Caffeine Activates a Ca^{2+}-permeable, Nonselective Cation Channel in Smooth Muscle Cells

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ABSTRACT The effects of caffeine on cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and plasma membrane currents were studied in single gastric smooth muscle cells dissociated from the toad, *Bufo marinus*. Experiments were carried out using Fura-2 for measuring $[Ca^{2+}]_i$ and tight-seal voltage-clamp techniques for recording membrane currents. When the membrane potential was held at −80 mV, in 15% of the cells studied caffeine increased $[Ca^{2+}]_i$ without having any effect on membrane currents. In these cells ryanodine completely abolished any caffeine induced increase in $[Ca^{2+}]_i$. In the other cells caffeine caused both an increase in $[Ca^{2+}]_i$ and activation of an 80-pS nonselective cation channel. In this group of cells ryanodine only partially blocked the increase in $[Ca^{2+}]_i$ induced by caffeine; moreover, the change in $[Ca^{2+}]_i$ that did occur was tightly coupled to the time course and magnitude of the cation current through these channels. In the presence of ryanodine, blockade of the 80-pS channel by $GdCl_3$ or decreasing the driving force for Ca^{2+} influx through the plasma membrane by holding the membrane potential at +60 mV almost completely blocked the increase in $[Ca^{2+}]_i$ induced by caffeine. Thus, the channel activated by caffeine appears to be permeable to Ca^{2+}. Caffeine activated the cation channel even when $[Ca^{2+}]_i$ was clamped to below 10 nM when the patch pipette contained 10 mM BAPTA suggesting that caffeine directly activates the channel and that it is not being activated by the increase in Ca^{2+} that occurs when caffeine is applied to the cell. Corroborating this suggestion were additional results showing that when the membrane was depolarized to activate voltage-gated Ca^{2+} channels or when Ca^{2+} was released from carbachol-sensitive internal Ca^{2+} stores, the 80-pS channel was not activated. Moreover, caffeine was able to activate the channel in the presence of ryanodine at both positive and negative potentials, both conditions preventing release of Ca^{2+} from stores and the former preventing its influx. In summary, in gastric smooth muscle cells caffeine transiently releases Ca^{2+} from a ryanodine-sensitive internal store and also increases Ca^{2+} influx through the plasma membrane by activating an 80-pS cation channel by a mechanism which does not seem to involve an elevation of $[Ca^{2+}]_i$.

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INTRODUCTION

Internal Ca^{2+} stores play a role in regulating the levels of cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) by means of pumps that remove Ca^{2+} from the cytoplasm and Ca^{2+} permeable ion channels that in turn can open and release the accumulated Ca^{2+} into the cytoplasm (for a review see Tsien and Tsien, 1990). Two types of Ca^{2+} release channels have been associated with the internal stores: the IP_3 receptor and the ryanodine receptor, named for the agents that bind to the channels and alter their activity. The former has been shown to play a role in agonist mediated release of Ca^{2+} from internal stores in smooth muscle cells (Somlyo, Bond, Somylo, and Scarpa, 1985). The physiological role of the latter in smooth muscle cells remains unclear.

In cardiac cells, Ca^{2+} itself has been shown to be a physiological activator of the ryanodine receptor. This activation presumably underlies the phenomenon of calcium-induced calcium release (CICR) in these cells (for a review see Fabiato, 1989). Caffeine also activates these channels, but the mechanism by which this is accomplished is not known. Because caffeine increases the Ca^{2+} sensitivity of ryanodine receptors incorporated into planar lipid bilayers, presumably it could release Ca^{2+} from internal stores by activating the ryanodine receptor even at resting Ca^{2+} levels (Rousseau and Meissner, 1989; O'Neil and Eisner, 1990). However, at higher concentrations caffeine can activate the ryanodine receptors incorporated in planar bilayers independent of the concentration of internal Ca^{2+} (Sitsapesan and Williams, 1990). Moreover, caffeine is known to have other effects. For example, it increases cAMP levels by inhibiting cyclic nucleotide phosphodiesterase (Williams and Jarvis, 1988) and blocks adenosine receptors (Williams and Jarvis, 1988). On the other hand, ryanodine serves to functionally remove the caffeine-sensitive internal stores in smooth muscle (Iino et al., 1988). Depending on the cell type and/or the concentration ryanodine has been shown either to "lock" the channel into an open, low conductance state as is the case in toad smooth muscle cells (Xu, Lai, Cohn, Etter, Guerrero, Fay, and Meissner, 1994) or to completely block the channel (McPherson, Kim, Valdivia, Knudson, Takekura, Franzini-Armstrong, Coronado, and Campbell, 1991). The former would cause depletion of Ca^{2+} from the stores while the latter would prevent its release.

To study the role of the ryanodine receptor and its associated internal stores in smooth muscle cell contraction we examined the effects of caffeine and ryanodine on $[Ca^{2+}]_i$ in voltage clamped smooth muscle cells isolated from the stomach of the toad, *Bufo marinus*. Ryanodine receptors have been found in these smooth muscle cells (Xu et al., 1994) so as would be expected from studies of caffeine in other cell types, caffeine transiently increased cytoplasmic Ca^{2+} by releasing it from internal stores. However, caffeine also had an unexpected and interesting additional effect: it increased Ca^{2+} influx through the plasma membrane by opening a Ca^{2+} permeable nonselective cation channel whose activation seemed to be independent of $[Ca^{2+}]_i$.

In this paper we discuss the general effects of caffeine on the release of Ca^{2+} from internal stores and on the activation of the cation channel in isolated smooth muscle cells from toad stomach. We also characterize the channel opened by this agent since it provides a new mechanism by which Ca^{2+} can enter the cell. In the accompanying paper (Guerrero, Singer, and Fay, 1994) we provide a quantitative analysis of the

relative contribution of these two pathways to the increase in $[Ca^{2+}]_i$ induced by caffeine. We do this using a method developed in that paper to calculate the fraction of the cation current carried by Ca^{2+}. A brief, preliminary report of this work has been published elsewhere (Guerrero, Singer, and Fay, 1992).

MATERIALS AND METHODS

Cell Isolation and Solutions

Single smooth muscle cells were freshly isolated from the stomach of the toad, *Bufo marinus* as previously described (Fay, Hoffman, LeClair, and Merriam, 1982). For these experiments the extracellular solution contained (in millimolar): 120 NaCl, 3 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 TEACl, 5 HEPES pH 7.6 (NaOH). The pipette solution, which was designed to allow the isolation and recording of Ca^{2+} currents and the measurement of free $[Ca^{2+}]_i$ at the same time, usually contained (in millimolar): 130 CsCl, 4 $MgCl_2$, 3 Na_2ATP, 1 Na_3GTP, 0.05 K_5Fura-2 and 20 HEPES, pH 7.2 (CsOH). For pipette solutions containing low $[Cl^-]$, 130 mM Cesium-glutamate (Csglutamate) was substituted for CsCl. Sometimes 5–10 mM BAPTA was included in the pipette solution to prevent any increases in $[Ca^{2+}]_i$. BAPTA also seemed to improve the access to the cytoplasm. BAPTA had no obvious effect on single channel conductance or reversal potential of the caffeine activated channel. All experiments were carried out at room temperature.

