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Functional Coupling of Secretion and Capacitative Calcium Entry in PC12 Cells

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The caffeine-evoked effects on the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and on the release of dopamine by PC12 cells were investigated. Stimulation by caffeine resulted in a transient Ca^{2+} release which was followed by a sustained phase of Ca^{2+} entry through a non-voltage dependent pathway. Treatment with cyclopiazonic acid (CPA) or thapsigargin, inhibitors of the Ca^{2+}ATPase pump of the endoplasmic reticulum, resulted in only a sustained rise in $[Ca^{2+}]i$ in the presence of extracellular Ca^{2+}. Pretreatment of cells with CPA or thapsigargin abolished the subsequent Ca^{2+} responses to caffeine. Caffeine also evoked the release of dopamine from the cells only in the presence of extracellular Ca^{2+}, which was mimicked by CPA. These results suggest that store-dependent Ca^{2+} entry evoked by caffeine has an indispensable role in the secretory response in an excitable cell line, PC12 cells.
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Over ten years have passed since the original idea that Ca^{2+} can enter cells through the plasma membrane in response to intracellular store depletion was first proposed (1). Recently, much attention has been focused on this so-called capacitative Ca^{2+} entry (CCE) because it has become clear that CCE plays a central role in many aspects of cell signaling (2, 3). In excitable cells, however, the existence of CCE is controversial, in part because it is unclear whether ryanodine receptor (RyR)-linked Ca^{2+} stores activate CCE in a manner similar to that which results from depletion of inositol 1,4,5-trisphosphate receptor ($InsP_3R$)-linked stores (2). We previously reported that activation of P_{2U}-purinoceptors in an excitable cell line, rat pheochromocytoma PC12 cells, by uridine 5′-triphosphate (UTP) leads to a transient rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) resulting from inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} mobilization, which was followed by a sustained rise in $[Ca^{2+}]i$ resulting from non-voltage dependent Ca^{2+} influx, presumably CCE (4). Besides $InsP_3$-sensitive Ca^{2+} stores, caffeine/ryanodine sensitive Ca^{2+} stores are present in PC12 cells (5), which enables us to investigate whether RyR-linked Ca^{2+} stores activate CCE via an $InsP_3$-independent mechanisms.

As for the physiological significance of CCE in excitable cells, it is far from clear. UTP could stimulate the release of dopamine only in the presence of extracellular Ca^{2+} in PC12 cells (4), which raises the possibility that CCE activated by $InsP_3R$-mediated Ca^{2+} release and the secretory response are closely linked.

In this study, we demonstrate the existence of CCE in an excitable cell line, PC12 cells, and report that, in addition to $InsP_3Rs$ activation (4), CCE can be stimulated by activation of RyRs. Furthermore, we also show the role played by CCE in the exocytotic secretory response of the cells.

MATERIAL AND METHODS

Cell culture. Culture conditions of PC12 cells were as described previously (6). All experiments described in this manuscript were performed with cells at passages 53 through 68. Cells were plated onto collagen-coated 35 mm polystyrene dishes (1×10^6 cells/dish) for measuring the release of dopamine, or poly-L-lysine (Sigma, St. Louis, U.S.A.)-coated glass coverslips, placed in silicon rubber walls (Flexiperm, W.C. GmbH, Germany) for measuring the increase in $[Ca^{2+}]i$. Cells were cultured an additional 2 days in a humidified atmosphere of 90 % air and 10 % CO_2 at 37 °C.

Measurement of $[Ca^{2+}]i$ in single cells. Changes in $[Ca^{2+}]i$ were measured by the fura-2 method as described previously (7) with minor modifications (4). In brief, 30 min after incubation with 5 μM fura-2 acetoxymethylester (fura-2 AM) at 37 °C, cells were washed with a balanced salt solution (BSS) of the following composition (mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, D-glucose 10 and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 25 (pH adjusted to 7.4 with NaOH) and mounted on an inverted fluorescence

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Abbreviations: $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; CCE, capacitative Ca^{2+} entry; CPA, cyclopiazonic acid; $InsP_3$, inositol 1,4,5-trisphosphate; RyR, ryanodine receptor.

