Ca²⁺ entry into PC12 cells initiated by ryanodine receptors or inositol 1,4,5-trisphosphate receptors

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Capacitative Ca²⁺ entry (CCE) is a universal mechanism for refilling intracellular Ca²⁺ stores in electrically non-excitable cells. The situation in excitable cells is less clear, however, since they may rely on other entry mechanisms for Ca²⁺-store refilling. In the present study we investigated CCE in intact PC12 cells, using acetylcholine to bring about activation of InsP₃ receptors (InsP₃Rs), caffeine to activate ryanodine receptors (RyRs) and thapsigargin to inhibit sarco/endoplasmic reticulum Ca²⁺-ATPase pumps. We found that depletion of the InsP₃-, caffeine- or thapsigargin-sensitive stores promoted Ca²⁺ entry, suggesting that stimulation of either InsP₃Rs or RyRs can activate CCE. The CCE pathways activated by InsP₃Rs, RyRs and thapsigargin appeared to be independent at least in part, since their effects were found to be additive. However, CCE triggered by caffeine,

acetylcholine or thapsigargin progressively diminished with time. The decay of CCE caused by one agent also inhibited subsequent responses to the others, suggesting that some component of the CCE pathway is common to all intracellular Ca²⁺ stores. The magnitude of CCE stimulated by InsP₃Rs or RyRs was related to the size of the stores; the InsP₃-sensitive store was smaller than the RyR-sensitive store and triggered a smaller entry component. However, both stores filled with a similar half time (about 1 min), and both could be filled more rapidly by depolarization-induced Ca²⁺ entry through voltage-operated channels. A significant basal Ca²⁺ influx was apparent in PC12 cells. The basal entry component may be under the control of the InsP₃-sensitive Ca²⁺ store, since short incubations in Ca²⁺-free medium depleted this store.

INTRODUCTION

In electrically non-excitable cells, agonist-evoked intracellular Ca²⁺ ([Ca²⁺]_i) signals are often recorded as biphasic responses, reflecting an initial phase of Ca²⁺ release from intracellular stores followed by a more prolonged signal that is dependent on Ca²⁺ entry [1,2]. In the absence of extracellular Ca²⁺ (Ca²⁺_o), the response is limited to a transient [Ca²⁺]_i rise, until Ca²⁺_o is replenished [1]. This entry of Ca²⁺ into the cell is a universal feature of non-excitable cells and is commonly activated by depletion of Ca²⁺ stores sensitive to the intracellular messenger InsP₃. Classically, this capacitative Ca²⁺-entry (CCE) pathway is activated for the duration of store emptying; once triggered it is independent of the presence or absence of agonist and is inhibited on repletion of the stores [1,3].

In electrically excitable cells, the regulation of [Ca²⁺]_i is often more complex than in electrically non-excitable cells, since these cells possess additional mechanisms for elevating and reducing [Ca²⁺]_i, such as voltage-operated Ca²⁺ channels (VOCs) and Na⁺/Ca²⁺ exchangers [4]. Furthermore, ryanodine receptors (RyRs) are more ubiquitous in excitable cells [5]. Indeed, it has been suggested that electrically excitable cells may not rely on a CCE mechanism [1], since their intracellular stores can refill from voltage-dependent Ca²⁺ entry [6,7]. If this suggestion is correct, then the Ca²⁺-releasing channels in excitable tissues, whether they are InsP₃ receptors (InsP₃Rs) or RyRs, cannot be coupled to the classical CCE pathway. Evidence that this may be the case has come from studies on gonadotrophs, where refilling of the

intracellular Ca²⁺ stores in response to gonadotropin-releasing hormone can be totally accounted for by Ca²⁺ entry through VOCs [8,9]. In other excitable cell types, depletion of intracellular stores has been shown to evoke only transient [Ca²⁺]_i rises that are suggestive of Ca²⁺ mobilization without CCE [10–12]. Furthermore the intracellular stores in some excitable cells do not give agonist-evoked [Ca²⁺]_i rises without a prior depolarizing pulse to allow store refilling [11,13,14]. In contrast with these data, however, several other studies suggest that excitable cell types do display prolonged [Ca²⁺]_i rises and store refilling after agonist activation [15–18] consistent with CCE.

A further point of interest concerning CCE in excitable cells is whether it can be triggered by InsP₃Rs or RyRs. Since many electrically excitable cells express both types of receptor, only one of the two, or both, could be coupled to Ca²⁺ entry. In porcine coronary artery smooth-muscle cells, for example, caffeine evoked a transient [Ca²⁺]_i rise, without the prolonged response indicative of Ca²⁺ entry. Furthermore, whereas ryanodine blocked the initial response to acetylcholine (ACh), it had no effect on the subsequent prolonged response [19], suggesting that in these cells CCE is regulated primarily by InsP₃-sensitive stores. In mouse anococcygeus smooth-muscle cells, however, it may be that CCE is triggered independently by either InsP₃Rs or RyRs, since carbachol and caffeine both activated CCE and blockade of one receptor did not inhibit CCE activated by the other [17].