Voltage Clamp and Fura-2 Loading

Whole-cell membrane currents were recorded using patch pipettes (4–7 MΩ) with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). With the average access resistance of 10 MΩ, 5 min were usually allowed for Fura-2 to enter the cell before beginning the experimental protocols. Input resistance was measured from the current-voltage relationship obtained over a range of −110 to −80 mV using 10 mV pulses. Three second depolarizing pulses to 0 mV from a holding potential of −80 mV were used to obtain simultaneous measurements of Ca^{2+} currents and $[Ca^{2+}]_i$ for determining the Ca^{2+} buffer capacity and the rate of cytoplasmic Ca^{2+} removal for each cell (see Guerrero, Singer, and Fay, 1994 for details). Membrane potential and currents were sampled each 3 ms and stored in a PDP 11/73 microcomputer for further analysis as described previously (Becker, Singer, Walsh, and Fay, 1989; Moore, Becker, Fogarty, Williams, and Fay, 1990).

$[Ca^{2+}]_i$ Measurements

Fura-2 fluorescence was measured using a system consisting of an inverted Zeiss microscope with a fast rotating filter wheel as described previously (Yagi, Becker, and Fay, 1988; Becker et al., 1989; Moore et al., 1990). Fluorescence was collected each 3 ms and stored in a PDP 11/73 microcomputer for further analysis. An image mask was used to exclude the field without the cell to reduce the background contribution of the non-cell regions to the overall signal thereby increasing the signal to noise ratio. Fura-2 ratios corrected for background fluorescence (measured just after gigaseal formation), were converted to $[Ca^{2+}]_i$ using the ratio method (see Grynkiewicz, Poenie, and Tsien, 1985) by a custom written program employing a calibration chamber and a kD of 200 nM. Maximum (R_{max}) and minimum (R_{min}) 340/380 fluorescence ratios were reduced by 15% to correct for viscosity (Poenie, 1990). The R_{max} used was 14.6; R_{min}, 0.4; and β (the ratio of the 380 fluorescence of the Ca^{2+} free to Ca^{2+} bound forms of Fura-2), 12.8.

Chemicals and Drugs

Caffeine (20 mM) or Carbachol (100 μM) were applied by pressure ejection from a micropipette positioned about 100 μm from the cell using a Picospritzer II (General Valve, Fairfield, NJ) (Lassignal, Singer, and Walsh, 1986). The concentrations given are those in the pressure ejection pipette; the concentration at the cell surface would tend to be lower even though the chamber was not continuously perfused with bathing solution. Successive applications of an agent might not provide the same time course in the local concentration at the cell due to variabilities in the application pressure and in the flow pattern at the tip of the pipette. Ryanodine (final concentration 100 μM) (Agrisystems International) was added to the pipette solution from a 10 mM aqueous stock solution or added to the bath (2.4 ml) from a 100-mM DMSO stock solution. We found that 5 min after breaking into the cell was a sufficient period of time for ryanodine to inhibit the effect of caffeine on internal stores. K_5Fura-2 was obtained from Molecular Probes, Inc. (Eugene, OR). The other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Statistics

Unless otherwise indicated all statistical measurements are given as the mean ± the standard error of the mean (SEM) with the number (n) of cells indicated.

RESULTS

Caffeine Releases Ca^{2+} from a Ryanodine-sensitive Internal Store

Application of caffeine (20 mM) to single smooth muscle cells with the membrane potential held at −80 mV causes a transient increase in $[Ca^{2+}]_i$ (Figs. 1 *A* and 2). In ~15% of the cells studied (n = 71) there was no change in the holding membrane current accompanying this response (Fig. 1). In the remaining cells an inward current was activated (see next section below and Fig. 2).

When there was no change in the membrane current, caffeine produced an abrupt increase in $[Ca^{2+}]_i$ followed by a slower return to the resting level (Fig. 1). In these cells, recovery occurred even in the continued presence of caffeine (see Guerrero et al., 1994, for other examples). $[Ca^{2+}]_i$ increased by 364 ± 36 nM (n = 4) at the peak of the Ca^{2+} transient from a resting level of 58.6 ± 7.3 nM (n = 14).

If caffeine is acting by releasing Ca^{2+} from internal stores by its action on the ryanodine receptor (Iino et al., 1988), then in the presence of ryanodine the effect of caffeine should be diminished or blocked. Consistent with this mechanism of action, when ryanodine (100 μM) was added to the pipette solution, caffeine failed to produce any significant change in $[Ca^{2+}]_i$ in the cells that did not show any change in the membrane current (Fig. 1 *B*). For these cells $[Ca^{2+}]_i$ was changed by 5.0 ± 7.4 nM (n = 3) from a resting level of 65.3 ± 5.5 nM (n = 10).

The absence of an effect of caffeine in the presence of ryanodine strongly suggests that in those cells where caffeine had no effect on the holding current the caffeine-induced increase in $[Ca^{2+}]_i$ was due exclusively to release from internal stores. However, because the cells were bathed in solutions containing 1.8 mM $[Ca^{2+}]$, it is possible that a small current (less than the 1-pA limit of resolution of our recording system) carrying Ca^{2+} could produce the caffeine-induced change in $[Ca^{2+}]_i$. From the time course of the increase in Ca^{2+}, the cytoplasmic Ca^{2+} buffer capacity and the removal of Ca^{2+} (Guerrero et al., 1994) we calculated the size of a

pure Ca^{2+} current required to produce the observed change in $[Ca^{2+}]_i$. This is indicated as the dashed line in Fig. 1 *A*. As can be seen, a Ca^{2+} current with a peak greater than 100 pA would be required to produce the caffeine induced Ca^{2+} transient. For four cells where this exercise was carried out the peak current required was 86 ± 13 pA. Since for this subset of gastric smooth muscle cells there was no