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microscope (TMD-300, Nikon, Tokyo, Japan) equipped with a Xenon-lamp and band-pass filters of 340 and 360 nm. For the Ca^{2+}-depleted experiments, we used a medium in which Ca^{2+} was removed and 1 mM ethylenediaminetetraacetic acid (EGTA) was added (Ca^{2+}-free BSS). Image data, recorded by a high-sensitivity silicon intensifier target camera (C-2741-08, Hamamatsu Photonics, Hamamatsu, Japan), were processed by a Ca^{2+}-analyzing system (Furusawa Lab. Appliance. Co., Kawagoe, Japan). Caffeine was applied to the cells two or three times for 1 min at intervals of 4 min. The absolute $[Ca^{2+}]i$ was estimated from the ratio of emitted fluorescence (F340/F360) according to the calibration curve obtained by standard Ca^{2+}-buffer.

Measurement of dopamine release. The procedures for the measurement of released dopamine were basically the same as those described by Koizumi et al. (8). Cells were stimulated by various concentrations of caffeine and CPA dissolved in BSS for 1 and 3 min, respectively. For Ca^{2+}-free experiments, the dishes were washed twice with Ca^{2+}-free BSS for 1 min before caffeine application. The amount of dopamine released into the superfusate and that remaining in the cells were determined with high performance liquid chromatography (HPLC) coupled with an electrochemical detector (ECD)(LC-4B, Bioanalytical systems, West Lafayette, U.S.A.). The percentage of release was calculated by dividing the supernatant values by the sum of the supernatant and pellet values.

Chemicals. Drugs used were as follows. Caffeine, cyclopiazonic acid (CPA), thapsigargin, nicardipine hydrochloride, cadmium chloride, zinc acetate and ω-conotoxin GVIA (ω-CTX) were purchased from Sigma. Fura-2 AM and HEPES were from (Dojin, Kumamoto, Japan). Other chemicals are purchased from Wako Purechemicals (Tokyo, Japan). Nicardipine, CPA and thapsigargin were dissolved in dimethyl sulphoxide at a concentration of 10 mM (nicardipine and CPA) or 1 mM (thapsigargin), and then dissolved in BSS to appropriate concentrations. Other drugs were directly dissolved in BSS or Ca^{2+}-free BSS. All data are mean±s.e.m.

Statistics. Statistical differences in the values of dopamine release or the increase in $[Ca^{2+}]i$ were determined using an analysis of variance followed by Dunnet's test for multiple comparisons.

RESULTS

Caffeine (30 mM) produced a significant rise in $[Ca^{2+}]i$ in about 80 % of the PC12 cells (188 out of 231 cells tested) and the average amplitude was 312±40.1 nM. When caffeine was applied to the cells twice for 1 min separated by 3 min, the Ca^{2+} response to the 2 nd caffeine was almost the same as that of the 1 st caffeine (data not shown). Fig. 1 (a, b and c) shows the time-course of the caffeine-evoked increase in $[Ca^{2+}]i$ in the cells. Caffeine evoked a transient $[Ca^{2+}]i$ rise in the absence of extracellular Ca^{2+} (b), whereas it produced a transient $[Ca^{2+}]i$ rise, followed by a sustained rise in $[Ca^{2+}]i$, in the presence of extracellular Ca^{2+} (a). The individual traces were superimposed and the time-courses were compared (Fig. 1c). The maximal $[Ca^{2+}]i$ level evoked by caffeine (h_{max}) in the absence of extracellular Ca^{2+} was comparable to that in the presence of external Ca^{2+} (Fig. 1d). The $[Ca^{2+}]i$ at 1 min after the caffeine application (marked #) was defined as $h^{\#}$ and the ratio of $h^{\#}/h_{max}$ was calculated in the absence and presence of extracellular Ca^{2+}. As shown in Fig. 1e, the $h^{\#}/h_{max}$ ratio was dramatically decreased by the depletion of extracellular Ca^{2+}, suggesting that the sustained component results from Ca^{2+} influx from extracellular spaces. Incubation of the cells with 30 μM CPA (Fig. 1f) or 1 μM thapsigargin (Fig. 1g), inhibitors of the Ca^{2+}ATPase pump of the endoplasmic reticulum, resulted in a sustained rise in $[Ca^{2+}]i$, which almost completely inhibited the subsequent Ca^{2+} responses to caffeine (30 mM). Thapsigargin evoked only a transient and slight rise in $[Ca^{2+}]i$ in the absence of extracellular Ca^{2+}, but induced a long-lasting $[Ca^{2+}]i$ elevation upon replacement of the Ca^{2+}-free medium with 1.8 mM Ca^{2+} (Fig. 1h). When the cells were repetitively exposed to 30 mM caffeine for periods of 1 min separated by 3 min in the presence of 10 μM ryanodine, the Ca^{2+} responses to caffeine were dramatically inhibited in a use-dependent manner and the 3 rd series of Ca^{2+} responses to caffeine were almost all abolished (data not shown).