In the present study, we used population measurements and single-cell imaging of fura-2-loaded rat pheochromocytoma (PC12) cells to investigate the characteristics of CCE triggered by

Abbreviations used: ACh, acetylcholine; Ca²⁺_o, extracellular Ca²⁺; [Ca²⁺]_i, concentration of intracellular free Ca²⁺; CCE, capacitative Ca²⁺ entry; EM, extracellular medium; InsP₃R, InsP₃ receptor; RyR, ryanodine receptor; VOC, voltage-operated Ca²⁺ channel; AM, acetoxymethyl ester.

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depletion of caffeine- and InsP_3 -sensitive stores. PC12 cells have advantages for such a study, since they display robust Ca^{2+} -entry responses to both caffeine [18] and InsP_3 -generating stimuli [20], although whether or not this entry is mediated by a CCE mechanism is not known. Our data indicate that both the caffeine- and InsP_3 -sensitive stores activated CCE in an additive manner and with similar characteristics.

EXPERIMENTAL

Cell culture

PC12 cells were grown in RPMI 1640 (Gibco, Paisley, Scotland, U.K.) supplemented with 2 mM glutamine, 10% horse serum and 5% fetal bovine serum, in a humidified atmosphere (5% CO_2 , 95% air). For imaging, the PC12 cells were transferred from plastic culture dishes to glass coverslips (22 mm diameter; Chance Propper Ltd, Smethwick, Warley, U.K.) coated with poly-D-lysine (Sigma). To ensure firm attachment, the cells were allowed to grow on the coverslips for at least 48 h before use.

Single-cell fura-2 imaging

The culture medium was replaced with an extracellular medium (EM) containing (mM): NaCl, 145; KCl, 5; MgCl_2 , 1; CaCl_2 , 3; glucose, 10; Hepes, 20; pH 7.4. All solutions used were supplemented with 100 μM sulphipyrazone to inhibit the rapid clearance of fura 2. Cells were loaded with fura 2 by incubation with 1 μM fura 2 acetoxymethyl ester (AM) (Molecular Probes, Eugene, OR, U.S.A.) for 45 min at 37 °C, followed by an EM wash and a further 40 min incubation to allow de-esterification of the loaded dye. A single glass coverslip was mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope, and the cells were visualized using a glycerol-immersion $\times 40$ objective. Fluorescent images were obtained by alternate excitation at 340 and 380 nm (40 ms at each wavelength) using twin xenon-arc lamps (Spex Industries). The emission signal at 510 nm was collected using an intensified charge-coupled device video camera (Extended ISIS; Photonic Science). The video signal from the camera was digitized and stored in an Imagine image-processing unit (Synoptics Ltd., Cambridge, U.K.). The excitation wavelength was switched by a rotating mirror (Glen Spectra Instruments, Stanmore, Middx., U.K.), synchronized with the video time-base to give alternate TV frames at each of the two wavelengths. The Imagine video-rate array processor was programmed to form a 'live' ratio image, from each successive pair of frames. The ratio image was filtered with a 200 ms time constant, and stored on videotape for subsequent processing.

$[\text{Ca}^{2+}]_i$ was calculated using the formula of Grynkiewicz et al. [21]. Minimal and maximal fluorescence ratios were determined empirically under standard operating conditions using bulk solutions containing 100 mM KCl, 10 mM EGTA and 10 mM Mops, pH 7.2, with 20 μM fura 2 (pentapotassium salt; Molecular Probes) added. The $[\text{Ca}^{2+}]_{\text{free}}$ was adjusted by adding CaCl_2 . Recorded data were played back through a frame-grabber and high-speed image processor (Sprynt; Synoptics Ltd.) to provide a false-colour rendering of image intensity. The Sprynt card was programmed using the Semper language (Synoptics Ltd.) to capture images at regular intervals and to calculate $[\text{Ca}^{2+}]_i$ as a function of time.

Cell population fura-2 measurements

Cells grown in suspension were harvested from tissue culture dishes, centrifuged (1000 g; 5 min) and resuspended at a density

of $5 \times 10^6/\text{ml}$ in EM supplemented with 100 μM sulphipyrazone and 1 μM fura 2 AM. Cells were loaded with fura 2 AM for 45 min at 37 °C with constant stirring, washed twice in EM, and then resuspended at a density of $10 \times 10^6/\text{ml}$ in EM and incubated at 37 °C for a further 40 min. Cell suspensions (1 ml) were transferred to a stirred cuvette at room temperature, and fura 2 fluorescence was continuously monitored using a Perkin-Elmer LS-50 fluorimeter with excitation and emission wavelengths of 340 and 510 nm respectively, or for the experiments using Mn^{2+} ([20]; Figure 6) (excitation 360 nm and emission 510 nm).

Caffeine-induced $[\text{Ca}^{2+}]_i$ decrease

The decrease in fluorescence observed on application of caffeine to caffeine+ryanodine-pretreated cell populations (e.g. Figure 4b) could be ascribed to a rightward shift in the fura-2 excitation spectrum induced by caffeine (Figure 4 inset). For this reason, $[\text{Ca}^{2+}]_i$ was not determined in these experiments.