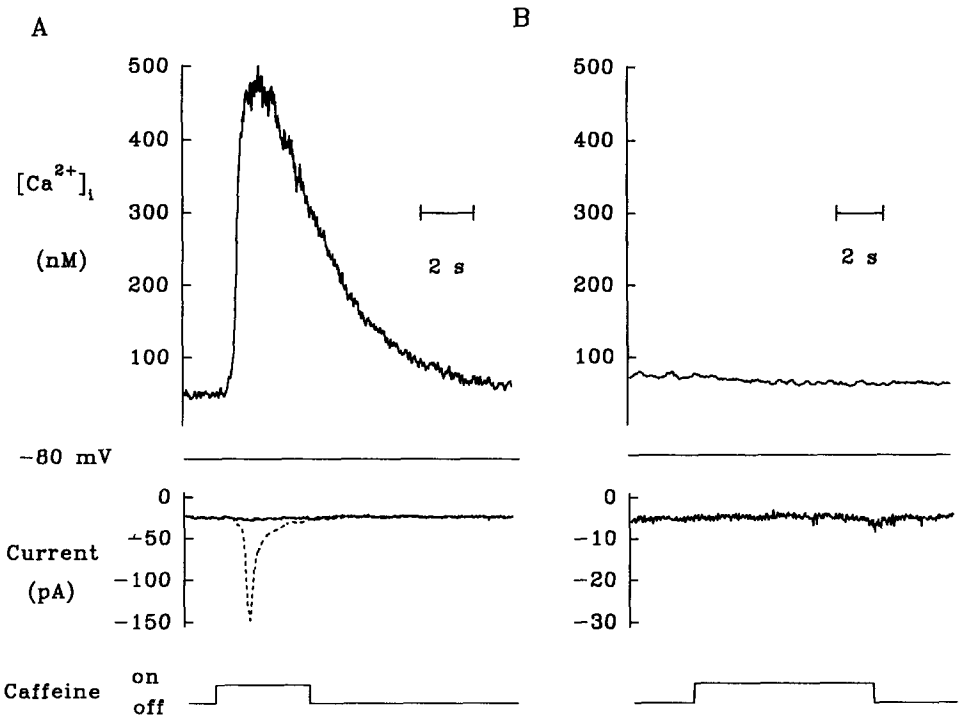

FIGURE 1. Caffeine induces an increase in $[Ca^{2+}]_i$ which is completely blocked by ryanodine only in those cells without a caffeine-induced inward current. (*A*) Application of caffeine (*bottom trace*) to a cell when the membrane potential (*upper middle trace*) was held at −80 mV caused an increase in $[Ca^{2+}]_i$ (*top trace*). In ~15% of the cells there was no change in the membrane current (*lower middle trace*). Note the fast rising phase of the change in $[Ca^{2+}]_i$ and the return of $[Ca^{2+}]_i$ toward the resting level even in the continuous presence of caffeine. Dashed line in the current trace is an estimate of a pure Ca^{2+} current that would have been required to produce the observed caffeine-induced increase in $[Ca^{2+}]_i$ if the rise in Ca^{2+} was entirely due to Ca^{2+} influx. This estimated current was obtained by differentiating the change in $[Ca^{2+}]_i$ (after adding back Ca^{2+} removed from the cytoplasm) taking into account cytoplasmic Ca^{2+} buffering and assuming a cell volume of 6 pL (see Guerrero et al., 1994). (*B*) The caffeine-induced change in $[Ca^{2+}]_i$ was completely blocked (in another cell) when 100 μM ryanodine was present in the pipette solution.

significant change in the holding membrane current observed with the application of caffeine and since the effect of caffeine on $[Ca^{2+}]_i$ was blocked by ryanodine, the Ca^{2+} transient induced by caffeine in these cells is most likely due only to release of Ca^{2+} from internal stores by activation of the ryanodine receptor.

Caffeine Releases Ca^{2+} from Internal Stores and Activates a Nonselective Cation Channel

In 85% of the cells caffeine not only increased $[Ca^{2+}]_i$, but, in addition, it induced an inward current (peak 80 ± 6.7 pA; n = 20) at −80 mV (Fig. 2). In these cells where caffeine induced an inward current, peak $[Ca^{2+}]_i$ was similar to that found in cells without the inward current [peak $[Ca^{2+}]_i$ increased by 342 ± 26 nM (n = 8)], but the transient was more prolonged (see Guerrero et al., 1994). Moreover, $[Ca^{2+}]_i$ did not

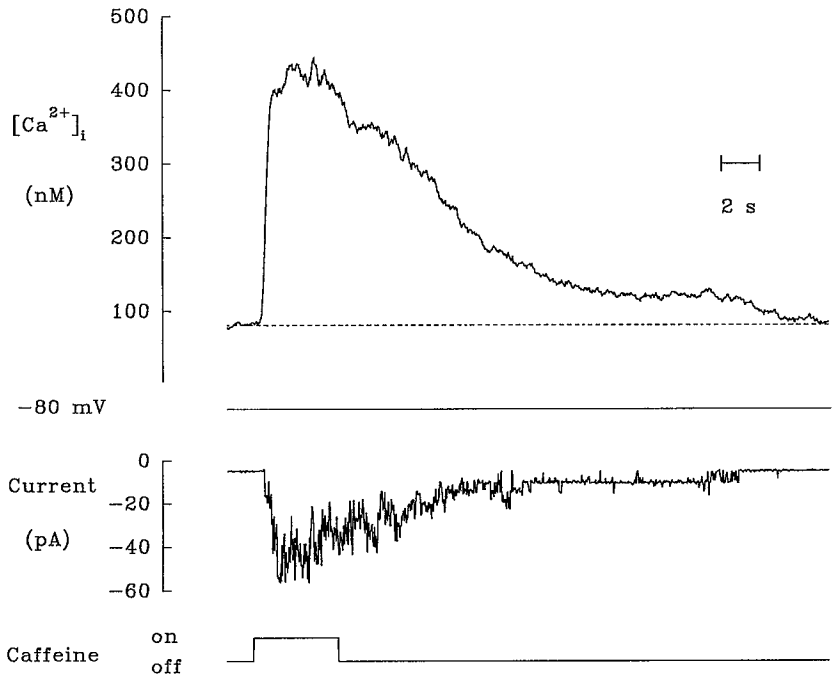

FIGURE 2. Caffeine (*bottom trace*) increases $[Ca^{2+}]_i$ (*top trace*) and activates channels producing inward currents (*lower middle trace*) in a cell with the membrane potential (*upper middle trace*) held at −80 mV. The change in $[Ca^{2+}]_i$ was much more prolonged in those cells with the caffeine-induced inward current (compare to Fig. 1 *A*). $[Ca^{2+}]_i$ remained elevated above the resting level (*dashed line*) until the caffeine-activated current returned to zero. Even the current (recorded in the tight-seal, whole-cell configuration) due essentially to one channel being open at a time (at the end of the current trace) was able to maintain the $[Ca^{2+}]_i$ above resting level in this cell. The rate of rise of the current for this cell was somewhat faster than usual.

completely return to its resting level as long as the current was present suggesting that some Ca^{2+} influx was associated with the activation of this current. For the particular response illustrated in Fig. 2, the inward current associated with a single open channel (beginning at ~14 s after the onset of caffeine application) could maintain the elevation of $[Ca^{2+}]_i$. Furthermore, responses were similar with prolonged application of caffeine (see Guerrero et al., 1994) in that as long as the channels were active (which they usually were even with prolonged application), $[Ca^{2+}]_i$ remained elevated. Since this channel could provide another pathway in

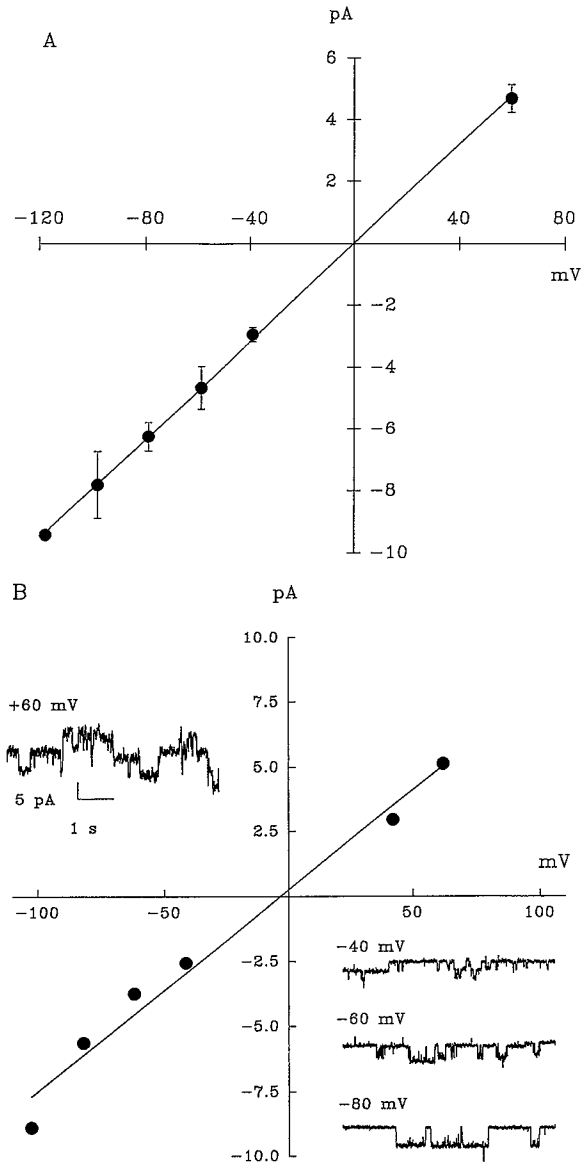

FIGURE 3. Current-voltage relationships of the caffeine-activated channel have a reversal potential near zero mV as expected for a cation channel. Because of the low average number of channels open, by using short (< 1 s) applications of caffeine (20 mM) it was possible to record clearly resolvable unitary currents in the tight-seal, whole-cell configuration. Amplitudes of the unitary currents were measured at the potentials indicated. (*A*) The data shown is the mean ± SD for six different cells with the usual pipette solution containing CsCl. For these cells the single channel conductance was 79.8 ± 0.6 pS and the interpolated reversal potential, −0.2 ± 1.2 mV. (*B*) Current-voltage relationship for a cell where Csglutamate replaced CsCl in the pipette solution. For this cell the single channel conductance was 77 pS and the interpolated reversal potential −3 mV. Sample traces are shown for the potentials indicated.