Similar to caffeine, theophylline (30 mM) produced a biphasic rise in $[Ca^{2+}]i$, i.e. a transient elevation in $[Ca^{2+}]i$ was followed by a sustained one (data not shown). The sustained $[Ca^{2+}]i$ rise was totally depen-

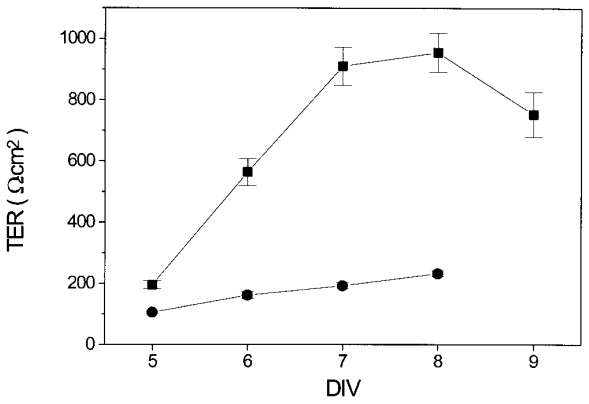

FIG. 1. The time-course of the caffeine-evoked increase in $[Ca^{2+}]i$ in PC12 cells. *a and b.* Typical Ca^{2+} responses to 30 mM caffeine in the presence (*a*) and absence (*b*) of extracellular Ca^{2+}. Caffeine was applied to the cells for 1 min (horizontal bars) at 4 min intervals. Traces *a* and *b* were obtained from the same cell. Vertical and horizontal scale bars show 100 nM and 1 min, respectively. *c.* The traces shown in *a* and *b* are superimposed and their time-courses were compared. The maximal Ca^{2+} responses to caffeine (h_{max}) are summarized in *d* (control, n=36; $-Ca^{2+}$, n=42). #s show the magnitude of $[Ca^{2+}]i$ ($h^{\#}$) at 1 min after caffeine application in the presence (control) and absence ($-Ca^{2+}$) of extracellular Ca^{2+}, and the responses are summarized in *e.* Values show the ratio of $h^{\#}$ over h_{max} in the presence (n=36; open column) and absence (n=42; dotted column) of extracellular Ca^{2+}. *f and g.* The effects of depletion of intracellular Ca^{2+} stores by CPA (cyclopiazonic acid)(*f*) and thapsigargin (*g*), on the caffeine-evoked rise in $[Ca^{2+}]i$. After caffeine (30 mM) stimulation, cells were incubated with CPA (30 μM; hatched column) for 3 min (*f*), or thapsigargin (1 μM; dotted column) for 2 min (*g*), and then caffeine was applied to the cells in the presence of CPA (n=47) or thapsigargin (n=31). *h.* Cells were incubated with thapsigargin (1 μM; dotted column) in the absence of extracellular Ca^{2+} (broken line) for 2 min, which resulted in the abolishment of the caffeine-evoked rise in $[Ca^{2+}]i$. Subsequently applied external Ca^{2+} produced a large and sustained $[Ca^{2+}]i$ increase (n=28). Vertical and horizontal scale bars show 100 nM and 1 min, respectively.