Determination of relative Ca^{2+} -store size

After changing to Ca^{2+} -free medium, fura-2-loaded PC12 cell populations were stimulated with caffeine (40 mM), ACh (100 μM) or thapsigargin (500 nM) either alone (control) or in varying orders. The relative amount of Ca^{2+} released by each treatment was assessed by both determining maximum fluorescence achieved and integrating the total fluorescence signal achieved in response to that treatment, and then subtracting these values from control (see Figure 4d).

RESULTS

Caffeine-stimulated Ca^{2+} responses in PC12 cells

Application of caffeine (40 mM) to a single fura-2-loaded PC12 cells stimulated a 'biphasic' response (Figure 1); an initial rapid $[\text{Ca}^{2+}]_i$ increase that was independent of Ca^{2+}_o (Figure 1a) was followed by a more prolonged $[\text{Ca}^{2+}]_i$ signal that was acutely dependent on Ca^{2+}_o (Figure 1b). Biphasic responses such as that shown in Figure 1 could be stimulated by a range of caffeine concentrations (1–40 mM) (Figure 2). The threshold for detectable caffeine-stimulated responses varied between cells; 32% of cells did not respond to 1 mM caffeine (the lowest concentration tested), whereas all cells responded to caffeine concentrations of 5 mM or above ($n = 38$). The Figure shows that the peak $[\text{Ca}^{2+}]_i$ increase was concentration-dependent, but the plateau phase was progressively inactivated. A similar result was observed on reversal of the order of addition of the caffeine concentrations shown (results not shown). The Ca^{2+} signals stimulated by caffeine were due to activation of RyRs on intracellular stores [5], since the effects of caffeine were inhibited by pretreatment with caffeine+ryanodine (see Figure 4b). Furthermore, the Ca^{2+} -influx phase of the response could be sustained by ryanodine alone (10 or 100 μM), after channel activation by caffeine (Figure 3a). Theophylline (40 mM), which has previously been shown to release Ca^{2+} from intracellular stores in our PC12 clone [14], evoked similar Ca^{2+} signals to those obtained with caffeine (results not shown).

A possible contribution to the caffeine-evoked Ca^{2+} signals from the VOCs expressed on these cells was investigated by comparing the effect of L- and N-type VOC blockers on depolarization- and caffeine-induced signals. A cocktail of 10 μM verapamil and 100 $\mu\text{g}/\text{ml}$ ω -conotoxin was found to reduce the Ca^{2+} signal in response to depolarization with 55 mM K^+ by $77 \pm 5\%$ ($n = 39$) (Figure 3b). However, in the presence of the same blocker concentrations, the caffeine response was unaffected (Figure 3b). In contrast, the plateau phase of the caffeine response

Figure 1 Caffeine-evoked sustained $[\text{Ca}^{2+}]_i$ increases in PC12 cells

(a) Application of 40 mM caffeine to a single fura-2-loaded cell. Responses shown are from a single cell stimulated with 40 mM caffeine in the presence (ai) and absence (a.ii) of Ca^{2+}_o . The caffeine applications were separated by 15 min intervals to allow cell recovery. In Ca^{2+}_o -free EM the caffeine response was reduced to the initial rapid transient. (b) The plateau phase of the response was acutely dependent on the presence of Ca^{2+}_o .

Figure 2 Concentration-dependence of caffeine-stimulated $[\text{Ca}^{2+}]_i$ increases

Caffeine at the concentration shown by the filled bars was superfused on to single fura-2-loaded cells in Ca^{2+}_o -containing EM. The caffeine applications were interspersed by 15 min rest intervals. The data are representative of six independent experiments with more than 20 cells per experiment.

was inhibited by multivalent cations in the order $\text{La}^{3+} \gg \text{Zn}^{2+} > \text{Cd}^{2+}$ (results not shown).

Caffeine-, ACh- and thapsigargin-sensitive Ca^{2+} stores in PC12 cells

In addition to expressing caffeine-sensitive Ca^{2+} stores, the PC12 cells were sensitive to ACh (100 μM) and thapsigargin (500 nM). Application of ACh, caffeine and thapsigargin in varying orders to PC12 cells in Ca^{2+}_o -free medium revealed that both the

Figure 3 Caffeine-stimulated Ca^{2+} entry is due to activation of RyRs and is unaffected by inhibitors of VOCs

(a) A single fura-2-loaded cell was treated with 40 mM caffeine and ryanodine (10 or 100 μM) as shown by the filled bars. Removal of caffeine caused a small artifactual increase in $[\text{Ca}^{2+}]_i$ (see Figure 4 inset). (b) A single fura-2-loaded cell was treated with 40 mM caffeine or 55 mM K^+ as shown, in the presence or absence of 10 μM verapamil + 100 $\mu\text{g/ml}$ ω -conotoxin (ω -CgTx). Each Figure is a representative of more than 50 cells imaged in three independent experiments.

