Simultaneous Measurement of Ca^{2+} Release and Influx into Smooth Muscle Cells in Response to Caffeine

A Novel Approach for Calculating the Fraction of Current Carried by Calcium

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ABSTRACT Activation of ryanodine receptors on the sarcoplasmic reticulum of single smooth muscle cells from the stomach muscularis of *Bufo marinus* by caffeine is accompanied by a rise in cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$), and the opening of nonselective cationic plasma membrane channels. To understand how each of these pathways contributes to the rise in $[Ca^{2+}]_i$, one needs to separately monitor Ca^{2+} entry through them. Such information was obtained from simultaneous measurements of ionic currents and $[Ca^{2+}]_i$ by the development of a novel and general method to assess the fraction of current induced by an agonist that is carried by Ca^{2+}. Application of this method to the currents induced in these smooth muscle cells by caffeine revealed that ~20% of the current passing through the membrane channels activated following caffeine application is carried by Ca^{2+}. Based on this information we found that while Ca^{2+} entry through these channels rises slowly, release of Ca^{2+} from stores, while starting at the same time, is much faster and briefer. Detailed quantitative analysis of the Ca^{2+} release from stores suggests that it most likely decays due to depletion of Ca^{2+} in those stores. When caffeine was applied twice to a cell with only a brief (30 s) interval in between, the amount of Ca^{2+} released from stores was markedly diminished following the second caffeine application whereas the current carried in part by Ca^{2+} entry across the plasma membrane was not significantly affected. These and other studies described in the preceding paper indicate that activation of the nonselective cation plasma membrane channels in response to caffeine was not caused as a consequence of emptying of internal Ca^{2+} stores. Rather, it is proposed that caffeine activates these membrane channels either by direct interaction or alternatively by a linkage between

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ryanodine receptors on the sarcoplasmic reticulum and the nonselective cation channels on the surface membrane.

INTRODUCTION

The effect of many agonists on many types of smooth muscle is to cause a biphasic contractile response—a large initial transient contraction followed by a smaller sustained response. The initial brief transient phase is believed to reflect release of Ca^{2+} from internal stores (Williams and Fay, 1986) whereas the secondary sustained phase coincides with an increased Ca^{2+} influx across the plasma membrane. The relative magnitude of these two phases varies both with regard to the stimulus as well as the smooth muscle source (Iino, Kobayashi, and Endo, 1988). Similar biphasic responses have also been observed in other cell types in response to stimuli that trigger a wide range of cell processes (Tsien and Tsien, 1990). The mechanism of the dual action of agonists whose action is at least in part mediated by IP_3 has been the subject of intense recent investigation. While release of Ca^{2+} from internal stores due to such agonists results from the action of IP_3 on receptors on those stores (Somlyo, Bond, Somylo, and Scarpa, 1985), the influx of Ca^{2+} across the plasma membrane may be due to activation of ion channels by either voltage or some second messenger (Tsien and Tsien, 1990). It has recently been suggested that some, as yet unknown, second messenger is generated as a result of the emptying of internal stores of Ca^{2+} independently of how the stores are emptied (Putney, 1990; Meldolesi, Clementi, Fasolato, Zachetti, and Pozzan, 1991; Randriamampita and Tsien, 1993). Ca^{2+} may also be released by the activation of ryanodine receptors present on internal stores and agonists like caffeine that activate those receptors often cause cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) to rise in a biphasic manner (Pacaud and Bolton, 1991). In the case of smooth muscle cells from the stomach of the toad, the sustained phase of the response to agonists like caffeine has been attributed to the activation of plasma membrane channels that are at least partially permeable to Ca^{2+} (Guerrero, Fay, and Singer, 1994). The contribution of Ca^{2+} entry via such channels to the overall increase in $[Ca^{2+}]_i$ induced by agonists like caffeine is not well understood nor is there much information about the mechanism possibly linking the dual action of caffeine on $[Ca^{2+}]_i$.

For the most part, the assessment of the extent to which $[Ca^{2+}]_i$ increases are due to Ca^{2+} release from internal stores versus entry across the plasma membrane as well as coupling of these two Ca^{2+} entry pathways has been based on the effects of changing the $[Ca^{2+}]$ gradient across the plasma membrane. The interpretation of effects observed following changes in the $[Ca^{2+}]$ gradient may not be entirely straightforward as not only does a change in extracellular $[Ca^{2+}]$ cause a change in the driving force for Ca^{2+} entry across the plasma membrane, but it also can affect the state of filling of internal Ca^{2+} stores (Matsumoto, Kanaide, Shogakiuchi, and Nakamura, 1990) and the resulting changes in intracellular and extracellular $[Ca^{2+}]$ are likely to influence other processes involved in Ca^{2+} homeostasis which are themselves subject to feedback control by Ca^{2+} (Becker, Singer, Walsh, and Fay, 1989; McCarron, McGeown, Reardon, Ikebe, Fay, and Walsh, 1992).

Combination of microspectrofluorimetry using Ca^{2+} sensitive dyes and electrophysiology in single cells provides a means for more directly assessing the contribu-

tion of Ca^{2+} entry across the surface and internal membranes in response to a specific stimulus. If we knew the fraction of the current induced by a given stimulus carried by Ca^{2+}, we could, by subtraction, determine the time course and magnitude of Ca^{2+} release from internal stores. In this paper we develop methods to determine from measurements of ionic currents and $[Ca^{2+}]_i$ the fraction of the current that is carried by Ca^{2+} that in this case is induced by caffeine. We then use these methods to dissect the effect of caffeine on $[Ca^{2+}]_i$ into components due to activation of Ca^{2+} permeable channels on internal stores and on the plasma membrane. This information is in turn used to investigate if and how these two effects of caffeine might be linked. Some of these studies have been previously reported in abstract form (Guerrero, Singer, and Fay, 1992).

METHODS

Cell Isolation and Solutions

Single smooth muscle cells were freshly isolated from the stomach of the toad as previously described (Fay, Hoffman, LeClair, and Merriam, 1982). The extracellular solution contained (in millimolar): 120 NaCl, 3 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 TEACl, and 5 HEPES pH 7.6 with NaOH. Elevated external $[Ca^{2+}]$ solution was prepared by increasing $CaCl_2$ to 20 mM and reducing NaCl to 94 mM in the extracellular solution leaving the rest of its components the same. 0 Na^+ bathing solution contained (in millimolar): 130 TEACl, 1.8 $CaCl_2$, 1.0 $MgCl_2$ and 5 HEPES pH 7.6 with KOH yielding a final $[K^+]$ of 2.8 mM. Pipette solution contained (in millimolar): 130 CsCl, 4 $MgCl_2$, 3 Na_2ATP, 1 Na_3GTP, 0.05 K_5 Fura-2, and 20 Hepes, pH 7.2 with CsOH. All experiments were carried out at room temperature. Voltage clamp and $[Ca^{2+}]_i$ measurements were made as indicated in preceding paper (Guerrero et al., 1994).

Perforated Patch

In some experiments, whole cell membrane currents were recorded using the perforated patch (Horn and Marty, 1988) as described by Rae, Cooper, Gates, and Watsky (1991) using amphotericin B (Sigma Chemical Co., St. Louis, MO). Only cells with a series resistance of less than 30 MΩ were utilized, which in general was achieved in a period of 10–30 min. Pipette solution contained (in millimolar): 130 CsCl, 1 $MgCl_2$, 0.1 BAPTA, 20 Hepes pH 7.6 with NaOH and 300 μg/ml of amphotericin B from a stock solution of 60 mg/ml in dry DMSO. Fura-2 was loaded by incubating around 10^4 cells/ml with 200 nM Fura-2/AM for at least 1 h at room temperature. For these recordings, background fluorescence was measured in a region devoid of a cell and thus did not include cell autofluorescence. Ryanodine (100 μM) when used with the perforated patch recording method was added to the bath for at least 10 min, but otherwise was applied by inclusion in the patch pipette.

Data Analysis

Rates of cytoplasmic Ca^{2+} removal were determined as previously described (Becker et al., 1989). Analysis of the changes in $[Ca^{2+}]_i$ induced by caffeine were compared to the *trans*-plasma membrane ionic entry detected by the caffeine-activated current which was integrated at time intervals of 12 ms. Unless otherwise indicated, all statistical measurements are given as the mean ± SEM with the number (n) of cells indicated.