addition to voltage-gated Ca^{2+} channels (Nelson, Standen, Brayden, and Worley, 1988), stretch-activated channels (Kirber, Walsh, and Singer, 1988; Franco and Lansman, 1990; Wellner and Isenberg, 1993), and Na^+/Ca^{2+} exchange (Aaronson and Benham, 1989) for Ca^{2+} entry across the plasma membrane into the cell we decided to characterize it in more detail.

Channel Characteristics

Current-voltage plots obtained for the caffeine-activated channel were nearly linear between +60 and −60 mV with a single channel conductance of ~80 pS (79.6 ± 0.6;

mean ± SD; $n = 6$) in normal bathing solution (1.8 mM $[Ca^{2+}]_e$) and with an interpolated reversal (or zero current) potential near 0 mV (Fig. 3 *A*). Reversal potentials were actually a little more positive than zero since application of caffeine to cells held at 0 mV caused a small inward current accompanied by a small increase in $[Ca^{2+}]_i$ in the presence of ryanodine [not shown]). The reversal potential and single channel conductance was not significantly affected by replacing CsCl with Csglutamate ($n = 2$) (see Fig. 3 *B*) or with NaCl ($n = 3$) (conductance = 77.5 ± 3.5 pS; reversal potential = −1.5 ± 0.1 mV) or with KCl ($n = 3$) (conductance = 85 ± 4 pS;

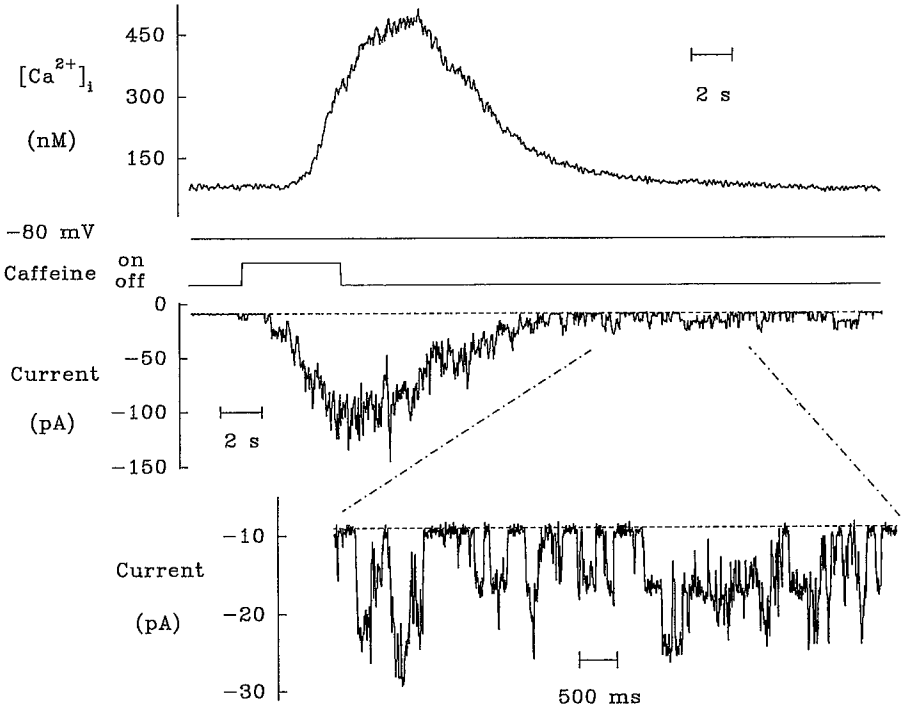

FIGURE 4. In those cells with a caffeine-induced inward current (*bottom two traces*) caffeine caused an increase in $[Ca^{2+}]_i$ (*top trace*) in the presence of ryanodine (100 μM) (compare with Fig. 1 *B*) but the rate of rise of $[Ca^{2+}]_i$ was decreased (compare with Figs. 1 *A*, and 2). Caffeine (20 mM) was applied for the time indicated to a cell held at −80 mV which had been exposed to ryanodine (in the pipette solution) for at least 5 min. (*Bottom trace*) Part of the current trace at expanded current and time scales to provide a clear view of the unitary currents.

reversal potential = −4.1 ± 1.0 mV) in the pipette solution suggesting that the channel activated by caffeine is a cation selective channel. In the absence of external Ca^{2+} (Mg^{2+} replacing Ca^{2+} but no chelator added) the single channel conductance was increased (to 110 pS) ($n = 2$). On the other hand, the whole-cell currents obtained in higher concentrations of external Ca^{2+} (20 mM) were smaller and unitary currents were difficult to discern (see Fig. 3 in Guerrero et al., 1994). These effects of changes in the external Ca^{2+} on conductance are consistent with results obtained in other types of Ca^{2+} permeable cation channels (see Kirber et al., 1988, for another

example in toad smooth muscle cells). As is the case for many other nonselective cation channels, the channel studied here did not seem to be voltage-activated; that is, changes in the membrane potential did not cause channel openings. However, we did not determine whether membrane potential could modulate the activity of channels already activated by caffeine.

The Caffeine-activated Cation Channel Is Ca^{2+} Permeable

The prolonged $[Ca^{2+}]_i$ transient in those cells where caffeine activated cation channels suggested that the channel was Ca^{2+} permeable. That this was indeed the

