_i$ in intact cells (Figure 2). Secondly, after pretreatment of intact cells with ryanodine and caffeine to deplete and prevent refilling of the caffeine-sensitive store, ATP was still able to induce an, albeit decreased, elevation in $[\text{Ca}^{2+}]_i$ (Figure 4b), and thirdly, permeabilized cells that had been pretreated with a supramaximal concentration of caffeine still responded with a

rise in $[Ca^{2+}]_i$ to the addition of $InsP_3$ (Figure 3). All these data suggest that the PC12 cells in this study contain a Ca^{2+} store that is uniquely $InsP_3$ -sensitive, and an additional, larger, Ca^{2+} store that is sensitive to both $InsP_3$ and caffeine.

Ca^{2+} release through the caffeine-sensitive RyR contributes to the ATP-induced $[Ca^{2+}]_i$ signal

Pretreatment of intact PC12-cell populations with ryanodine and ATP depleted and prevented the refilling of caffeine-sensitive Ca^{2+} stores, suggesting that ATP-induced Ca^{2+} release had triggered the opening of, and therefore the binding of ryanodine to, caffeine-sensitive RyRs (Figures 5a and 5b). The significance of this finding is two-fold. Firstly, it indicated that ATP released Ca^{2+} from a Ca^{2+} store sensitive to caffeine. Secondly, and most importantly, it indicated that this Ca^{2+} release was mediated by the activation of RyRs. We have previously shown that extracellular ATP triggers a propagating Ca^{2+} wave that initiates from a discrete subcellular locus in PC12 cells (Cheek and Barry, 1993; Barry and Cheek, 1994), and a similar effect has been reported in response to the alternative $InsP_3$ -mobilizing stimulus bradykinin in another PC12 clone (Reber et al., 1993). The propagation of intracellular Ca^{2+} waves may involve the process of CICR mediated by the $InsP_3$ R alone, the caffeine-sensitive RyR alone, or a combination of these receptors (Berridge, 1993a; Galione et al., 1993). Our finding that ATP stimulation leads to activation of RyR in PC12 cells suggests that RyR may play an important role in propagating Ca^{2+} waves in these cells. One possibility is that ATP-mediated intracellular Ca^{2+} waves are initiated by Ca^{2+} release from the uniquely $InsP_3$ -sensitive Ca^{2+} store, that has a restricted subcellular localization, and are then propagated through the cytoplasm by CICR through caffeine-sensitive RyRs (and perhaps also $InsP_3$ R) that are present on a more diffusely distributed Ca^{2+} store. There is evidence to suggest that $InsP_3$ - and caffeine-sensitive Ca^{2+} stores are differentially localized in this way within adrenal chromaffin cells (Burgoyne et al., 1989), but whether this is also the case in PC12 cells remains to be tested.

The caffeine-sensitive Ca^{2+} store acts as a Ca^{2+} sink, not a Ca^{2+} source, after depolarization with 55 mM K^+

In view of the fact that Ca^{2+} release mediated by the RyR contributes to ATP-induced Ca^{2+} signals in these cells, it was intriguing to find that RyR-mediated Ca^{2+} release did not similarly contribute to the Ca^{2+} signal induced by depolarization with 55 mM K^+ (Figure 6). The reasons for this are unclear, but one possibility is that Ca^{2+} may only act a co-agonist at the neuronal RyR, as at the $InsP_3$ R (Bezprozvanny et al., 1991; Finch et al., 1991). It may be that full activation of the RyR requires the presence of an additional activating molecule, such as cyclic ADP-ribose (Galione, 1992; Galione et al., 1993; Cheung-Lee et al., 1993; Berridge, 1993b), and that this is only generated after cell-surface receptor activation. Evidence reporting a positive contribution of the RyR to depolarization-induced $[Ca^{2+}]_i$ signals is contradictory. For example, studies using sympathetic neurons (Thayer et al., 1988a), sensory ganglion cells (Thayer and Miller, 1990) and another PC12 clone (Reber et al., 1993) have indicated, as reported here, that CICR is weak, if present at all, after depolarization. Conversely, other studies using sympathetic and spinal neurons have indicated that Ca^{2+} release from the RyR can increase the rate of rise of $[Ca^{2+}]_i$ (Friel and Tsien, 1992a) and potentiate the peak $[Ca^{2+}]_i$ (Holliday