RESULTS

Framework for Determination of the Fraction of the Caffeine-activated Current Carried by Ca^{2+}

In the preceding article (Guerrero et al., 1994) we demonstrated that caffeine activates a Ca^{2+} permeable nonselective cation channel in addition to causing Ca^{2+} release from internal stores in gastric smooth muscle cells of the toad. Furthermore, we showed that this channel is not activated by caffeine in direct response to a caffeine induced increase in $[Ca^{2+}]_i$ per se. Thus, as can be seen in Fig. 4 of the preceding paper (Guerrero et al., 1994), in the presence of ryanodine, where the release of Ca^{2+} from internal stores in response to caffeine is completely blocked, caffeine induces an increase in $[Ca^{2+}]_i$ which is tightly associated with the activation of a caffeine activated cation channel (Guerrero et al., 1994).

To further characterize the ability of the caffeine induced cation channel to influence $[Ca^{2+}]_i$ and thus to determine the relative contribution of this pathway to the rise in $[Ca^{2+}]_i$ induced by caffeine we determined what fraction of the current induced by caffeine was carried by Ca^{2+}. For that purpose we consider that the caffeine induced increase in $[Ca^{2+}]_i$ to be the balance of four different processes: (*a*) release of Ca^{2+} from internal stores; (*b*) Ca^{2+} influx through the caffeine activated cation channel; (*c*) rapid binding of Ca^{2+} by cytoplasmic Ca^{2+} buffers, and (*d*) removal of cytoplasmic Ca^{2+} by various mechanisms. By carrying out the experiments in the presence of ryanodine we can eliminate the contribution from intracellular stores to changes in $[Ca^{2+}]_i$ induced by caffeine. Under these conditions the change in $[Ca^{2+}]_i$ induced by caffeine is given by the following equation:

$$\Delta[Ca^{2+}]_i(t) = [f\Sigma(-I_{caff}\Delta t)/(BZFV_c)] - [\Sigma R(Ca^{2+})\Delta t]. \quad (1)$$

The term on the left, $\Delta[Ca^{2+}]_i(t)$, is the increase in the free Ca^{2+} concentration in nanomoles per liter over baseline at time (t) after the application of caffeine. The first term on the right, $[f\Sigma(-I_{caff}\Delta t)/(BZFV_c)]$, represents the contribution of Ca^{2+} influx through cation channels to the increase in the $[Ca^{2+}]_i$ and is the numerical integral of the cation current times the fraction of the current carried by Ca^{2+} (f) times a term taking into account internal Ca^{2+} buffering capacity (B), the last with dimensionless units indicating the ratio of the change in total $[Ca^{2+}]$ relative to free $[Ca^{2+}]$; V_c is the volume of the cell which is assumed to be 6 pL. As indicated below the buffer capacity, B is calculated from a series of measured cell parameters divided by V_c. Hence any errors involved in an assumed cell volume cancel out in our estimates of f, the fraction of the caffeine current carried by Ca^{2+}. F is the Faraday constant, and Z is 2, the charge on Ca^{2+}. The second term on the right, $[\Sigma R(Ca^{2+})\Delta t]$, represents the contribution of the Ca^{2+} removal mechanisms to the $[Ca^{2+}]_i$. The removal rate, R, in nmol·liter^{-1}·s^{-1}, is a function of $[Ca^{2+}]_i$ which changes with time (see Becker et al., 1989). B and $R(Ca^{2+})$ can be obtained in the same cell (in the presence of ryanodine) by measuring the change in the $[Ca^{2+}]_i$ following the rise in $[Ca^{2+}]_i$ due to activation and deactivation of the voltage-gated Ca^{2+} current.

Under conditions where the entire Ca^{2+} influx is from current through voltage gated Ca^{2+} channels, B can be obtained from the relationship between the rise in $[Ca^{2+}]_i$ and the integral of the Ca^{2+} current during the first 100 ms after the

membrane potential has been stepped from −80 mV to 0 mV, according to the expression:

$$B = \sum_{t=0}^{100\ \text{ms}} (-I_{\text{depol}}\Delta t)/(ZFV_c\Delta[Ca^{2+}]_i). \quad (2)$$

In this expression I_{depol} is the increase in current at time t relative to that at the end of the 3-s depolarization. The assumption here is that under the conditions of this experiment all current that flows across the membrane in response to depolarization is carried by Ca^{2+}. The other major current in these cells carried by K^+ was blocked in these experiments by using Cs^+ in the pipette and TEA^+ in the bathing medium. The magnitude of the currents induced by depolarization were measured relative to that at the end of a 3-s depolarizing pulse to 0 mV, where inward Ca^{2+} currents are expected to have been almost fully inactivated. This assumption was checked using 100 μM $CdCl_2$, a good inhibitor of high voltage activated Ca^{2+} channels (Bolton, Mackenzie, and Aaronson, 1988; Bean and Knudson, 1988; Liu, DeFelice, and Mazzanti, 1992). There was no more than a 2–3 pA Cd^{2+} sensitive current seen at the end of a 3-s depolarizing pulse; the existence of this small residual Ca^{2+} current did not significantly affect our estimates of cellular buffering.

The rate of cytoplasmic Ca^{2+} removal and its relationship to $[Ca^{2+}]_i$ was obtained from the decline in $[Ca^{2+}]_i$ after the membrane potential had been jumped back to −80 mV. Any Ca^{2+} channels not in the inactivated state would be deactivated virtually instantaneously relative to the measurements of $[Ca^{2+}]_i$ when the membrane potential is stepped back to −80 mV (Swandulla and Armstrong, 1988). Once the buffering and the removal are known, f can be obtained from the relationship of cation current and $[Ca^{2+}]_i$ during application of caffeine (Eq. 1).

Measurements of Ca^{2+} Buffering and Ca^{2+} Removal

Fig. 1 shows the typical effect of a 3-s membrane depolarization on $[Ca^{2+}]_i$ in the presence of ryanodine. As can be seen in Fig. 1 *B*, in the presence of ryanodine the relationship between the integral of the inward current and the change in $[Ca^{2+}]_i$ was linear up to 300 nM. The Ca^{2+} buffering capacity (B) calculated according to Eq. 2 from these data for the first 100 ms was 69. During the initial 100 ms $[Ca^{2+}]_i$ rose at a peak rate of 1.4 ± 0.16 μM/s (n = 15). The average cytoplasmic Ca^{2+} buffering calculated in this way in 11 cells was 82 ± 7 (n = 11) for the initial 100 ms. The integral of current and the change in $[Ca^{2+}]_i$ were related linearly, with a linear correlation coefficient of 0.981 ± 0.007 in these same 11 cells. The integral of the inward current continued to be linearly related to the measured $[Ca^{2+}]_i$ for considerably longer (up to 300 ms) in many (12 of 13) cells. At even longer times, however, when the current had declined significantly and $[Ca^{2+}]_i$ had risen to higher values, the change in $[Ca^{2+}]_i$ for a given amount of current diminished over that seen in the first 100 ms, presumably because now Ca^{2+} removal processes were removing a significant fraction of the Ca^{2+} flowing into the cell through voltage-gated Ca^{2+} channels. During the first 100 ms, the rate of $[Ca^{2+}]$ change due to Ca^{2+} entry following depolarization was sufficiently high that Ca^{2+} removal mechanisms do not significantly affect our estimates of B, however. During the first 100 ms of Ca^{2+} entry

after depolarization, the average peak rate of $[Ca^{2+}]_i$ rise was 1400 ± 160 nM/s (n = 15) as $[Ca^{2+}]_i$ rose from 56 nM at rest to ~200 nM, yet the maximal rate of $[Ca^{2+}]_i$ decline due to removal processes for that level of $[Ca^{2+}]_i$ is 56 ± 3.6 nM/s (n = 12). Hence, the effect of neglecting removal would result in a rather small overestimate of B, at most a 4% error.