caffeine- and ACh-sensitive stores were encompassed by the thapsigargin-sensitive Ca^{2+} pool (results not shown). To determine the degree of overlap between the different stores, aliquots from the same population of cells were subjected to different treatments. As described previously, addition of 40 mM caffeine in Ca^{2+}_o -free EM resulted in a large transient $[\text{Ca}^{2+}]_i$ increase (Figure 4a) that amounted to 56% of the total thapsigargin-sensitive pool (Figure 4d). Pretreatment of cells with 40 mM caffeine + 10 μM ryanodine completely abolished the response to 40 mM caffeine (Figure 4b; see inset for explanation of fluorescence decrease) but not the response to 100 μM ACh (Figure 4c). This response to ACh was approx. 70% of that observed in control cells (results not shown) and indicated a partial overlap of the caffeine- and ACh-sensitive Ca^{2+} stores that amounted to 10% of the total thapsigargin-sensitive pool (Figure 4d). The ability of preincubation with ryanodine to provide a 'memory' of those RyRs that become activated [12] was used to determine whether the ACh- and caffeine-sensitive Ca^{2+} stores physically overlap or whether ACh-induced Ca^{2+} release triggers RyR opening. Preincubation of cells with 100 μM ACh + 10 μM ryanodine did not dramatically affect the magnitude of the Ca^{2+} response to a subsequent challenge with 40 mM caffeine (results not shown). These observations indicate that ACh does not activate RyRs, which suggests that the ACh-evoked $[\text{Ca}^{2+}]_i$ signal arises solely from the opening of InsP_3 Rs.

Figure 4 Caffeine- and ACh-sensitive Ca^{2+} stores physically overlap

(a) Addition of caffeine to populations of fura-2-loaded PC12 cells in Ca^{2+}_o -free EM caused a large transient $[\text{Ca}^{2+}]_i$ increase. [Recovery of this response to a level that was sub-basal was due to a combination of the shift in the fura-2 excitation spectrum (inset) and dilution of the cells.] (b) Another aliquot of cells from the population used in (a) was incubated for 5 min with 40 mM caffeine + 10 μM ryanodine in control EM. The cells were then washed in control EM and resuspended in Ca^{2+}_o -free EM. These cells did not respond with a rise in $[\text{Ca}^{2+}]_i$ to the subsequent application of 40 mM caffeine, suggesting that the ryanodine had caused depletion of the caffeine-sensitive Ca^{2+} store (see inset for explanation of fluorescence decrease). (c) A third aliquot of cells pretreated with caffeine + ryanodine as in (b) responded to 100 μM ACh with a signal that was approx. 50% of that in control cells (results not shown), indicating a partial overlap of the caffeine- and ACh-sensitive Ca^{2+} stores. (d) Summary of the relationship between thapsigargin-, caffeine- and ACh-sensitive Ca^{2+} stores in PC12 cells. The percentages were obtained by subtraction of the integrated $[\text{Ca}^{2+}]_i$ signals. Inset, Rightward shift of the fura-2 excitation spectrum by caffeine. Excitation spectra were recorded from fura-2-loaded cells in control (a) or 40 mM caffeine-containing (b) EM. The shift in the excitation spectrum caused by caffeine addition causes an apparent decrease in $[\text{Ca}^{2+}]_i$ (e.g. b). N.B. At the single-cell level, all cells responded to 100 μM ACh, 40 mM caffeine or 500 nM thapsigargin (results not shown). The population responses shown above are therefore not complicated by subpopulations of cells with differential sensitivities to these stimuli.

Therefore there appears to be physical overlap between the ACh- and caffeine-sensitive Ca^{2+} stores.

Caffeine- and ACh-stimulated Ca^{2+} entry

The aim of the present study was to characterize CCE in PC12 cells, and in particular to investigate whether the caffeine- and

ryanodine-sensitive store activates a CCE pathway similar to that classically activated by InsP_3 -generating stimuli. The biphasic Ca^{2+} signals evoked by caffeine, e.g. Figure 1, are similar to Ca^{2+} signals recorded from many cell types challenged with InsP_3 -mobilizing hormones. A universal feature of CCE is that it can be activated by store depletion alone, and does not require the continued presence of the stimulating agent (e.g. ryanodine in

Figure 5 Caffeine-stimulated Ca^{2+} entry does not require the continued presence of caffeine

The response of a single fura-2-loaded PC12 cell during switching from control to Ca^{2+} -free EM is shown. The data are representative of six independent experiments with more than 20 cells per experiment.

Figure 6 Mn^{2+} -quench reveals activation of a bivalent-cation-entry pathway by caffeine and ACh

Populations of fura-2-loaded PC12 cells were either unstimulated or stimulated with ACh ($100 \mu\text{M}$), caffeine (40 mM) or ATP ($100 \mu\text{M}$). Approx. 15 s after addition of the stimulus, $100 \mu\text{M}$ MnCl_2 was added to the EM, and the subsequent quench of the fura-2 fluorescence was monitored at 360 nm excitation. The ATP-induced quench gives an estimation of the maximal extent of the quench in the cells, since ATP has been shown to both open a Mn^{2+} -permeable receptor-operated channel and stimulate InsP_3 production in these cells [20]. Traces are representative of those obtained from four independent experiments (see also Table 1).