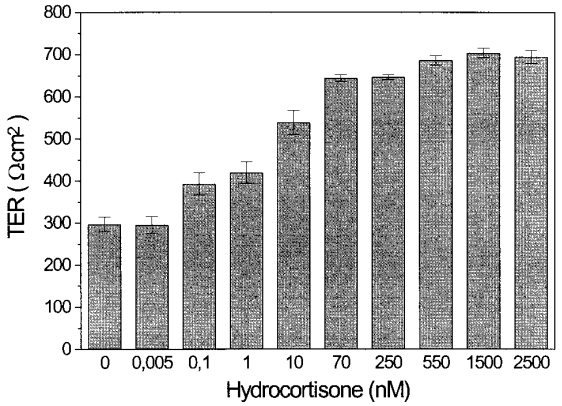

FIG. 2. The effects of Zn^{2+}, Cd^{2+}, La^{3+}, ω-CTX and nicardipine on the caffeine-evoked rise in $[Ca^{2+}]i$ in PC12 cells. *a and b.* Typical Ca^{2+} responses to 30 mM caffeine in the absence (*a*) and presence (*b*) of 30 μM Zn^{2+}. Caffeine was applied to the cells two times for 1 min at 4 min intervals (filled horizontal bars). Zn^{2+} (dotted column) and caffeine were applied to the cells simultaneously (*b*). Vertical and horizontal bars show 100 nM and 60 s, respectively. The traces shown in *a* and *b* were superimposed and their time-courses were compared in *c*. The maximal Ca^{2+} responses to caffeine (h_{max}) in the absence (open column) and presence (filled column) of 30 μM Zn^{2+} are summarized in *d*. #s show the magnitude of $[Ca^{2+}]i$ ($h^{\#}$) at 1 min after caffeine application in the absence (control, n=143) and presence (+Zn^{2+} 30 μM, n=37-58) of Zn^{2+}, and the responses are summarized in *e*. The values show the ratio of $h^{\#}$ over h_{max} in the absence (n=143; open column) and presence (n=37-58; filled column) of Zn^{2+}. In addition to Zn^{2+}, the effects of Cd^{2+} (n=48-61; hatched columns), La^{3+} (n=68; double hatched column), ω-CTX (n=49, dotted column) and nicardipine (n=41; striped column) on the ratio of $h^{\#}/h_{max}$ were examined and summarized in *e*. Asterisks show a significant difference from the ratio of control (*$p<0.05$, **$p<0.01$).

dent on extracellular Ca^{2+}. Forskolin (10 μM) had no effects on either the resting $[Ca^{2+}]i$ or the theophylline-evoked rise in $[Ca^{2+}]i$ (n=48). Pretreatment of cells with nicardipine (30 μM) + ω-conotoxin (ω-CTX, 1 μM) for 1 min before and during the theophylline application had no effects on the sustained Ca^{2+} responses to theophylline (the amplitude of the sustained response 1 min after theophylline application in the presence of nicardipine + ω-CTX was 104.4±9.1 % of theophylline alone, n=32), though these chemicals inhibited the KCl (53 mM)-evoked rise in $[Ca^{2+}]i$ by 82.4±6.2% (n=26).

The effects of various compounds on the rise in $[Ca^{2+}]i$ were examined (Fig. 2). Zn^{2+}, an inhibitor of CCE (9), almost completely inhibited the sustained component of the $[Ca^{2+}]i$ rise without affecting the transient component (Fig. 2b, d and e). Traces from Fig. 2a and b were superimposed and the time-courses were compared (Fig. 2c). The h_{max} in the presence of Zn^{2+} was not different from that in the absence of extracellular Zn^{2+} (Fig. 2c and d). However, Zn^{2+} at 30 μM dramatically inhibited the sustained component of the $[Ca^{2+}]i$ rise and significantly inhibited the $h^{\#}/h_{max}$ ratio (Fig. 2e). Other cations ($\leq$100 μM) failed to inhibit the sustained component significantly (Fig. 2e) though La^{3+} showed inhibitory actions when its concentration was raised to 300 μM ($h^{\#}/h_{max}$ ratio: 0.14±0.04, $p<0.01$, n=38). Incubation of cells with nicardipine (30 μM) or ω-CTX (1 μM) for 1 min before and during the caffeine stimulation had no effect on the sustained Ca^{2+} response to caffeine. Aminophylline (100 μM) had no effect on the caffeine-evoked sustained rise in $[Ca^{2+}]i$ ($h^{\#}/h_{max}$:0.41±0.03, n=19). Similar observations were reported in response to UTP (4). In addition, the sustained $[Ca^{2+}]i$ elevation evoked by CPA (30 μM) was also inhibited by Zn^{2+} (30 μM) but not by Cd^{2+} (100 μM), La^{3+} (100 μM) or nicardipine (30 μM) (data not shown). Thus, these findings strongly suggest that, in PC12 cells, caffeine induces Ca^{2+} influx by a mechanism dependent upon Ca^{2+} depletion.