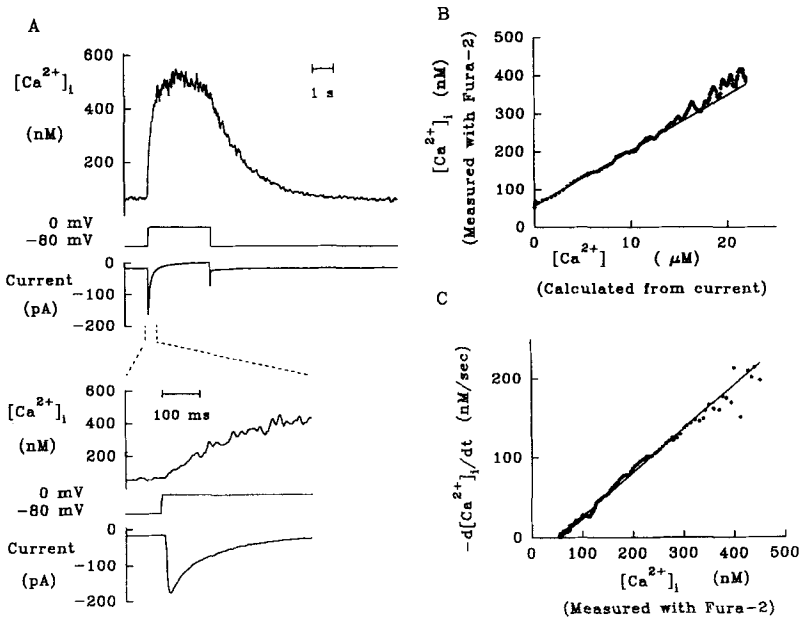

FIGURE 1. Measurement of Ca^{2+} buffer capacity and Ca^{2+} dependence of cytoplasmic Ca^{2+} removal processes in the presence of ryanodine. (*A*) Membrane potential (*middle trace*) was stepped to 0 mV for 3 s from a holding potential of −80 mV. The bottom panel shows the inward current and its associated change in $[Ca^{2+}]_i$ during the initial 400 ms of the membrane depolarization on an expanded time scale. (*B*) $[Ca^{2+}]_i$ measured with Fura-2 during the initial 100 ms depolarization was compared with the $[Ca^{2+}]$ change predicted from the numerical integral of the sampled inward current. The slope obtained was used as our measure of the cytoplasmic Ca^{2+} buffering capacity, as described in the text. The data shown are for the initial 500 ms of the integral of the inward current, the line drawn is the least squares fit to the first 100 ms of data. Note that this line describes the relation between $[Ca^{2+}]_i$ and current for a considerable time beyond this point. (*C*) The rate of $[Ca^{2+}]_i$ recovery showed a linear dependence on $[Ca^{2+}]_i$ and was determined from the slope of the relationship of $[Ca^{2+}]_i$ versus time using 100 points on either side of a given $[Ca^{2+}]_i$.

Because the caffeine induced increase in $[Ca^{2+}]_i$ in the presence of ryanodine was rather slow in contrast to the rise in $[Ca^{2+}]_i$ induced by depolarization, we corrected the former for Ca^{2+} losses from the cytoplasm due to Ca^{2+} removal mechanisms. For this purpose we analyzed the rate of $[Ca^{2+}]_i$ decline for the voltage induced Ca^{2+} transient as a function of $[Ca^{2+}]_i$ after repolarization to −80 mV. The rate of Ca^{2+} removal was always measured at the same holding potential at which caffeine was

applied. Fig. 1 *C.* shows that the rate of removal was linearly related to $[Ca^{2+}]_i$ with a $t_{1/2}$ of 1.68 ± 0.13 s (n = 11) in the presence of ryanodine.

It is important to point out that both the Ca^{2+} buffering and the rate of cytoplasmic Ca^{2+} removal were calculated for each particular cell and used to estimate the fraction of the caffeine current carried by Ca^{2+} for only that cell. The rate of Ca^{2+} removal at a given $[Ca^{2+}]_i$ did not vary significantly from cell to cell. However, that was not the case for the Ca^{2+} buffering power (B) which showed a wide range of values, from 33 to 200. Although variable from cell to cell, Ca^{2+} buffering was effectively constant for a given cell during the 30 s required to define its Ca^{2+} buffering power, removal characteristics and to determine the effect of caffeine on $[Ca^{2+}]_i$ and current. The variability in the estimate of Ca^{2+} buffering power could result from different amounts of Fura-2 that entered the cells or loss to a different extent of mobile cellular buffers with the whole cell patch clamp configuration. This latter possibility is unlikely, however, as indicated in the discussion.

Fraction of the Caffeine Current Carried by Ca^{2+}

Once the buffering and $[Ca^{2+}]_i$ dependence of Ca^{2+} removal have been characterized in a given cell, one can obtain an estimate of the fraction of the current induced by caffeine which is carried by Ca^{2+} as illustrated in Fig. 2. This is accomplished by: (*a*) adding back to the Fura 2 measured $[Ca^{2+}]_i$ record the Ca^{2+} that has been removed at each point in time; (*b*) numerically integrating the digitized current record to obtain an estimate of the accumulated ion entry and dividing that accumulated ion entry by the cell's cytoplasmic Ca^{2+} buffering power and the other constants indicated in Eq. 1. The $[Ca^{2+}]_i$ changes calculated in the latter step are then plotted against the change in $[Ca^{2+}]_i$ from the corrected Fura-2 signal over time (Fig. 2 *B*). To the extent that not all of the caffeine activated current is carried by Ca^{2+}, the $[Ca^{2+}]$ changes predicted from the integral of the current will be greater than the observed $[Ca^{2+}]_i$ changes even after correcting for Ca^{2+} removal processes. The ratio of the observed $[Ca^{2+}]_i$ changes to that predicted from the integral of the caffeine current provides an estimate of the fraction of the caffeine current carried by Ca^{2+}.

As expected and shown in Fig. 2, the observed $[Ca^{2+}]_i$ changes induced by caffeine were always less than that predicted for the integrated current. The mean ratio observed was 0.189 ± 0.017 (n = 5) indicating that on average about 20% of the caffeine activated current was carried by Ca^{2+} at −80 mV. As can be seen in Fig. 2 *C*, the fraction of the caffeine activated current carried by Ca^{2+} was quite constant throughout both rising and falling phases of the Ca^{2+} transient.

The absolute value of the fraction of caffeine activated current carried by Ca^{2+} is critically dependent on our estimate of Ca^{2+} buffer power of the cell (B). While B in cells used to estimate the fraction of the caffeine induced current carried by Ca^{2+} varied over ~3× range, there was no correlation whatsoever between estimates of f and the buffer power of individual cells (linear regression slope = −0.005, r^2 = 0.027, n = 8). As discussed subsequently, the variations in B are most likely due to variability in the amount of Fura 2 loaded into cells. Hence, the observed constancy of f in the face of variations in B suggest that our numerical estimate of the fraction of the caffeine current carried by Ca^{2+} is a property of the channels activated

by caffeine and not a complex parameter dependent on the cell's Ca^{2+} buffering power or assumptions inherent in our estimate of *B*.

Fraction of the Caffeine-activated Cation Current Carried by Ca^{2+} in the Perforated Patch Configuration

To determine if the properties of the caffeine activated channels may have been influenced by loss of cytoplasmic components during the dialysis that occurs in the

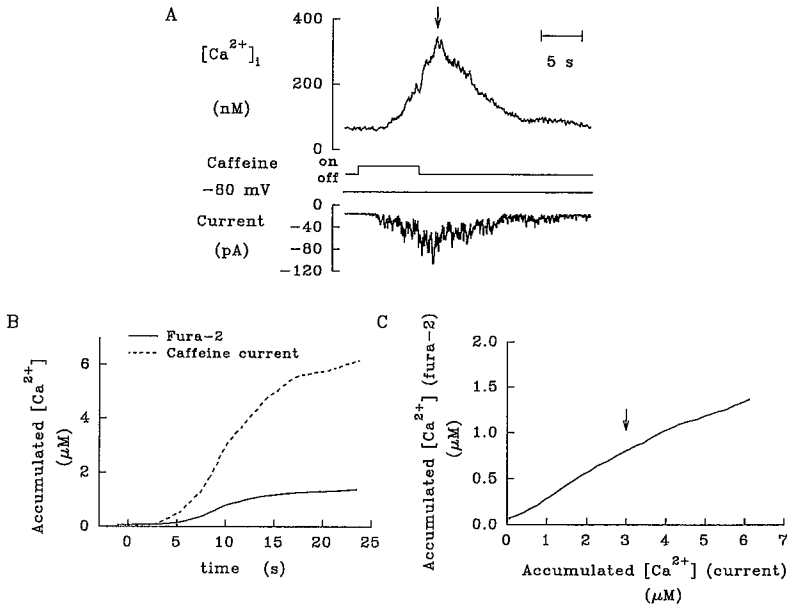

FIGURE 2. Measurement of the fraction of the caffeine-induced current carried by Ca^{2+}. (*A*) Cell in the presence of ryanodine was exposed to caffeine and $[Ca^{2+}]_i$ and membrane currents were measured. (*B*) With the cytoplasmic Ca^{2+} buffer capacity measured for this cell, the numerical integral of the caffeine-dependent inward current was transformed to $[Ca^{2+}]$ (*dashed line*) and its time course was compared with the removal-corrected change in $[Ca^{2+}]$ as calculated from $[Ca^{2+}]_i$ detected by Fura-2 (*continuous line*). Time zero is the beginning of caffeine application. (*C*) A linear relationship was found between Ca^{2+} accumulation in the cell as calculated from the inward current and the removal corrected $[Ca^{2+}]$ changes measured by Fura-2 with an average slope of 0.2 between 1.0 and 4.0 μM accumulated $[Ca^{2+}]$ (*current*). The fraction of the caffeine activated current carried by Ca^{2+} in four other cells under these conditions was calculated in an identical manner. The arrows in *A* and *C* point out equivalent points in time in the two graphs.