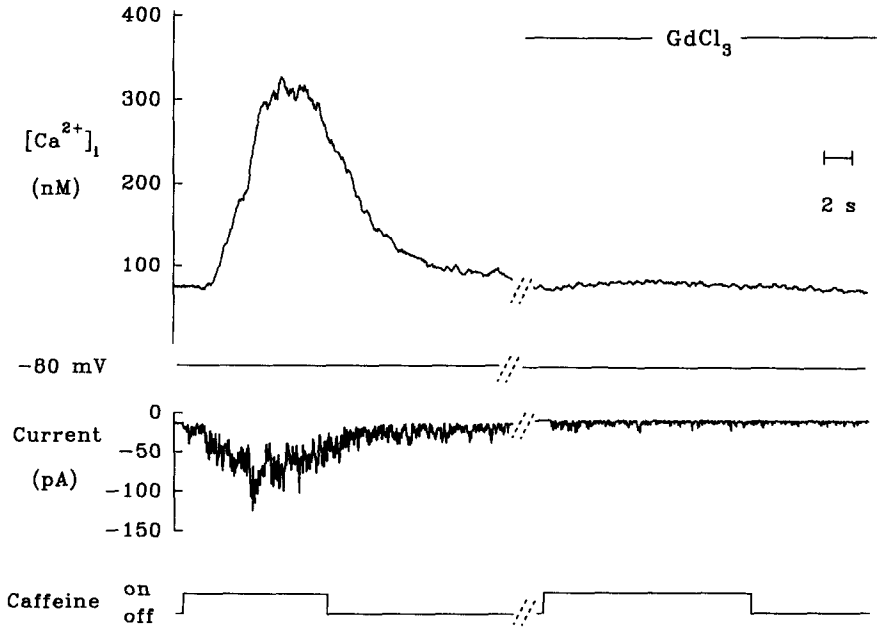

FIGURE 5. Blocking Ca^{2+} influx through the caffeine-activated cation channels (*lower middle trace*) with $GdCl_3$ (*right*) decreases the caffeine induced rise in $[Ca^{2+}]_i$ (*top trace*) in the presence of ryanodine. Caffeine (20 mM) was applied for the time indicated to a cell held at −80 mV which had been exposed to ryanodine (100 μM in the pipette solution) for at least 5 min. $GdCl_3$ was added to the bath (*right*) from a 100 mM stock solution (final concentration 100 μM) ~5 min after the first application of caffeine. Caffeine was again applied to the cell as indicated (*bottom trace*) after verifying that Gd^{3+} was indeed blocking channels by demonstrating blockade of Ca^{2+} current (not shown) in the same cell.

case was shown by adding 100 μM ryanodine to the pipette solution to eliminate the contribution of internal stores to the caffeine-induced increase in $[Ca^{2+}]_i$. Unlike the cells described above where caffeine did not activate cation channels at −80 mV (Fig. 1 *B*), in the presence of ryanodine, application of caffeine to cells with cation channels increased $[Ca^{2+}]_i$ (Fig. 4). However, the increase in $[Ca^{2+}]_i$ occurred at a much slower rate than when ryanodine was not present; and the time course followed the activity of the channels, increasing with increased channel activity and falling as

channel activity returned to zero (compare with Fig. 2). These results are consistent with caffeine activating a cation channel permeable to Ca^{2+}. We carried out two additional sets of experiments using GdCl3 and changes in driving force to further verify this conclusion.

If the channel activated by caffeine was permeable to Ca^{2+} and the associated influx of Ca^{2+} through this channel was one source of the elevation in $[Ca^{2+}]_i$, then, in the presence of ryanodine to eliminate the contribution of internal stores, blocking the channel currents should suppress the Ca^{2+} transient. We used $GdCl_3$, which blocks both cation channels and voltage-gated Ca^{2+} channels (Franco and Lansman, 1990; Lansman, 1990) to block the caffeine-induced cation current (Fig. 5). In the

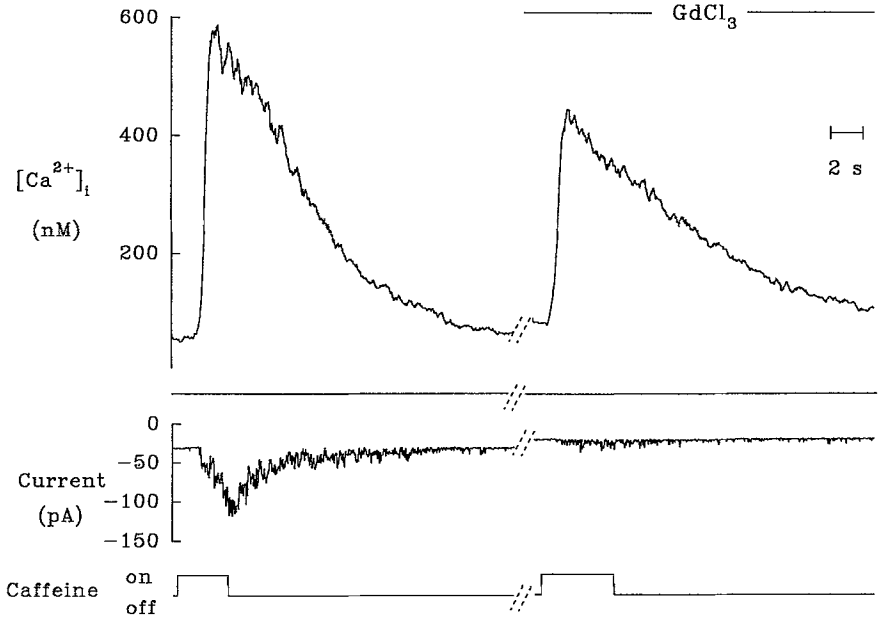

FIGURE 6. $GdCl_3$ does not interfere with the ability of caffeine to release Ca^{2+} from internal stores. In the absence of ryanodine, caffeine was applied to the cell before and after exposure of the cell to $GdCl_3$. The protocol was the same as for Fig. 5, but here there was a significant rise in the internal Ca^{2+} (*upper trace*) even though the caffeine-induced current (*lower middle trace*) was substantially inhibited by $GdCl_3$. Compare with Fig. 5.

presence of ryanodine 89.6 ± 1.3% (n = 4) of the caffeine-induced current was blocked by $GdCl_3$ (100 μM) (when 10-s currents integrals were compared in the same cell before and after $GdCl_3$). As expected, this inhibition of the caffeine-induced current caused an almost complete block of the Ca^{2+} transient in response to caffeine (Fig. 5) ($[Ca^{2+}]_i$ increased only 13 ± 6.8 nM, n = 3).

To demonstrate that $GdCl_3$ had no major effect on the release of Ca^{2+} from internal stores (or on Fura-2 fluorescence), we applied $GdCl_3$ in the absence of ryanodine (Fig. 6). Under this condition the caffeine-induced current was blocked as indicated before. However, the change in peak $[Ca^{2+}]_i$ was reduced only by 14.75 ± 2.0% (n = 4), and the rate of rise was not discernably affected (Fig. 6). This result

indicates that in experiments like those illustrated in Fig. 5, Gd^{3+} blocked the change in $[Ca^{2+}]_i$ in the presence of ryanodine by blocking Ca^{2+} influx and not by interfering with Fura-2 fluorescence or by itself interfering with release of Ca^{2+} from internal stores. It also suggests that the fast initial phase of the rise in Ca^{2+} is due to release of Ca^{2+} from internal stores (see Guerrero et al., 1994).

If caffeine is increasing Ca^{2+} influx through the plasma membrane by opening cation channels, then this influx should be affected by alterations in the driving force for Ca^{2+}. When the membrane potential was held at +60 mV in the presence of