Fig. 3a shows the concentration-dependence of the caffeine-stimulated release of dopamine and the effects of extracellular Ca^{2+} on the stimulation. Caffeine stimulated the release of dopamine in a concentration-dependent manner over a concentration range from 10 to 50 mM only in the presence of extracellular Ca^{2+} (Fig. 3a). The release of dopamine was mimicked by 30 μM CPA, and this was totally dependent on extracellular Ca^{2+} [CPA: 313.4±16.3 (n=6, $+Ca^{2+}$), 102.2±6.2 (n=6, $-Ca^{2+}$) % of spontaneous release]. Aminophylline, an antagonist of non-selective adenosine receptors, had no effects on the release of dopamine evoked by caffeine (aminophylline 100 μM: 102.1±6.5 % of caffeine alone, n=6).

The effects of various cations and an organic compound on the caffeine-evoked dopamine release were examined in the PC12 cells (Fig. 3b). Zn^{2+} potently inhibited the release of dopamine with an IC_{50} value of about 4 μM. Other cations or nicardipine failed to inhibit the dopamine release significantly. The effects of these chemicals on the release were well in agreement with those of the chemicals on the sustained $[Ca^{2+}]i$ rise evoked by caffeine (Fig. 2 and 3b).

DISCUSSION

Caffeine acts at the RyR where it appears to shift the Ca^{2+} sensitivity of the channel to a lower concentration (10), thereby increasing the probability of channel opening, namely "calcium-induced Ca^{2+} release". In the PC12 cells, caffeine evoked Ca^{2+} release by stimulating RyRs since ryanodine (10 μM) abolished the Ca^{2+} responses to caffeine in a use-dependent fashion, which was well in accordance with previous results obtained from chromaffin cells (11) and PC12 cells (5). In addition to Ca^{2+} release from RyR, we showed here that caffeine stimulates a sustained Ca^{2+} entry in the cells (Fig. 1). The caffeine-evoked Ca^{2+} entry could be activated by a decrease in the stored Ca^{2+} concentration because (1) a similarly sustained Ca^{2+} entry was mimicked by both CPA and thapsigargin, inhibitors of the endoplasmic reticu-

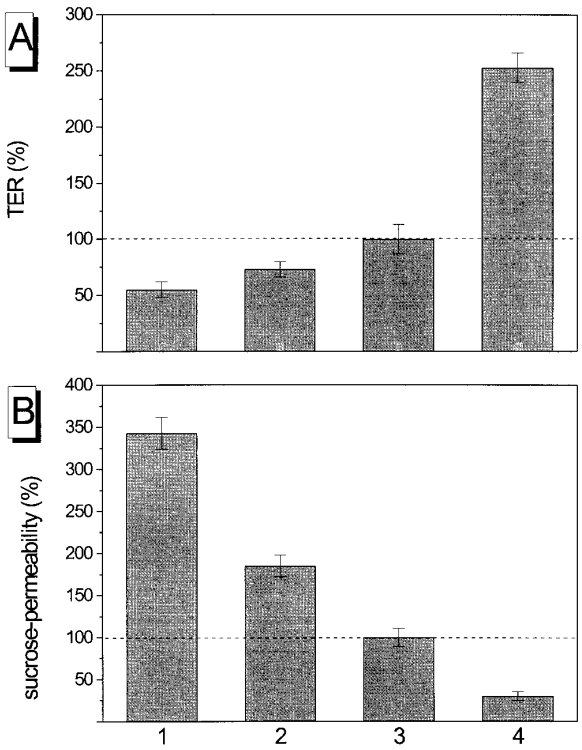

FIG. 3. *a*. Concentration-dependence of the caffeine-evoked dopamine release from PC12 cells. Open and closed circles represent the release of dopamine evoked by caffeine in the presence (open circles) and absence (closed circles) of extracellular Ca^{2+}. These are results from a typical experiment with each data point being the mean±s.e.mean. of triplicate measurements. Four such experiments with similar results were performed. *b*. The effects of Zn^{2+}, nicardipine, Cd^{2+} and La^{3+} on the caffeine-evoked dopamine release from PC12 cells. Values represent % of dopamine release evoked by 30 mM caffeine alone (open circle). The effects of Zn^{2+}, nicardipine, Cd^{2+} and La^{3+} on the caffeine-evoked responses are indicated: Zn^{2+} (closed circles), nicardipine (open triangle), Cd^{2+} (closed triangles) and La^{3+} (closed squares). These are results from a typical experiment and each data point is the mean±s.e.mean. of triplicate measurements. At least three such experiments were performed, and similar results were obtained. Asterisks show a significant difference from the response evoked by caffeine alone (*$p<0.05$, **$p<0.01$).