conventional whole cell configuration (with disrupted patch) the experiments were repeated using the perforated patch recording technique (Horn and Marty, 1988). That we had a true perforated patch was evident by the slower run down of voltage gated Ca^{2+} current compared to cells recorded in the conventional whole cell mode (not shown). Single cells previously loaded with Fura-2 using its membrane perme-

able form and exposed to 100 μM ryanodine in the bath for at least 10 min were voltage clamped using identical protocols to those utilized for the previous studies. Under these conditions caffeine was applied to cells held at −80 mV and the current and changes in $[Ca^{2+}]_i$ were compared. The average fraction calculated in three cells under these conditions was 0.194 ± 0.01 (not shown). Hence the fraction of the current carried by Ca^{2+} was virtually the same as that recorded using conventional whole cell recording methods. We did not find a significant difference in the estimates of Ca^{2+} buffering nor the rate of Ca^{2+} removal between cells studied with the perforated patch and cells recorded in the conventional whole cell configuration.

Effect of Increased External $[Ca^{2+}]$ and of Na^+ Substitution on the Fraction of the Caffeine-activated Cation Current Carried by Calcium

To further test the ability of our method to estimate the fraction of the caffeine current that is carried by Ca^{2+}, we carried out additional experiments under other ionic conditions where Ca^{2+} would be expected to carry a larger fraction of the current, i.e., when extracellular $[Ca^{2+}]$ was increased or $[Na^+]$ decreased. We found that when extracellular $[Ca^{2+}]$ was increased to 20 mM the changes in $[Ca^{2+}]_i$ induced by caffeine were of similar magnitude to those observed in 1.8 mM external $[Ca^{2+}]$ however, they were associated with a substantially reduced current (Fig. 3). This suggests that Ca^{2+} dwells in the channel longer than monovalent cations (Tsien, Hess, McCleskey, and Rosenberg, 1987). As can be seen in Fig. 3 *B* the removal-corrected change in $[Ca^{2+}]_i$ was very similar to that calculated in normal external $[Ca^{2+}]$ however the numerical integral of the caffeine activated current was reduced by 50% compared to that in normal external $[Ca^{2+}]$. Fig. 3 also shows that the relationship between the numerical integral and the removal-corrected change in $[Ca^{2+}]_i$ was relatively constant throughout the caffeine induced change in $[Ca^{2+}]_i$. The average fraction of the current carried by Ca^{2+} calculated in four cells under these conditions was 0.433 ± 0.014, more than twice as high as in 1.8 mM external $[Ca^{2+}]$.

We also removed Na^+ from the extracellular solution and substituted for it an impermeant cation (TEA^+) as another test of the method for estimating f. It is to be expected that while the current induced by caffeine would go down, the $[Ca^{2+}]_i$ increase might not and hence f should increase. Thus, caffeine was applied to cells in otherwise normal bathing solution with TEA^+ replacing Na^+ in the presence of ryanodine. Under these conditions the rates of cytoplasmic Ca^{2+} removal were slower ($t_{1/2}$ = 3.0 ± 0.4 s, n = 8) than with normal Na^+ ($t_{1/2}$ = 1.7 ± 0.1 s, n = 11). While the Ca^{2+} buffering power was lower in the absence of extracellular Na^+ (50.1 ± 4.4, n = 7) than in the presence of extracellular Na^+ (82 ± 7, n = 11) the difference in the means between groups was not statistically significant. Fig. 4 shows a typical response of a cell to caffeine in the absence of extracellular Na^+. As can be seen the magnitude of the current activated by caffeine was markedly reduced. Despite the reduction in current the caffeine-induced changes in $[Ca^{2+}]_i$ were of comparable size to that obtained in normal Na^+. Comparison of the time course of the removal corrected change in $[Ca^{2+}]_i$ with the integral of the caffeine induced current indicated that now the fraction of the caffeine-activated current carried by Ca^{2+} was 0.59 ± 0.03 (n = 3), almost three times that seen when Na^+ was in the medium. The increase in the fraction of the current carried by Ca^{2+} presumably reflects that Na^+

can pass through this channel whereas TEA^+ cannot. Under these conditions Ca^{2+} movement through caffeine gated channels is now the major contributor to the observed current with K^+ (present in the bath but not in the pipette) and possibly Mg^{2+} presumably responsible for the remainder of the inward current. All of these data clearly show that f is a good indicator of the fraction of the caffeine activated current carried by Ca^{2+}.

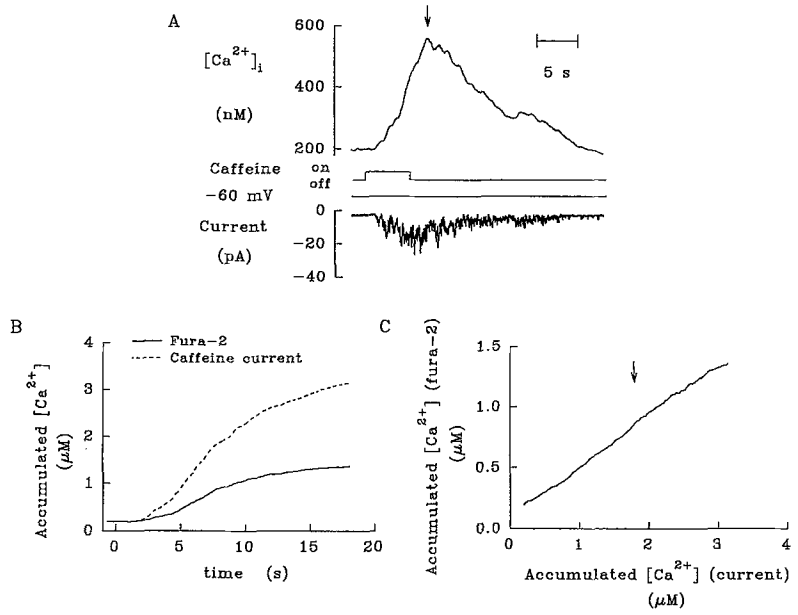

FIGURE 3. Measurement of the fraction of the caffeine-dependent current carried by Ca^{2+} in the presence of elevated external Ca (20 mM). (*A*) Cell in the presence of ryanodine was exposed to caffeine and $[Ca^{2+}]_i$ and membrane currents were measured. Notice that the caffeine-activated current is one third of that in Fig. 2. Nevertheless the increments in $[Ca^{2+}]_i$ were similar. (*B*) Time course of the removal-corrected change in $[Ca^{2+}]_i$ as detected by Fura-2 (*continuous line*) and the numerical integral of the caffeine-activated current transformed to $[Ca^{2+}]$ as described in the text (*dashed line*). (*C*) A linear relationship was found between the removal corrected change in $[Ca^{2+}]$ and the numerical integral of the caffeine-activated current with an average slope of 0.420 from 1.0 to 3.0 μM of accumulated $[Ca^{2+}]_i$ (*current*). The arrows in *A* and *C* point out equivalent points in time in the two graphs.