et al., 1991; Hua, et al., 1993) attained after depolarization. The reasons for these differences are unknown. One possibility is that these studies reveal the critical dependence of CICR on the delivery of trigger Ca^{2+} . In those studies that revealed CICR (Holliday et al., 1991; Friel and Tsien, 1992a; Hua et al., 1993), trigger Ca^{2+} was delivered by small depolarizing pulses, whereas in the present study and that of Reber et al. (1993) high K^+ was applied continually, resulting in a greater depolarizing signal. Chad and Eckert (1984) and Simon and Llinas (1985) used quantitative modelling to show that small depolarizations activate few Ca^{2+} channels, with a large Ca^{2+} influx through each opened channel, whereas larger depolarizations activate a greater number of Ca^{2+} channels, but with less Ca^{2+} influx through individual channels (although total Ca^{2+} influx is greater). Consequently, the larger Ca^{2+} entry induced by small depolarizations may be less buffered in localized regions centred on the inner mouth of the Ca^{2+} channel, because of localized cytoplasmic Ca^{2+} buffers approaching saturation (Neher and Augustine, 1992; Zhou and Neher, 1993), and may therefore contribute more to a unit $[Ca^{2+}]_i$ transient. Conversely, the decreased single-channel Ca^{2+} entry induced after larger depolarizations, although mediated by more Ca^{2+} channels in high density, may be more strongly buffered and lead to a smaller unit $[Ca^{2+}]_i$ transient that may be an insufficient trigger for CICR. A similar explanation may account for the observation that CICR was observed after modest (35 and 45 mM) K^+ -induced depolarizations, but not after maximal (70 mM) K^+ depolarization, in nerve-growth-factor-differentiated PC12 cells (Reber and Reuter, 1991).

There is more general agreement that the caffeine-sensitive store can play an important role in Ca^{2+} homeostasis by acting as a Ca^{2+} sink to buffer cytoplasmic Ca^{2+} after depolarization (Lipscombe et al., 1988; Thayer et al., 1988a; Cheek et al., 1990; Friel and Tsien, 1992a; Hua et al., 1993; Simpson et al., 1993). This is illustrated in Figure 6, in which ensuring that the caffeine-sensitive store was either replete (Figure 6a), or empty and unable to refill (Figure 6b), resulted in the increase in $[Ca^{2+}]_i$ induced by 55 mM K^+ remaining at an elevated plateau level, in contrast with the more transient $[Ca^{2+}]_i$ signal in control cells. Similarly, emptying the caffeine-sensitive Ca^{2+} store by treating cells with caffeine just before depolarization with K^+ decreased the elevated Ca^{2+} plateau to basal $[Ca^{2+}]_i$ levels much more rapidly than in control cells (Figure 7). A similar effect, a more rapid recovery of the K^+ -induced rise in $[Ca^{2+}]_i$ in the presence of caffeine, has been reported in sympathetic neurons, where the effect was ascribed to enhanced uptake into the empty Ca^{2+} stores (Friel and Tsien, 1992a). In agreement, the rapid transient increase in $[Ca^{2+}]_i$ followed by a rapid decrease to basal $[Ca^{2+}]_i$ levels observed when caffeine was applied after K^+ (Figure 7a) could be explained by the depletion of caffeine-sensitive stores stimulating Ca^{2+} re-uptake. Using sympathetic neurons, Hua et al. (1993) observed a similar phenomenon: activation of Ca^{2+} release from the RyR in the midst of depolarization resulted in a rapid rise in $[Ca^{2+}]_i$ followed by an enhanced rate of decay that was due to an increase in the Ca^{2+} -buffering capacity. All these data indicate that, in a number of different neuronal cell types, the role of the caffeine-sensitive Ca^{2+} store in Ca^{2+} homeostasis after depolarization is certainly to act as a Ca^{2+} sink and that, under certain conditions, it may also act as a Ca^{2+} source by releasing Ca^{2+} through the RyR.

In conclusion, the caffeine- and ryanodine-sensitive Ca^{2+} store can act as a Ca^{2+} source and a Ca^{2+} sink in PC12 cells, and its role may in part be governed by the nature of the stimulating agent.

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