Figure 3a). This was also true for the caffeine-activated Ca^{2+} -entry pathway in the present study. Depletion of stores by a brief application of caffeine (40 mM) in Ca^{2+} -free medium activated a Ca^{2+} -permeable pathway that was revealed by the appearance of a 'refilling transient' on re-addition of Ca^{2+} (Figure 5).

To demonstrate RyR-stimulated CCE in PC12 cells directly, we used the Mn^{2+} -quench technique [20], where the rate and extent of quench of intracellularly loaded fura 2 by Mn^{2+} provide a direct measure of cation entry. The rate constants for the quench of fura 2 triggered by ACh, caffeine and caffeine + ryanodine were significantly different from controls (Figure 6 and Table 1), suggesting the opening of additional cation-entry channels. For example, caffeine (40 mM) increased the Mn^{2+} -quench rate constant by approx. $24 \pm 4\%$ over controls (Table 1). A similar effect was also observed in cells pretreated with caffeine (40 mM) + ryanodine ($10 \mu\text{M}$), confirming that the increased rate of Mn^{2+} entry resulted from RyR activation. The extent of quench was smaller for ACh ($100 \mu\text{M}$) than the other

Table 1 Mn^{2+} -quench reveals activation of a bivalent-cation-entry pathway by caffeine and ACh

Averaged first-order rate constants are shown for the quench of fura 2 obtained by fitting single-exponential decay curves to quench traces as illustrated in Figure 6. The large error for the ATP data is due to a poor single exponential fit. The data are means $\pm$ S.E.M. for four experiments. * Indicates statistically different from control with $P < 0.05$.

Treatment	$10^{-2} \times$ Rate constant for quench (s^{-1}) ($n = 4$)
Control	1.70 ± 0.10
ACh ($100 \mu\text{M}$)	$2.05 \pm 0.10^*$
Caffeine (40 mM)	$2.10 \pm 0.08^*$
Caffeine (40 mM) + ryanodine ($10 \mu\text{M}$)	$2.50 \pm 0.05^*$
ATP ($100 \mu\text{M}$)	$3.60 \pm 0.90^*$

Figure 7 Time-dependent refilling of caffeine- and ACh-sensitive Ca^{2+} stores

(a) Response of a single fura-2-loaded cell to repetitive stimulation with 40 mM caffeine interspersed with various rest periods. A similar protocol, but using $100 \mu\text{M}$ ACh instead of caffeine, was used to examine the refilling of InsP_3 -sensitive Ca^{2+} stores. (b) Recovery of the caffeine- or ACh-stimulated $[\text{Ca}^{2+}]_i$ response with the duration of rest. Data are plotted as percentages of the initial response, and are means $\pm$ S.E.M. from more than 100 single-cell responses for both caffeine- and ACh-treated cells.

treatments, consistent with the action of a smaller ACh-sensitive Ca^{2+} store (Figure 6). The rapid entry of cation promoted by ATP was due to activation of a receptor-operated channel in addition to the generation of InsP_3 [20].

To assess further the characteristics of the entry pathways, we compared the refilling times of the caffeine- and ACh-sensitive Ca^{2+} stores, using the experimental protocol shown in Figure 7(a). The larger caffeine-sensitive Ca^{2+} store and the smaller

ACh-sensitive Ca^{2+} store refilled with similar time courses; $t_{1/2}$ for store refilling was 64 and 53 s for the caffeine- and ACh-sensitive Ca^{2+} store respectively. Full recharging of both stores took 10 min or more (Figure 7b). The rate-limiting step for refilling both the ACh- and caffeine-sensitive stores is probably the entry of Ca^{2+} across the plasma membrane, because both stores could refill to greater than their steady-state loading during a 3 min $[\text{Ca}^{2+}]_i$ increase caused by application of a 55 mM K^+ -containing solution (Figure 7a). Furthermore, rapid refilling of the stores by K^+ -induced depolarization revealed that the progressive increase in response magnitude was not due to a recovery from desensitization, since the cells were fully responsive to both caffeine and ACh in less than 3 min after the depolarization step.