Caffeine-induced Increase in $[Ca^{2+}]_i$ Can Be Explained by a Dual Mechanism, Release of Ca^{2+} from Stores and an Increased Ca^{2+} Influx through the Plasma Membrane

The estimate of the fraction of the caffeine activated current that is carried by Ca^{2+} based on studies in the presence of ryanodine can be used to understand the relative contributions of Ca^{2+} entry from stores versus that carried by the caffeine activated current in experiments in the absence of ryanodine. The only additional assumptions required are that the properties of caffeine activated plasma membrane channels are

not altered by ryanodine and that $[Ca^{2+}]_i$ changes induced by caffeine reflect the sum of these two pathways for Ca^{2+} entry into the cytoplasm. The Ca^{2+} buffering and Ca^{2+} removal characteristics were determined as described above, and were not found to be significantly different in the cells in the absence of ryanodine. The Ca^{2+} buffering value averaged 85.5 ± 6.3, (n = 20), virtually identical to that obtained in ryanodine treated cells (82.0 ± 7.0, n = 11). The $t_{1/2}$ for Ca^{2+} removal averaged 1.7 ± 0.1 s (n = 15), also very similar to the value obtained from ryanodine treated

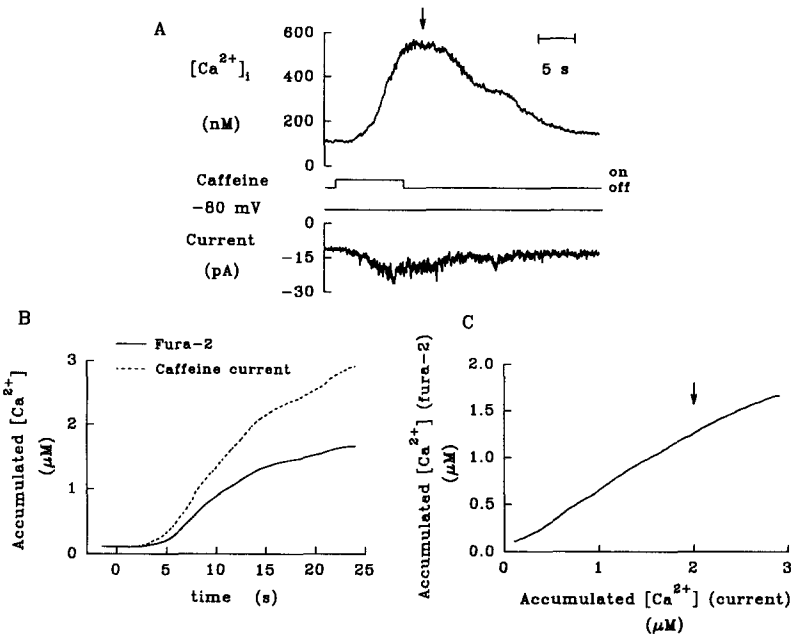

FIGURE 4. Measurement of the fraction of caffeine-induced current carried by Ca^{2+} in a cell where Na^+ was substituted with TEA^+. (*A*) Caffeine applied to a cell held at −80 mV in the absence of external Na^+ but with ryanodine increased $[Ca^{2+}]_i$ (*top trace*) to a similar extent as in normal Na^+ but with a much smaller current (*bottom trace*). (*B*) Cytoplasmic Ca^{2+} buffer capacity and rate of Ca^{2+} removal were calculated for this cell as indicated in Fig. 1, and the numerical integral of the inward current transformed to $[Ca^{2+}]$ and removal-corrected changes in $[Ca^{2+}]$ calculated from Fura-2 measurements of $[Ca^{2+}]_i$. (*C*) The relationship between the $[Ca^{2+}]$ calculated from the numerical integral of the inward current and removal corrected $[Ca^{2+}]$ is still linear with a slope close to 0.6 between 0.5 and 2.5 μM accumulated $[Ca^{2+}]$ (*current*). The arrows in *A* and *C* point out equivalent points in time in the two graphs.

cells (1.68 ± 0.13 s, n = 11). Finally, the peak current induced by caffeine in the absence of ryanodine (85.0 ± 11.1 pA, n = 13) was statistically indistinguishable from that induced in the presence of ryanodine (78.0 ± 9.5 pA, n = 11). This result provides support for the assumption that in these cells ryanodine has no effect per se on the action of caffeine on Ca^{2+} entry due to the current induced by caffeine.

As can be seen in Figs. 5, 6, and 7 (part *b*) using estimates of the Ca^{2+} buffering and Ca^{2+} removal for each cell studied in conjunction with measurements of changes

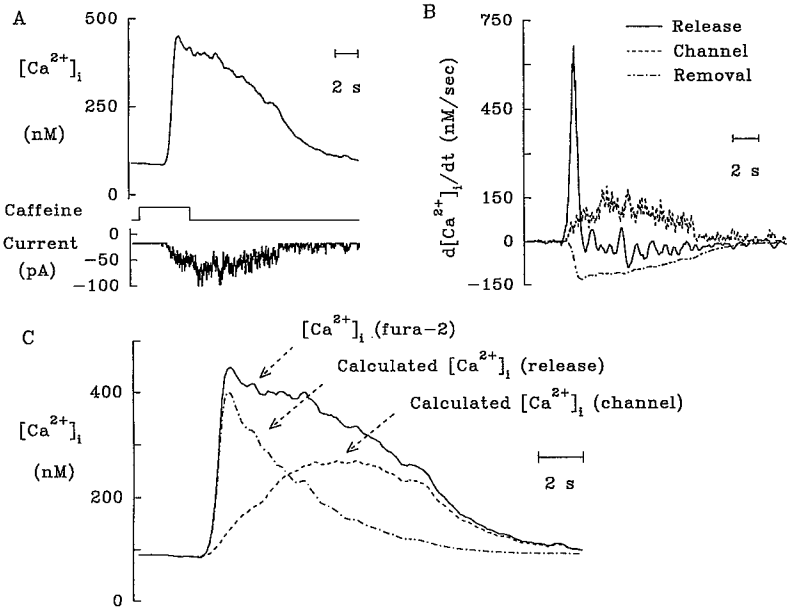

FIGURE 5. Caffeine increases $[Ca^{2+}]_i$ by both release from internal stores and by increasing Ca^{2+} influx through the plasma membrane. (*A*) Caffeine applied for the time indicated (*middle trace*) to a cell held at −80 mV in the absence of ryanodine caused a rise in $[Ca^{2+}]_i$ (*upper trace*) measured with Fura-2. (*B*) Rate of $[Ca^{2+}]_i$ changes in the cytoplasm during response to caffeine due to Ca^{2+} entry through caffeine activated channels (*channel*), Ca^{2+} removal mechanisms (*removal*), and Ca^{2+} entry from internal stores (*release*). Rate of $[Ca^{2+}]$ change due to channels calculated by dividing the current record by the measured value for the cell's buffer capacity determined as in Fig. 1, by constants as described in text relating current to ionic equivalents in moles, and an estimated cell volume of 6 pL. To analyze results obtained in the absence of ryanodine we assumed that f, the fraction of the caffeine activated current carried by Ca^{2+}, was 0.19, the value calculated from experiments in the presence of ryanodine. The rate of $[Ca^{2+}]_i$ change due to removal during the caffeine response was determined from the relationship between the rate of $[Ca^{2+}]_i$ decline and $[Ca^{2+}]_i$ determined for this cell as described in Fig. 1. The rate of $[Ca^{2+}]_i$ change due to release from internal stores was determined by subtracting the rate of $[Ca^{2+}]_i$ change due to channel mediated entry and removal from the observed rate of change of $[Ca^{2+}]_i$ obtained by differentiating the measured $[Ca^{2+}]_i$ with respect to time. (*C*) Decomposition of the measured change in $[Ca^{2+}]_i$ during the response to caffeine into contributions due to channel mediated Ca^{2+} entry (*channel*) and internal stores induced Ca^{2+} release (*release*). These traces were calculated by numerically integrating the rates of $[Ca^{2+}]_i$ change versus time as presented in *B* for these two sources of Ca^{2+} influx and then correcting each trace for Ca^{2+} removal. This correction was performed by calculating the rate of removal at each measured $[Ca^{2+}]_i$. The effect of removal mechanisms on $[Ca^{2+}]_i$ at any point in time was then apportioned between the expected $[Ca^{2+}]_i$ changes due to Ca^{2+} release from internal stores and channel mediated entry according to the relative magnitude of the $[Ca^{2+}]_i$ change attributable to these two sources. Notice that the calculated release component of the caffeine induced $[Ca^{2+}]_i$ transient was very similar to the $[Ca^{2+}]_i$ transient measured in Fig. 8 in a cell with no caffeine activated channels.

in $[Ca^{2+}]_i$ and current induced by caffeine one can calculate the time course and magnitude of the rate of Ca^{2+} entry into the cytoplasm due to release of Ca^{2+} from internal stores as well as that due to opening of plasma membrane non-selective cation channels. One can also calculate the time course of Ca^{2+} removal from the cell due to Ca^{2+} removal mechanisms. One can in turn also integrate the estimates of the variation in time of the rate of Ca^{2+} entry from the stores and across the plasma membrane to obtain an estimate of the contribution of Ca^{2+} entry from these two sources to the changes in $[Ca^{2+}]_i$ induced by caffeine (part *c*, Figs. 5, 6, and 7). Fig. 5