lum Ca^{2+}ATPase pump, (2) pretreatment of cells with CPA or thapsigargin abolished the subsequent Ca^{2+} responses to caffeine, which indicates an overlap of Ca^{2+} entry mechanisms and (3) the sustained $[Ca^{2+}]$i rises evoked by both caffeine and CPA were non-voltage dependent but were sensitive to Zn^{2+}. These results strongly suggest that the caffeine-evoked sustained rise in $[Ca^{2+}]$i is dependent upon the filling state of the intracellular Ca^{2+} stores. A similar phenomenon was observed upon UTP stimulation of these cells: UTP stimulates P_{2U}-purinoceptors, which leads to $InsP_3$ formation and the resultant mobilization of Ca^{2+} from intracellular Ca^{2+} stores via $InsP_3R$. The initial Ca^{2+} mobilization is transient, and this is then followed by a sustained $[Ca^{2+}]$i elevation resulting from Ca^{2+} influx, which is also highly sensitive to Zn^{2+} (4). It is therefore likely that PC12 cells possess a Ca^{2+} entry pathway(s) which can be activated by Ca^{2+} store depletion induced by the activation of either $InsP_3$ or RyR.

We demonstrated here a possible link between CCE and exocytosis in PC12 cells. As shown in Fig. 1, caffeine could stimulate Ca^{2+} release in the absence of extracellular Ca^{2+}, and the amplitude was almost the same as that in the presence of extracellular Ca^{2+}, but secretion could be produced only in the presence of extracellular Ca^{2+} (Fig. 2). This property described above is in agreement with that of the UTP-evoked responses in the cells. UTP evoked the release of dopamine only in the presence of extracellular Ca^{2+} (4), indicating that the UTP-evoked release of dopamine was not due to Ca^{2+} release via $InsP_3Rs$ but due to the Ca^{2+} entry. In addition, the release of dopamine was mimicked by CPA, which was totally dependent on extracellular Ca^{2+}. All these findings support the hypothesis that CCE and secretion are functionally linked in PC12 cells. Moreover, both the sustained $[Ca^{2+}]$i rise and the release of dopamine evoked by caffeine were inhibited by Zn^{2+}, an inhibitor of CCE (9), without affecting the transient rise in $[Ca^{2+}]$i. These similarities also support the hypothesis.

Caffeine has various non-specific actions, such as the inhibition of phosphodiesterase and adenosine receptors. This raises the possibility that caffeine evoked CCE not by Ca^{2+} release through RyRs directly but as an indirect result of elevating cAMP or inhibiting adenosine receptors. Furthermore, the caffeine-evoked secretion may not be due to CCE but due to such non-specific actions of caffeine. However, both CPA and thapsigargin produced a sustained rise in $[Ca^{2+}]$i which overlapped with that evoked by caffeine (Fig. 1f and g). Theophylline also produced a biphasic $[Ca^{2+}]$i rise only in the presence of extracellular Ca^{2+}. Forskolin had no effect on either the resting or the theophylline-evoked rise in $[Ca^{2+}]$i. Aminophylline (100 μM), an antagonist of non-selective adenosine receptors, had no effect on the caffeine-evoked changes in $[Ca^{2+}]$i. These findings argue against the involvement of either cAMP-dependent or adenosine receptor-mediated mechanisms in the triggering of caffeine-evoked Ca^{2+} entry. With regard to secretion, the CPA-evoked release of dopamine was totally dependent upon the presence of extracellular Ca^{2+}. UTP, which does not affect cAMP formation or adenosine receptors, can also stimulate the release of dopamine only in the presence of extracellular Ca^{2+} (4). Aminophylline had no effects on the release of dopamine evoked by caffeine. These findings strongly suggest that neither cAMP-dependent mechanisms nor the inhibition of adenosine receptors contribute to the caffeine-evoked secretion in the cells.

We have shown that CCE is present in an excitable cell line, PC12 cells, and that it can be stimulated by

both $InsP_3R$- (4) and RyR-activation. Furthermore, CCE has an indispensable role in the secretory response evoked by activation of either $InsP_3R$- or RyR. Although the mechanisms by which RyR activation triggers CCE and CCE promotes the secretory response remain to be examined, our present results indicate a new direction for the study of CCE in relation to secretion.

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