Additivity of ACh-, caffeine- and thapsigargin-stimulated Ca^{2+} entry

One of the difficulties in trying to assess additivity between ACh- and caffeine-stimulated Ca^{2+} entry is that 40 mM caffeine inhibits the response to ACh in PC12 cells (Figure 8a). When ACh was given first, there was clear evidence that caffeine was able to induce an additional entry of Ca^{2+} (Figure 8b). Another way to demonstrate additivity is to lock the RyRs open using a caffeine + ryanodine pretreatment (see Figure 4b), as shown in Figures 8(c) and 8(d). Pretreatment of cells with 40 mM caffeine + 10 μM ryanodine in Ca^{2+}_o -free EM led to the activation of a Ca^{2+} -entry pathway which was revealed on readdition of Ca^{2+}_o (Figure 8c). Application of ACh (100 μM) to the cells displaying the caffeine- and ryanodine-activated Ca^{2+} entry evoked an additional sustained $[\text{Ca}^{2+}]_i$ rise (Figure 8d), suggesting that the solely ACh-sensitive store activated an additional component of Ca^{2+} entry not under the control of RyRs. Application of thapsigargin (500 nM) to cells under conditions where the caffeine- and ACh-stimulated CCE was maximal evoked an additional entry component (Figure 8di), consistent with the notion that maximal concentrations of ACh and caffeine are unable to deplete the thapsigargin-sensitive pool completely. This may be because either not all of the Ca^{2+} in this store is available for release through the receptor channel or this store is not under the control of InsP_3Rs or RyRs. Application of caffeine caused no increase in $[\text{Ca}^{2+}]_i$ (Figure 8dii; see Figure 4 inset for explanation of fluorescence decrease), confirming that the caffeine- and ryanodine-sensitive Ca^{2+} store had been depleted in this experiment.

Run-down of caffeine-stimulated Ca^{2+} signals

Caffeine-stimulated Ca^{2+} -entry signals decayed back to basal levels with a half-time of approx. 8 min, despite the continued presence of Ca^{2+}_o and caffeine (Figure 9a). A 1 h incubation in 40 mM caffeine gave a full inhibition of Ca^{2+} entry in most cells, and, after removal of caffeine, the inhibitory effect was slowly reversed ($t_{1/2} \sim 20$ min; results not shown). These data suggest that either Ca^{2+} release via RyRs, or Ca^{2+} influx, was progressively inactivated by prolonged stimulation.

Although RyRs have been suggested to undergo a form of desensitization [22], it is unlikely that the caffeine- or ryanodine-induced Ca^{2+} signals were diminished because of RyR inactivation, since thapsigargin, which does not work via RyRs, gave only a transient $[\text{Ca}^{2+}]_i$ increase when applied after caffeine (Figures 9bi and 9bii). ACh (100 μM) application showed a similar lack of effect after caffeine (results not shown). The observation that thapsigargin and ACh failed to elicit a significant prolonged $[\text{Ca}^{2+}]_i$ response suggests that the thapsigargin- and

Figure 8 Additivity of ACh-, caffeine- and thapsigargin-stimulated Ca^{2+} entry

It was necessary to show the additivity between ACh- and caffeine-stimulated Ca^{2+} entry using the caffeine + ryanodine-blocking protocol shown earlier (Figure 3a), since this maintains the RyR-dependent Ca^{2+} entry in the absence of caffeine. For traces (a)–(d), populations of fura-2-loaded cells were suspended in Ca^{2+}_o -free medium before the start of the experiment. (a) and (b) Responses from cells that were not preincubated with caffeine + ryanodine. (c) and (d) show the effect of similar experimental protocols to those in (a) and (b) on cells that were treated with 40 mM caffeine + 100 μM ryanodine for 3 min to lock open the RyRs. (c) Reapplication of Ca^{2+}_o to cells preincubated with caffeine + ryanodine evoked a large $[\text{Ca}^{2+}]_i$ rise because of the previous activation of the RyR-dependent Ca^{2+} -entry pathway. Subsequent application of caffeine caused an artifactual fall in $[\text{Ca}^{2+}]_i$ (see Figure 4 inset) and inhibited any response to 100 μM ACh addition. (d) Addition of 100 μM ACh to cells preincubated with caffeine + ryanodine evoked an additional Ca^{2+} -entry component (cf. Figure 4c), and further addition of 500 nM thapsigargin elicited another increase in Ca^{2+} entry (di). Application of caffeine caused an artifactual fall in $[\text{Ca}^{2+}]_i$ (dii, see Figure 4 inset). The traces shown are representative of at least three independent experiments.

ACh-stimulated Ca^{2+} -entry pathways were also inhibited. Furthermore, after prolonged caffeine application, the RyR-sensitive Ca^{2+} store could be refilled by a brief (3 min) $[\text{Ca}^{2+}]_i$ increase caused by depolarization with K^+ -containing solution (Figure 9c). The Ca^{2+} that entered the intracellular stores could be released by application of caffeine (Figure 9c); however, no Ca^{2+} entry was triggered. These data indicate that the Ca^{2+} -entry pathway, and not the RyRs, was inactivated by caffeine.

The inhibition of Ca^{2+} entry was also observed with threshold caffeine concentrations (5 mM; Figure 9d). However, after Ca^{2+} -entry inhibition with submaximal caffeine concentrations, application of a maximal caffeine concentration (40 mM) gave a transient $[\text{Ca}^{2+}]_i$ increase (Figure 9d). This observation indicates that the RyRs were not inactivated, and that the intracellular stores could retain Ca^{2+} during prolonged caffeine applications.