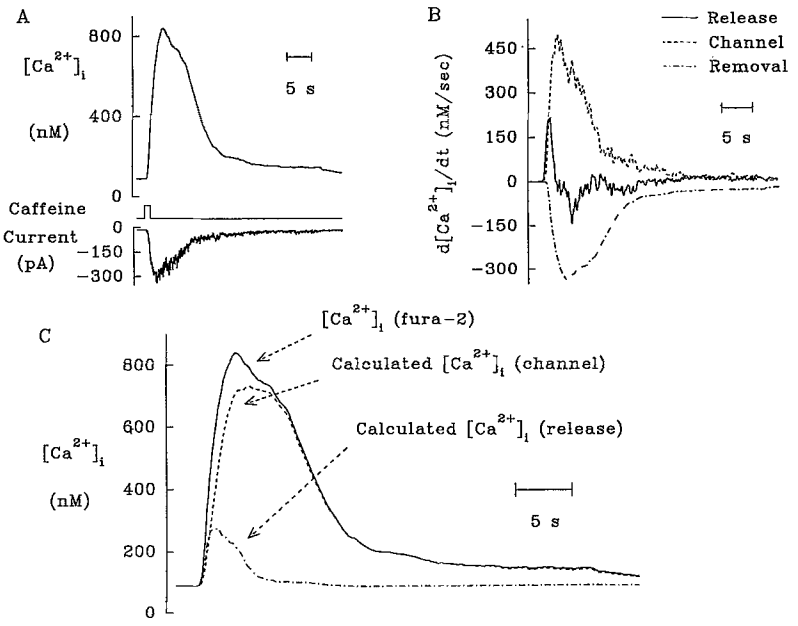

FIGURE 6. Analysis of $[Ca^{2+}]_i$ changes induced by caffeine in a cell where the caffeine induced increase in $[Ca^{2+}]$ was due almost completely to activation of the caffeine-dependent cation channel. (*A*) Cell held at −80 mV and in the absence of ryanodine was exposed to caffeine for the time indicated (*middle trace*) and $[Ca^{2+}]_i$ (*top trace*) and membrane currents (*lower trace*) were measured. (*B*) Rate of $[Ca^{2+}]_i$ change due to release from internal stores, Ca^{2+} influx through plasma membrane channels and the rate of $[Ca^{2+}]_i$ decline due to removal mechanisms calculated as in Fig. 5. (*C*) Calculated contribution of internal Ca^{2+} stores (*dot-dashed line*) and external Ca^{2+} (*dashed line*), to the caffeine induced change in $[Ca^{2+}]_i$ calculated as in Fig. 5.

shows data from a cell whose response to caffeine was similar to that most frequently observed. Ca^{2+} influx into the cytoplasm contributes almost equally with Ca^{2+} release from stores to the rise in $[Ca^{2+}]_i$ in response to caffeine (Fig. 5 *C*). Fig. 6 shows data from a cell where the caffeine induced rise in $[Ca^{2+}]_i$ was dominated by Ca^{2+} entry across the plasma membrane. In the 10 cells subject to this analysis, Ca^{2+} entry across the plasma membrane accounted for 48.6 ± 7.5% of the $[Ca^{2+}]_i$ rise seen during the Ca^{2+} transient induced by caffeine stimulation which lasted 4 s on average. There was

considerable variability in the relative contribution of Ca^{2+} release from internal stores to the total entry of Ca^{2+} in response to caffeine (e.g., Fig. 5 vs Fig. 6).

While on average Ca^{2+} release from internal stores contributed about half of the Ca^{2+} entering the cytoplasm in response to caffeine during the 4 s average exposure to caffeine, at early times Ca^{2+} release from internal stores was always the major contributor to the rise in $[Ca^{2+}]_i$. For example, at 1 s into the Ca^{2+} transient Ca^{2+} release from internal stores was responsible for 87 ± 2.3% (n = 10) of the $[Ca^{2+}]_i$ rise seen. As can be seen from part *b* of Fig. 5, the dominance of Ca^{2+} entry from stores at

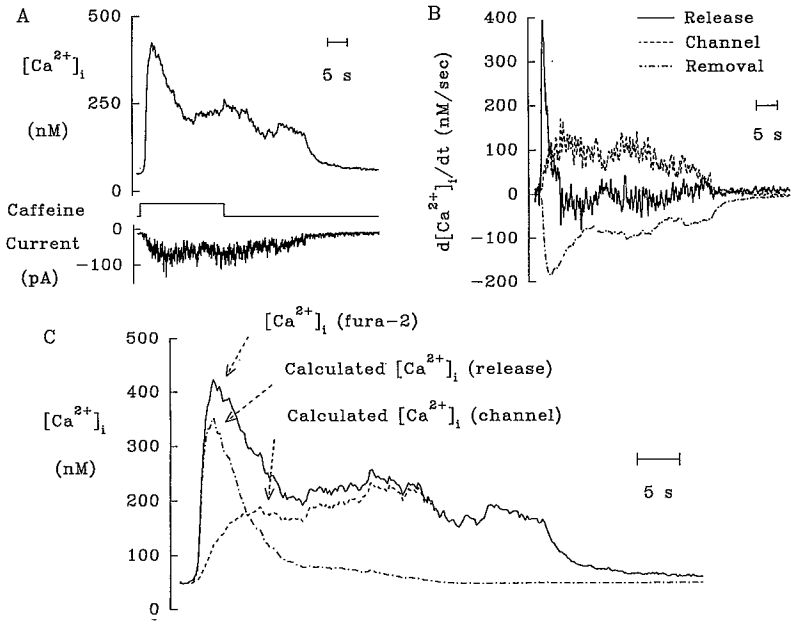

FIGURE 7. Analysis of $[Ca^{2+}]_i$ changes induced by prolonged application of caffeine. (*A*) Cell held at −80 mV and in the absence of ryanodine was exposed to caffeine for the time indicated (*middle trace*) and $[Ca^{2+}]_i$ (*upper trace*) and membrane currents (*lower trace*) were measured. (*B*) Rate of $[Ca^{2+}]_i$ change due to release from internal stores, Ca^{2+} influx through caffeine activated channels, and rate of $[Ca^{2+}]_i$ decline due to removal mechanisms calculated as in Fig. 5. (*C*) Calculated contribution of release of Ca^{2+} from internal stores (*dot-dashed*) and Ca^{2+} influx through plasma membrane ion channels (*dashed line*) to the caffeine induced change in $[Ca^{2+}]_i$ calculated as in Fig. 5.

early times results from the fact that Ca^{2+} release from internal stores triggered by caffeine was transient, and brief, and the peak rate of Ca^{2+} entry was always much greater and reached its peak more quickly than that occurring across the plasma membrane. In the 10 cells subject to this analysis peak Ca^{2+} entry from internal stores was 613 ± 108 nM/s while that mediated by the caffeine activated membrane channels was 253 ± 49 nM/s. Ca^{2+} entry from internal stores was maximal at 0.60 ± 0.06 s after the onset of the $[Ca^{2+}]_i$ transient while entry across the plasma membrane reached its maximum level on average at 3.0 ± 0.4 s after the onset of the $[Ca^{2+}]_i$

transient. Similar analysis carried out on four cells that exhibited no currents when stimulated by caffeine, revealed that the rate of Ca^{2+} entry into the cytoplasm due to release from internal stores was significantly ($P < .05$) larger (1048 ± 160 nM/s) than in cells in which caffeine also induced Ca^{2+} influx across the plasma membrane. The time for peak Ca^{2+} release (0.59 ± 0.11 s) was however not different in these four cells where caffeine failed to cause Ca^{2+} influx across the cell membrane.

As the average duration of the application of caffeine from the "puffer" pipette was 4.2 ± 0.8 s ($n = 10$), it appeared that plasma membrane gated Ca^{2+} entry may not have reached its maximal value. Hence, a series of experiments were carried out with longer duration caffeine applications. One of these is shown in Fig. 7. In this cell peak current activated by caffeine was observed at 5.6 s after the onset of the $[Ca^{2+}]_i$ transient. Although there was some fluctuation in the magnitude of the current thereafter there was no clear evidence that this response faded, the observed fluctuations may reflect variations in the flow of caffeine from the pipette.

In contrast to the effect of caffeine on Ca^{2+} entry through membrane channels which appears to be sustained, the effect of caffeine on internal stores is transient. Ca^{2+} entry into the cytoplasm returned to resting levels by 1.7 ± 0.4 s ($n = 10$) after the point of maximum release. The time course for the decay of Ca^{2+} release in different cells showed no obvious trend with either the amount of Ca^{2+} released, nor in the maximum rate of Ca^{2+} release. There was also no obvious difference in the decay of Ca^{2+} release that correlated with differences in the magnitude of the current induced by caffeine suggesting that the cessation of Ca^{2+} release from internal stores is not determined by Ca^{2+} entry across the cell membrane.