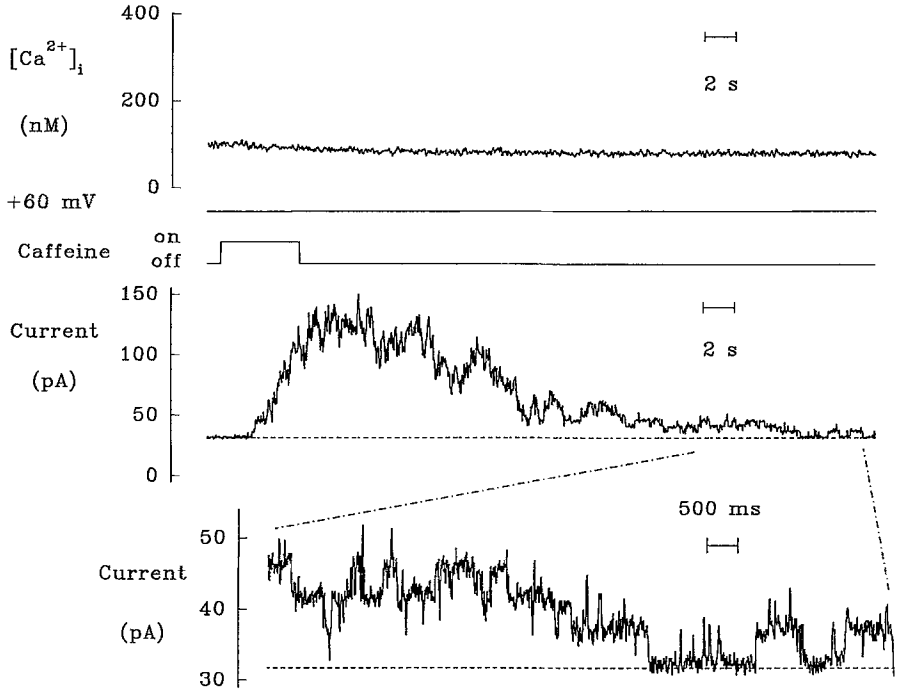

FIGURE 7. The change in $[Ca^{2+}]_i$ induced by caffeine in the presence of ryanodine was affected by the electrochemical gradient for Ca^{2+}. Application of caffeine (*middle trace*) did not increase $[Ca^{2+}]_i$ (*top trace*) when the cell was held at +60 mV and ryanodine was present in the pipette solution. This is to be expected since the driving force for Ca^{2+} entry through the cation channels would be close to zero. Bottom trace shows the current record on an expanded time scale so clear unitary currents can be observed.

ryanodine, caffeine still induced a current; but at this potential the current was outward (Fig. 7). As would be expected for a Ca^{2+} permeable channel with the membrane potential held close to the effective E_{Ca} some Ca^{2+} might pass through the channel but not enough to cause a detectable change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$ = 2.4 ± 8.2 nM, n = 7). When ryanodine was not present, caffeine applied to cells held at +60 mV increased free $[Ca^{2+}]_i$ by 422 ± 19.6 nM (n = 5, Fig. 8) with a time course and peak which was not very different from the response in those cells where the current was absent (Fig. 1 *A*) or inhibited by $GdCl_3$ (Fig. 6). Thus, the current passing

through the caffeine-activated cation channels appears to be at least partially carried by Ca^{2+} (see Guerrero et al. [1994] for a quantitative analysis), and it is this current that contributes to the elevation in $[Ca^{2+}]_i$ with the application of caffeine.

Caffeine-activated Cation Channel Does Not Require an Increase in Cyclic AMP or $[Ca^{2+}]_i$ to Open

We examined two mechanisms which might underlie the opening of cation channels by caffeine: elevation of cyclic AMP by the caffeine inhibition of phosphodiesterase

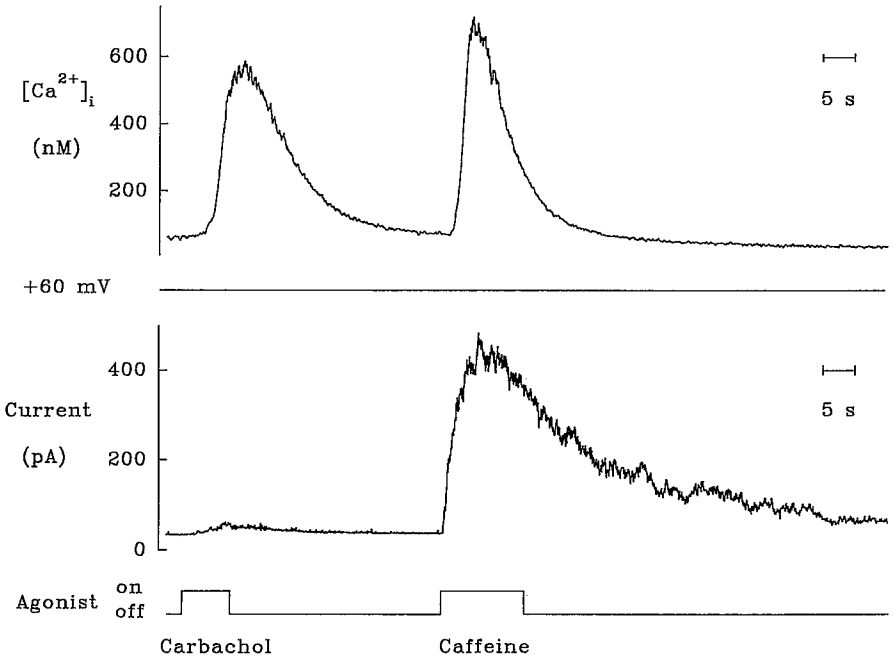

FIGURE 8. Release of Ca^{2+} from internal stores is not sufficient to activate the caffeine-activated cation channels. The cell membrane potential was held at +60 mV and exposed first to 100 μM carbachol and 30 s later to 20 mM caffeine (*bottom trace*). Both agents caused a similar increase in the internal Ca^{2+} by release from stores, but only caffeine was effective in activating the channel (*lower middle trace*). The origin of the small outward current with the application of carbachol is unclear (see Discussion). It is not activated at −80 mV (unlike cation channels activated by caffeine) suggesting that the source of the outward current is not from the caffeine-activated cation channel.

and/or elevation of $[Ca^{2+}]_i$. If caffeine was activating cation channels by elevating intracellular cyclic AMP, then it should be mimicked by application of the phosphodiesterase resistant cyclic AMP analogue, 8-Bromo-cAMP. When 10 mM of this analogue was included in the pipette solution, it did not activate the channel nor did it block channel activation by caffeine (n = 7). These results suggest that caffeine was not acting by this mechanism.

Because many nonselective cation channels are activated by elevation of $[Ca^{2+}]_i$ (Partridge and Swandulla, 1988), we carried out two types of experiments to

determine whether this is the case for the caffeine-activated channels in these smooth muscle cells. In one type of experiment we raised the internal Ca^{2+} by applying carbachol (100 μM) to the cell when the membrane potential was held at +60 mV. The increase in $[Ca^{2+}]_i$ that occurred presumably by release from internal stores (Becker, Fay, Singer, Montana, and Walsh, 1991) caused only a minimal change in the holding current (Fig. 8). However, 30 s later when the $[Ca^{2+}]_i$ levels were almost back to resting levels application of caffeine to the same cell also increased $[Ca^{2+}]_i$