Figure 9 Run-down of RyR-stimulated Ca^{2+} entry

(a) Single-cell response showing the progressive diminution of the Ca^{2+} -entry plateau phase during prolonged stimulation with 40 mM caffeine in Ca^{2+} -containing EM. (b) Run-down of caffeine-stimulated Ca^{2+} signals also inhibited subsequent responses to thapsigargin. (bii) Control single-cell response to 500 nM thapsigargin. (c) The run-down of Ca^{2+} entry was not due to inactivation of the RyRs, since refilling the stores by a 3 min depolarization provided caffeine-releasable Ca^{2+} . (d) Similar effects were seen with threshold caffeine concentrations, even though these doses did not deplete the entire caffeine-sensitive Ca^{2+} store. Each trace is representative of the responses in more than 40 cells tested in three independent experiments.

Figure 10 ACh-releasable Ca^{2+} store is sensitive to removal of Ca^{2+} .

(a) Caffeine response in populations of fura-2-loaded cells incubated either in control EM (ai) or for 1 min in Ca^{2+} -free EM (aii). (b) and (bii) The effect of the same preincubations as in (ai) and (aii) on responses evoked by 100 μM ACh.

Basal Ca^{2+} entry

A 'basal' influx was apparent from the sensitivity of $[\text{Ca}^{2+}]_i$ to removal of Ca^{2+}_o (Figure 5) and the rate of Mn^{2+} entry in unstimulated cells (Figure 6). This basal Ca^{2+} leak was unaffected by verapamil (10 μM) and ω -conotoxin (100 $\mu\text{g/ml}$) (results not

shown). We considered that this basal Ca^{2+} entry may play a role in regulating the repletion state of the intracellular Ca^{2+} stores in resting cells. To test this possibility, we investigated whether the Ca^{2+} stores would be depleted by incubation in Ca^{2+} -free medium. The initial response to caffeine (40 mM) was unaffected by incubation of the cells in Ca^{2+} -free medium for up to 10 min (Figure 10a). However, the response to ACh (100 μM) was significantly reduced by very short (< 1 min) incubations in Ca^{2+} -free medium (Figure 10b). A longer (10 min) incubation in Ca^{2+} -free medium did not deplete the ACh-sensitive Ca^{2+} stores completely (results not shown). The effect of very short incubations in Ca^{2+} -free medium on ACh-sensitive Ca^{2+} stores correlates with the rapid effect of removal of Ca^{2+}_o on $[\text{Ca}^{2+}]_i$ (Figure 5).

DISCUSSION

In this study we have shown that RyR activation (in addition to InsP_3 R activation) is able to promote CCE in PC12 cells. This result was somewhat surprising, particularly as previous studies on PC12 cells by us [14] and others [13] had suggested that these cells may not display a CCE mechanism, since their intracellular Ca^{2+} stores, in particular the caffeine-sensitive store, do not always appear to be replete. Furthermore, although caffeine-induced Ca^{2+} entry has been previously reported in PC12 cells, whether or not this was due to a CCE mechanism was not examined [18].

To substantiate the idea that RyRs can trigger CCE, we investigated the nature of the $[Ca^{2+}]_i$ rise triggered by activation of these receptors. Caffeine treatment resulted in a rapid $[Ca^{2+}]_i$ rise followed by a prolonged response which was acutely dependent on the presence of Ca^{2+}_o (Figure 1). This Ca^{2+} entry resulted from activation of RyRs, because the prolonged phase of the response was maintained by ryanodine in the absence of caffeine (Figure 3a). The increased rate of Mn^{2+} quench activated by caffeine and ryanodine directly demonstrates a RyR-induced entry of bivalent cations into these cells (Figure 6 and Table 1). The Ca^{2+} entry triggered by the caffeine-sensitive store is likely to be due to a classical CCE mechanism, since it required store depletion (Figure 1), was independent of the presence or absence of caffeine (Figure 5) and was inhibited when the stores had refilled (Figure 5). Furthermore, the Ca^{2+} entry triggered by caffeine was insensitive to a cocktail of VOC inhibitors (Figure 3b), but was inhibited by multivalent cations in the order $La^{3+} \gg Zn^{2+} > Cd^{2+}$, which is a similar sequence to that found to inhibit classical CCE [23].

The fact that the $[Ca^{2+}]_i$ signal associated with refilling the caffeine-sensitive store was transient (Figure 5), and that, when the CCE pathway had been inhibited by prolonged stimulation, a subsequent $[Ca^{2+}]_i$ rise did not trigger any further entry (Figures 9c and 9d) argue against the entry being triggered by the elevation in $[Ca^{2+}]_i$ itself, for example via a Ca^{2+} -activated non-specific bivalent-cation channel [24]. In addition, the lack of requirement for caffeine to be present rules out the activation of any RyRs expressed on the plasma membrane of our PC12 cells [25].