The time to onset of release of Ca^{2+} from internal stores and opening of membrane channels was virtually identical; it took on average 1.20 ± 0.2 s for the onset of Ca^{2+} release from internal stores and 1.17 ± 0.2 s for the apparent activation of membrane channels after onset of caffeine application. This delay does not appear to reflect entirely the time for caffeine to reach the cell from the puffer pipette, a process which we estimate to take from 100–200 ms under our conditions. One possible explanation for the apparent similarity in the time for opening of the plasma membrane and SR Ca^{2+} release channels is that the two processes are linked by some means.

Relationship between the Internal Stores and the Activity of the Caffeine-activated Cation Channel

To probe further for the possible linkage between Ca^{2+} release from internal stores and opening of channels on the plasma membrane in response to caffeine, we investigated the recovery kinetics of the two mechanisms following caffeine stimulation by applying caffeine twice with a relatively short interval (30 s) between applications. We have previously shown (Guerrero et al., 1994) that when caffeine is applied twice with at least a 3–5 min. pause in between applications the $[Ca^{2+}]_i$ transients induced by caffeine are virtually identical. As can be seen in Fig. 8 when only 30 s separates the first and second brief caffeine application the second exposure to caffeine elicits a much smaller Ca^{2+} transient; the peak of the second Ca^{2+} transient was 10% that due to the first caffeine application. In this particular cell the Ca^{2+} transient reflected principally release of Ca^{2+} from internal stores as there

was virtually no inward current elicited following either the first or the second exposure to caffeine. In cells where Ca^{2+} influx through caffeine activated channels was strongly reduced by holding them at +60 mV, the Ca^{2+} transient in response to a second application of caffeine was again markedly reduced by prior stimulation with caffeine (data not shown) similar to that seen in Fig. 8; the outward current elicited in these cells in response to caffeine was not noticeably different for the first versus the second exposure. Hence, while the first exposure to caffeine appears to

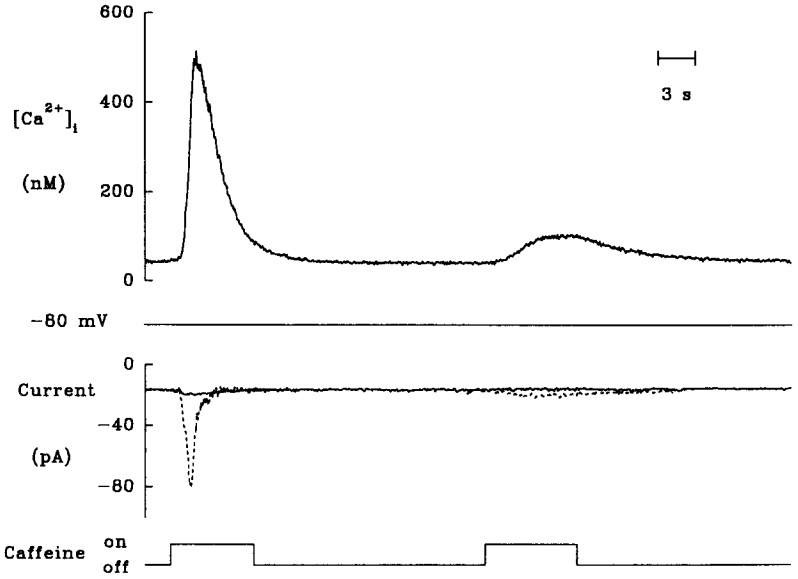

FIGURE 8. Response to dual application of caffeine in a cell where the increase in $[Ca^{2+}]_i$ in response to caffeine was due almost entirely to release from internal stores. Cell held at −80 mV was exposed to caffeine for the time indicated (*bottom trace*) and $[Ca^{2+}]_i$ (*top trace*) and membrane currents (*lower middle trace*) were measured. This cell appeared to have little or no caffeine activated cation channels. The change in $[Ca^{2+}]_i$ associated with the application of caffeine represents release from internal stores as indicated by the size of the Ca^{2+} current (*dashed line*) calculated using the cell's Ca^{2+} buffer capacity and rate of Ca^{2+} removal which would have been required to produce such increases in $[Ca^{2+}]_i$. Noise in the current trace was reduced using a moving average of five points in order to facilitate resolution of the calculated inward currents especially from the small $[Ca^{2+}]_i$ rise resulting from the second application of caffeine.

result in a persistent decline of the caffeine induced release of Ca^{2+} from internal stores, it seems to have almost no effect on the ability of caffeine to activate plasma membrane channels.

This can also be seen in Fig. 9, where results from a similar dual caffeine exposure experiment are presented for a smooth muscle cell now held at −80 mV. Under these conditions caffeine activation of the membrane channels results in significant Ca^{2+} influx from the medium. The relative contribution of Ca^{2+} release from stores versus

that due to Ca^{2+} entry through membrane channels calculated as described previously is also shown in Fig. 9. While the first response to caffeine reflects approximately equal total Ca^{2+} entry from these two Ca^{2+} sources, the rise in $[Ca^{2+}]_i$ in response to the second application of caffeine is largely due to entry of Ca^{2+} through caffeine activated membrane channels. There was relatively little entry of Ca^{2+} from internal stores during the second caffeine stimulus. Similar results were obtained in three other cells. On average, Ca^{2+} entry mediated by activation of membrane channels accounted for 65.7 ± 4% (n = 4) of the $[Ca^{2+}]_i$ rise during the first caffeine application, but accounted for 94.3 ± 2% (n = 4) during the second caffeine application which was on average 30 s after the first. This change reflected a decrease

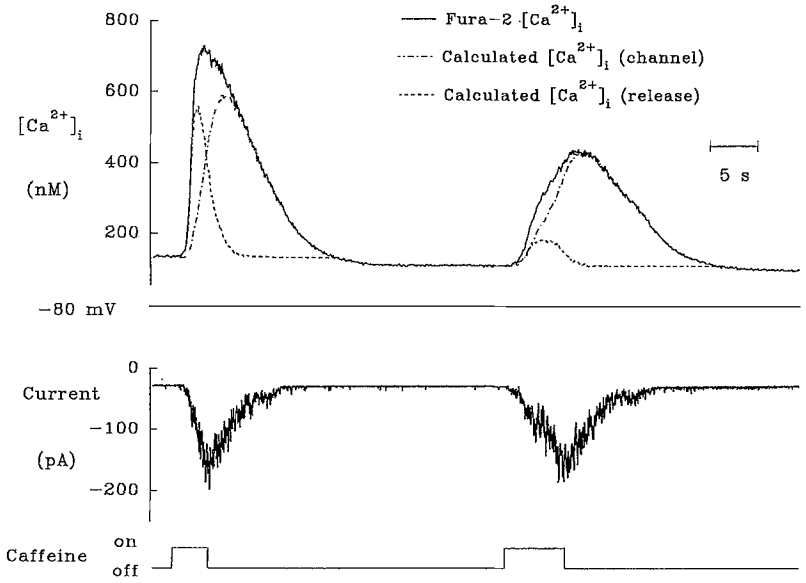

FIGURE 9. Analysis of the effect of dual application of caffeine on $[Ca^{2+}]$ changes resulting from Ca^{2+} release from internal stores and plasma membrane channels. Cell held at −80 mV was exposed to caffeine for the time indicated (*bottom trace*) and $[Ca^{2+}]_i$ (*top trace*) and membrane currents (*lower middle trace*) were measured. The contribution to the change in $[Ca^{2+}]_i$ of internal release (*dashed line*) and Ca^{2+} influx through the plasma membrane (*dot-dashed line*) were calculated as described in Fig. 5.

in Ca^{2+} release from internal stores whereas Ca^{2+} entry through membrane channels remained virtually unchanged. Hence, the results of these experiments clearly indicate that the extent of activation of membrane channels by caffeine does not appear to be correlated with the extent of Ca^{2+} release from internal stores.

DISCUSSION

The results presented in this paper have demonstrated that ~20% of the current induced by caffeine in single smooth muscle cells is carried by Ca^{2+}. The determination of the percentage of the current carried by Ca^{2+} required in turn that Ca^{2+}

buffering and Ca^{2+} removal characteristics of single smooth muscle cells be evaluated. Methods were thus developed to measure these parameters. With knowledge of the percentage of the caffeine induced current carried by Ca^{2+} we were able to extract from measurements of cytoplasmic $[Ca^{2+}]$ the characteristics of Ca^{2+} release from internal stores triggered by caffeine. The resulting detailed information regarding the time course and magnitude of Ca^{2+} entry into the cytoplasm has allowed us to simultaneously follow caffeine activated Ca^{2+} entry through channels on both the plasma membrane and sarcoplasmic reticulum and to assess possible linkages between these two pathways.