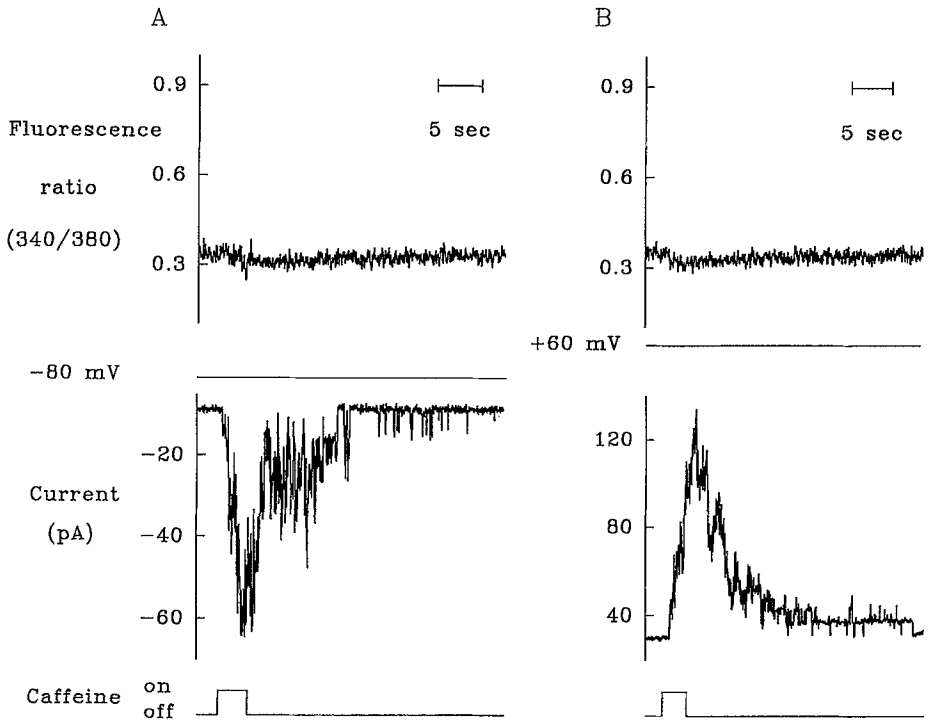

FIGURE 9. Activation of cation channels by caffeine does not require an increase in $[Ca^{2+}]_i$. Whether the membrane potential was held at either −80 mV (*A*) or +60 mV (*B*), application of caffeine caused activation of cation channels (*lower middle trace*) even with 10 mM BAPTA present in the pipette solution to chelate Ca^{2+} and prevent its increase (*top trace*). At least 5 min were allowed for BAPTA to pass into the cell before application of caffeine. BAPTA clamped $[Ca^{2+}]_i$ below 10 nM. The fluorescence ratio (*top trace*) was not converted to $[Ca^{2+}]_i$ because its value was so close to R_{min} (typical noise level in fluorescence measurements) that it caused the calculated estimates of Ca^{2+} to frequently go below zero.

but in this case there was substantial activation of the cation channels (n = 9). Thus, an increase in $[Ca^{2+}]_i$ per se does not activate cation channels.

In a second type of experiment caffeine was applied to cells voltage clamped (at either −80 or +60 mV; Fig. 9) with 10 mM BAPTA in the pipette solution to chelate $[Ca^{2+}]_i$. Caffeine caused activation of cation channels in the absence of any change in $[Ca^{2+}]_i$ (the value is lower than 10 nM because the fluorescence ratio is close to R_{min}) (n = 8). In light of these results, along with the results that caffeine activates the

cation channel in the presence of ryanodine at −80 mV (Fig. 4) and +60 mV (Fig. 7), a strong argument can be made against caffeine activating cation channels indirectly by releasing Ca^{2+} from internal stores. Instead, it appears that caffeine activates the channel directly or indirectly through some other mechanism. Various possibilities are discussed below.

DISCUSSION

The clear demonstration of ryanodine receptors in vascular smooth muscle cells (Herrmann-Frank, Darling, and Meissner, 1991) as well as in the smooth muscle cells from toad stomach (Xu et al., 1994) led us to carry out the studies described here and in a companion paper (Guerrero et al., 1994). Our goal was to better understand the role of these receptors and their associated internal Ca^{2+} store in the control of $[Ca^{2+}]_i$ in smooth muscle cells. Experiments were carried out in voltage clamped toad stomach smooth muscle cells, and as expected we found that caffeine, an agent known to affect ryanodine receptors, releases Ca^{2+} from a ryanodine sensitive internal store in these cells. Surprisingly, we found that caffeine also activates a nonselective Ca^{2+} permeable cation channel by a mechanism that does not appear to involve an increase in $[Ca^{2+}]_i$. Although caffeine-activated channels have been described in other cell types (see below), the mechanism of activation appears to be indirect due to the rise in $[Ca^{2+}]_i$ from the effect of caffeine on internal stores. Thus, the caffeine-activated channel found in toad smooth muscle cells provides a new mechanism by which Ca^{2+} can enter the cell, and as such, to our knowledge, has not been reported before in smooth muscle or any other preparation.

Release of Ca^{2+} from Internal Stores

Experiments claiming to demonstrate agonist induced release of Ca^{2+} from internal stores are usually carried out by applying the agonist in the absence of external Ca^{2+} so as to avoid the complication of influx across the plasma membrane. A major drawback of this approach is that the results are very sensitive to the time the cells have been exposed to zero Ca^{2+}. For example, too long an application of Ca^{2+}-free bathing solution to the smooth muscle preparation can by itself cause an unwanted depletion of the internal stores (Iino, 1990; Matsumoto, Kanaide, Shogakiuchi, and Nakamura, 1990) while too short an application may not sufficiently decrease the Ca^{2+} levels in the extracellular fluid adjacent to the cell membrane.

In the studies described here we have used a different approach. By maintaining the membrane potential near −80 mV using the voltage-clamp technique we have avoided any influence of voltage-gated Ca channels on our measurements of $[Ca^{2+}]_i$. Moreover, the experiments can be carried out at physiological concentrations of external Ca^{2+} so the $[Ca^{2+}]_i$ stores are not altered before the agonist (in our case, caffeine) is applied.

However, our ability to clearly demonstrate the contribution of internal stores to the change $[Ca^{2+}]_i$ relies on the use of ryanodine to completely eliminate that part of the response due to caffeine-sensitive internal Ca^{2+} stores. Three types of experiments described here provide evidence that ryanodine does indeed do so. First, ryanodine completely blocked the caffeine induced increase in $[Ca^{2+}]_i$ in cells where caffeine did not activate Ca^{2+} permeable plasma membrane cation channels. Second,

in those cells with caffeine-activated cation channels, in the presence of ryanodine caffeine had no effect on $[Ca^{2+}]_i$ when the membrane potential was held at +60 mV to reduce the driving force for Ca^{2+} influx to near zero. Third, again in those cells with caffeine-activated channels, in the presence of ryanodine caffeine caused a minimal increase in $[Ca^{2+}]_i$ with the membrane potential held at −80 mV if the current passing through the channels was almost completely blocked by $GdCl_3$. Thus, ryanodine appears to completely eliminate the contribution to the observed change in $[Ca^{2+}]_i$ of any release of Ca^{2+} from caffeine sensitive internal stores. Moreover, any caffeine induced increase in $[Ca^{2+}]_i$ in the presence of ryanodine could be attributed to the activation of Ca^{2+} permeable cation channels present in the plasma membrane of these cells. These three results are consistent with the demonstrated effect in bilayers of 10 μM ryanodine on the ryanodine receptor isolated from the same cells used in these studies (Xu et al., 1994). Ryanodine causes the channel to go into a maintained open subconductance state which would tend to cause depletion of stores in the intact cell. Ryanodine in 100 μM concentration may even close the channel after a few minutes (Rousseau and Meissner, 1989). Either way ryanodine removes stores from consideration in any studies of the effect of caffeine on $[Ca^{2+}]_i$.

Effect of Caffeine-induced Plasma Membrane Ion Currents on the Prolongation of the Rise in $[Ca^{2+}]_i$

In those cells with a caffeine activated inward current, the caffeine induced $[Ca^{2+}]_i$ transient was prolonged, with the duration and magnitude of the transient closely correlating with the magnitude and time course of the current. Others have also reported that caffeine produces a sustained or more prolonged increase in $[Ca^{2+}]_i$ in normal external $[Ca^{2+}]$ (Bals, Bechem, Paffhausen, and Pott, 1990; Baró and Eisner, 1992; Chen, Cannel, and van Breemen, 1992; Itoh et al., 1992; Marrion and Adams, 1992). Some of these investigators proposed that an important component of the prolonged time course of the Ca^{2+} transient was the continuous release of Ca^{2+} from internal stores which occurred because caffeine kept the Ca^{2+} release channels open (Bals et al., 1990; Chen et al., 1992; Marrion and Adams, 1992). However, a caffeine induced inward current at least partially carried by Ca^{2+} could also have contributed to their observed Ca^{2+} transient. It remains to be seen if such channels are identified in these or other preparations.