An interesting aspect of CCE in PC12 cells was the additivity of the Ca^{2+} -entry fluxes triggered by the different intracellular Ca^{2+} stores. Pharmacologically, our PC12 cell clone appears to express at least four different types of Ca^{2+} store; a uniquely $InsP_3R$ -sensitive store, a caffeine- and ryanodine-sensitive store, a third store with both receptor types and a fourth Ca^{2+} store that was only released by thapsigargin. We found that depletion of any of these stores could evoke CCE (Figure 8). It is not yet clear whether the same CCE pathway is recruited by each of the intracellular stores. However, the heterologous inhibition of CCE by prolonged application of ACh, caffeine or thapsigargin (Figure 9) would point to at least one step being common between each Ca^{2+} store.

The ability of each type of Ca^{2+} store to trigger CCE may give the PC12 cells a variable Ca^{2+} -entry signal. For example, the recruitment of RyRs in PC12 cells appears to be agonist-specific, in that ACh activates only $InsP_3Rs$ (Figure 4), while ATP brings about the activation of both $InsP_3Rs$ and RyRs (in addition to stimulating a receptor-operated channel; Figure 6 [14]). Thus RyR-mediated CCE would only be active with certain stimuli. Since the caffeine-sensitive Ca^{2+} store is larger than the ACh-sensitive store (Figure 4), and displayed a greater extent of cation entry than ACh (Figure 6), those stimuli that trigger CCE via depletion of the caffeine-sensitive store will trigger a larger $[Ca^{2+}]_i$ rise, which may, for example, have effects on secretion [26].

A similar situation of multiple independent CCE pathways may also exist in mouse anococcygeus smooth-muscle cells. In these cells, caffeine- and carbachol-activated CCE was found to be differentially blocked by ryanodine and heparin respectively [17]. However, it is unclear whether this effect was due to activation of distinct receptors or release of separate stores, since it was not determined whether $InsP_3Rs$ and RyRs were expressed on the same or different stores.

The mechanism underlying $InsP_3R$ - and RyR-activated CCE is unknown. Depletion of either store may release a diffusible

intracellular messenger such as Ca^{2+} -influx factor (CIF [27]) or activate a conformation-coupling mechanism [2]. Of particular interest was the ability of thapsigargin to promote Ca^{2+} entry when ACh- and caffeine-induced entry were on-going (Figure 8). Since a part of the thapsigargin-sensitive store (21%; Figure 4) was not depleted by ACh or caffeine stimulation, it may not express intracellular Ca^{2+} -release channels. This could rule out a direct interaction between Ca^{2+} -releasing channels and Ca^{2+} -entry channels on the plasma membrane [28]. Alternatively, CCE activated by the uniquely thapsigargin-sensitive store may rely on a protein-protein interaction between a non-conducting Ca^{2+} -release channel and a membrane channel [2]. Evidence for such a scheme has been recently demonstrated in *Xenopus* oocytes, where expression of type-III $InsP_3Rs$ enhanced CCE without changing the characteristics of Ca^{2+} release [29].

A notable feature of the PC12 cells used in the present study was significant Ca^{2+} entry in resting cells (Figures 5 and 6). These observations contrast with our experience of non-excitable cells, e.g. HeLa cells, in which removal of Ca^{2+}_o for prolonged periods had no detectable effect on $[Ca^{2+}]_i$, and in which the basal rate of Mn^{2+} quench was substantially lower than in PC12 cells (D. L. Bennett, M. D. Bootman, M. J. Berridge and T. R. Cheek, unpublished work). The basal Ca^{2+} entry in PC12 cells was unaffected by verapamil and ω -conotoxin, but was inhibited by multivalent cations in the order $La^{3+} \gg Zn^{2+} > Cd^{2+}$ (D. L. Bennett, M. D. Bootman, M. J. Berridge and T. R. Cheek, unpublished work). A basal Ca^{2+} leak, also insensitive to VOC blockers, has previously been described in another excitable secretory cell type, pancreatic β -cells [30]. We suggest that this basal Ca^{2+} entry may play a role in regulating the repletion state of the intracellular Ca^{2+} stores in resting cells, since the response to ACh was significantly reduced by very short (< 1 min) incubations in Ca^{2+}_o -free medium (Figure 10b). These data suggest that the uniquely $InsP_3$ -releasable store is particularly sensitive to the removal of Ca^{2+}_o . Given that this store also regulates CCE (Figure 8d), it is possible that the store may be 'leaky', and requires continual access to Ca^{2+}_o to remain full, thus evoking a constitutive CCE current.

In summary, we have shown that both $InsP_3Rs$ and RyRs, in addition to a store that does not possess any Ca^{2+} -releasing channels, activate CCE in PC12 cells. The CCE flux generated by these stores can be additive. Future work will be directed at the identification of the store-operated channel protein(s) responsible for this CCE. One likely candidate for the store-operated channel is the Trp protein [31–33]. Our preliminary reverse transcriptase PCR data have revealed the presence of up to six isoforms of Trp in PC12 cells (D. L. Bennett, M. D. Bootman, M. J. Berridge and T. R. Cheek, unpublished work), raising the possibility that different stores could couple to distinct Ca^{2+} -entry channels.

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