In toad smooth muscle cells missing the caffeine-induced inward current, $[Ca^{2+}]_i$ returns to resting levels even in the continuous presence of caffeine (Fig. 1 and Guerrero et al., 1994). Rat ventricular myocytes show a similar decline in $[Ca^{2+}]_i$ levels in the continued presence of caffeine (O'Neill and Eisner, 1990). We could not determine whether this was due to depletion of internal stores by caffeine or to some sort of desensitization of the Ca^{2+} release channel to caffeine or inactivation of the channels. In the accompanying paper (Guerrero et al., 1994) we provide some indication that the former may be the case in toad cells.

Caffeine-activated Channels

Cation channels activated by caffeine have also been found in smooth muscle cells isolated from rat portal vein (Loirand, Pacaud, Baron, Mironneau, and Morinneau, 1991), rabbit ear artery (Wang, Hogg, and Large, 1993), canine stomach (Sims, 1992), and canine and guinea-pig trachea (Janssen and Sims, 1992). In the first two

preparations single channel conductances were obtained and were different from the cation channel conductance in toad stomach cells being considerably larger (~200 pS) in portal vein or considerably smaller (28 pS) in ear artery. Moreover, it appears that the former is Ca^{2+} permeable whereas the latter is not. There is another important difference between the caffeine-activated channels found in all of these preparations and in toad stomach. The channels in these preparations appear not to be activated by caffeine in the same manner as occurs in toad stomach but instead appear to be activated indirectly by the rise in $[Ca^{2+}]_i$ caused by the caffeine-induced release of Ca^{2+} from internal stores. There has also been a report of a voltage-sensitive influx of Ca^{2+} in the presence of caffeine in guinea-pig jejunal smooth muscle cells (Pacaud and Bolton, 1991). In this preparation the nature of the channel activated by caffeine is not clear.

We have provided evidence here indicating that the channel in toad stomach is not a Ca^{2+}-activated channel and is instead activated by caffeine by some other mechanism. First, caffeine activated the channel in the presence of ryanodine which completely eliminated the caffeine induced release of Ca^{2+} from internal stores (Figs. 4 and 7). Second, caffeine activated the channel with 10 mM BAPTA inside the pipette which clamped $[Ca^{2+}]_i$ to below 10 nM (Fig. 9). Third, in the absence of caffeine when $[Ca^{2+}]_i$ was increased by activation of muscarinic receptors at +60 mV (Fig. 8) the cation channel was not activated. Other experiments also corroborate these findings: we have previously shown that application of acetylcholine causes an elevation in $[Ca^{2+}]_i$ without activating any inward current at −80 mV in 20 mM external $[Ca^{2+}]$ (Becker et al., 1991). Moreover, the elevation of $[Ca^{2+}]_i$ accompanying activation of voltage-gated Ca^{2+} channels does not activate cation channels since, when the membrane potential was repolarized back to −80 mV after eliciting the Ca^{2+} current at 0 mV, there was no inward current even though the $[Ca^{2+}]_i$ was still elevated (Becker et al., 1989).

Effects of Carbachol on Internal Stores and Membrane Current

An interesting feature of the dual application of carbachol and caffeine to the same cell (Fig. 8) is that while caffeine as expected (from Figs. 7 and 9) caused a marked outward current (at +60 mV in Fig. 8) carbachol also caused an outward current, though very small in size (seen in nine cells). This carbachol-activated outward current is not always seen, and its origin is unclear. Unlike as occurs with caffeine, this current does not appear at potentials near −80 mV in response to the application of acetylcholine (Becker et al., 1991) or 100 μM carbachol (12 cells) even when an accompanying increase in $[Ca^{2+}]_i$ occurs. Thus, it would appear that the outward current elicited by carbachol is not the same as the caffeine-activated current.

The Average Number of Channels Opened by Caffeine Is Small

As can be seen in Figs. 2, 4, 5, 6, and 9, the number of channels opened at any one time by caffeine is small. This is made all the more clear by realizing that the single channel currents are being recorded not in isolated patches of membrane but in the tightseal whole-cell recording configuration. The average peak inward currents of ~100 pA or less indicate that at one time only ~10–15 channels are open. This would occur if the density (or the number of channels [N]) of caffeine-activated cation

channels in the cell membrane were quite low or if in the presence of caffeine the channels have a low probability of being open (P_o). Luckily, the open times are sufficiently long so this activation even with only a few channels open on the average (low NP_o) can be observed. However, even the opening of a single channel can have a profound influence on the $[Ca^{2+}]_i$ levels (see Fig. 2, for example).

It is unlikely that the low NP_o observed is due to the loss (or addition) of cytosolic constituents from the dialysis that occurs after breaking into the cell with the patch pipette. The same results were obtained in the presumed absence of dialysis when perforated patch recordings were made (Guerrero et al., 1994). There are, however, a number of possible explanations for the observed low level of activity of the channels activated by caffeine: (*a*) caffeine may be mimicking the effect of some endogenous transmitter, second messenger, or metabolic product on a receptor site on or closely associated with the channel, but caffeine itself is only a weak activator of the channel. (*b*) It is not caffeine itself that activates the channel but caffeine acting indirectly by altering the level of a second messenger or metabolic product which, in turn, affects the channel. With caffeine having only a weak effect on the levels of these intermediaries, the average number of channels that will be open in response to caffeine is low. (*c*) Caffeine may be having a direct effect on the channel normally activated by some other agent but it acts at a site different from the endogenous or natural agent. (*d*) Any possible coupling between channel activity and the stores is weak or missing under our experimental conditions (see Guerrero et al., 1994).

Whatever reason for the low NP_o of the channel activated by caffeine, it is clear that it would be interesting to determine what the endogenous activator is. One obvious candidate, cAMP, does not appear to be a mediator as an analogue of cAMP, 8-Bromo-cAMP, in the pipette solution had no effect on the channel or on caffeine's effect on the channel. Moreover, 100 μM ATP, adenosine, serotonin, and carbachol applied extracellularly were ineffective. Thus, the natural agent remains to be determined.

Absence of the Channel in Some Cells

As should be clear when comparing the results in Figs. 1 and 2 there was a fraction of the cells that lack the caffeine-activated channel. Although we have not carried out an extensive survey of all the properties of those cells with and without the channels, so far other than the lack of the channel there appears to be no difference in the physiological properties of the cells. The lack of such a channel in a small population of cells could be due to the variability in the number of such channels expressed in each cell which is under the control of some as yet to be determined factors. Another possibility is that those cells without cation channels are localized to some part of the stomach that we do not differentiate during the isolation procedure. A third possibility is that the channels are damaged in some way during the isolation procedure. A fourth possibility is that these channels represent ryanodine receptors which when by mistake are inserted into the plasma membrane lose their ryanodine sensitivity but not their sensitivity to caffeine. This would explain their low number and also their absence in some cells where the insertion might not occur. Nevertheless, the fact that the cells lacking the channel can respond to caffeine's affect on the internal Ca^{2+} stores in a normal way suggests that the channel is not necessary for loading the stores with Ca^{2+} from the extracellular fluid. In the following paper

(Guerrero et al., 1994) we discuss the relationship between this channel and Ca^{2+} stores in controlling the level of $[Ca^{2+}]_i$.

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