Ca^{2+} Buffer Capacity and Removal Characteristics

The cytoplasmic Ca^{2+} buffering power of these smooth muscle cells was calculated from the relationship between inward current measured with whole cell patch clamp methodology and changes in $[Ca^{2+}]_i$ measured using Fura-2. These measurements indicate that for every 85 Ca^{2+} ions entering the cell only one remains free in the cytoplasm. The remaining 84 presumably bind to Ca^{2+} buffers. Similar estimates for the Ca^{2+} buffering power of the smooth muscle cytoplasm were obtained while recording with Fura-2/AM loaded cells with amphotericin-perforated patch electrodes. The similarity in results between recordings made in the conventional whole cell configuration and the perforated patch configuration where exchange between the electrode and the cytoplasm is more limited suggest that the cellular Ca^{2+} buffers in smooth muscle are not highly diffusible and that loading of Fura-2, most likely the major Ca^{2+} buffer over the range of $[Ca^{2+}]_i$ in these studies, is quite similar under these different experimental conditions. Recent studies on rat chromaffin cells reveal similar values for the Ca^{2+} buffering power in these cells after loading with Fura-2 with a large fraction of the observed Ca^{2+} buffering power around rest attributed to Fura-2 (Zhou and Neher, 1993). The rather wide range of values for the smooth muscle cells' Ca^{2+} buffering power in the current studies we believe results from different rates of diffusional equilibration of Fura-2 that resulted from differences in the access resistance among different individual recordings.

It should be remembered that the estimate of cytoplasmic Ca^{2+} buffering is obtained by dividing the Ca^{2+} influx measured from Ca^{2+} currents in response to depolarization by the resultant change in $[Ca^{2+}]_i$ over the first 100 ms after depolarization. Thus, the observed absence of an effect of ryanodine on this parameter suggests that amplification of Ca^{2+} entry into the cytoplasm by Ca^{2+} induced Ca^{2+} release does not contribute significantly to the aggregate Ca^{2+} entry into the cell over this time interval.

The relationship between the rate of $[Ca^{2+}]_i$ decline and $[Ca^{2+}]_i$ observed in the current study was in general adequately described by a linear relationship up to $[Ca^{2+}]_i$ of 400–500 nM. Previous studies (Becker et al., 1989) have indicated that at higher $[Ca^{2+}]_i$, the rate of Ca^{2+} removal does not continue to increase but rather levels off. The observed decrease in the rate of Ca^{2+} removal in the absence of extracellular Na^+ probably reflects inhibition of Ca^{2+} extrusion from the cytoplasm that is normally mediated by Na^+/Ca^{2+} exchange. We estimate that at -80 mV when $[Ca^{2+}]_i$ is 400 nM extrusion by the Na^+/Ca^{2+} exchanger accounts for $\sim 50\%$ of the overall rate of Ca^{2+} removal above rest (McCarron, Walsh, and Fay, 1994). The

observation that ryanodine had no significant effect on the $t_{1/2}$ for Ca^{2+} removal presumably reflects the existence in these smooth muscle cells of other pathways for Ca^{2+} removal that can compensate almost fully for the effective short circuiting of SR Ca^{2+} removal by ryanodine. This contrasts to the situation in mammalian cardiac cells (Barcenas-Ruiz and Wier, 1987) and guinea-pig urinary bladder smooth muscle cells (Ganitkevich and Isenberg, 1992*b*) where ryanodine slows considerably the rate of $[Ca^{2+}]$ decline.

Method of Analysis

The method described in the current paper for determining the fraction of the caffeine activated ionic current carried by Ca^{2+} should be applicable to other Ca^{2+} permeable channels and other cells. It is relatively straightforward to carry out requiring measurements on a single cell of ionic currents and measurements of $[Ca^{2+}]_i$ changes with fluorescent Ca^{2+} indicators. The method as described requires that the cell of interest have voltage-gated Ca^{2+} channels or some other pure Ca^{2+} channel in order to estimate Ca^{2+} buffering and removal characteristics. This is certainly the case for a wide range of cell types. In cells where this is not possible, the Ca^{2+} buffering and Ca^{2+} removal characteristics might be estimated by examining the response to the rapid delivery of a known amount of Ca^{2+} into the cytoplasm by iontophoresis as described by Belan, Kostyuk, Snitsarev, and Tepikan (1993). We believe, therefore, that this approach should be extremely powerful in uncovering the role played by channels activated by stimuli ranging from mechanical stretch to chemical neurotransmitters which affect cytoplasmic $[Ca^{2+}]_i$.

The method described in this paper is similar to one originally described by Benham (1989) in that it uses measurements of current and $[Ca^{2+}]_i$ to obtain an estimate of the fraction of the current passing through a given channel which is carried by Ca^{2+}. We believe that the method described in this paper provides a more accurate estimate, however, for two reasons. First, the buffer power of the cell under study is actually determined. Given the variability of the Ca^{2+} buffer capacity among cells (30–200), this is essential in the analysis of the relation of currents and $[Ca^{2+}]_i$. Second, in our approach we correct our estimate of the rise in $[Ca^{2+}]_i$ due to a given stimulus for removal of Ca^{2+} from the cytoplasm by cellular removal mechanisms. For stimuli that activate channels either slowly or that pass a relatively small amount of Ca^{2+}, this correction is essential for obtaining an accurate estimate of the fraction of the current that can be attributed to Ca^{2+}. As shown in Fig. 10 without this correction estimates of f would decline with time due to the underestimation of the delivery of Ca^{2+} into the cytoplasm due to the activation of a given channel. For example, in the current study, if we had not corrected for removal our estimate of f for the caffeine activated current would have been 0.09 ± 0.017 ($n = 4$) measuring the relationship between current and $[Ca^{2+}]$ in the first second after caffeine activated the channel, which would represent an error in our estimate of f of 55%.

The method we have described for estimating the Ca^{2+} permeation characteristics of the caffeine activated channels in single smooth muscle cells is more direct than that used widely to assess the permeation of other channels. The methods used principally to date derive an estimate of the relative permeability to different ions of a given channel from the effect of changes in the ionic composition of the medium on

the reversal potential using various models for ion permeation through channels (Benham and Tsien, 1987). These approaches involve numerous assumptions about the way in which ions permeate through channels which may not apply especially for Ca^{2+} permeation through a channel (Tsien et al., 1987; Ascher and Nowak, 1988). Furthermore, it requires often difficult to obtain knowledge of intracellular activities of ions passing through the channel of interest.

While the method presented in this paper requires relatively straight forward measurements and no assumptions about how individual ions move through membrane channels, the method does rely on a number of assumptions as well. Let us briefly consider these assumptions, their validity, and the effect of uncertainties associated with them on the final estimate of the role played by the caffeine activated channel in delivering Ca^{2+} into the cytoplasm.

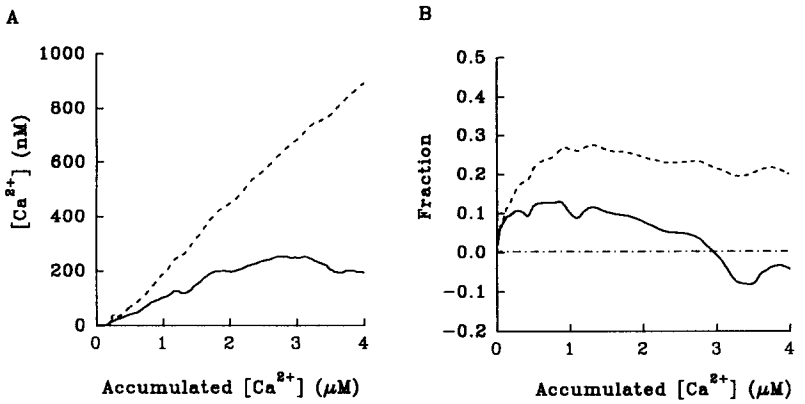

FIGURE 10. The effect of correction for Ca^{2+} removal during the response to caffeine on the estimate of the fraction of the caffeine induced current carried by Ca^{2+}. (*A*) Comparison of the numerical integral of the caffeine activated current with the removal-corrected change in $[Ca^{2+}]$ (*dashed line*) and the uncorrected change in $[Ca^{2+}]_i$ measured by Fura-2. (*B*) Fraction of current that is carried by Ca^{2+} for the relationships estimated from the data in *A*. The fraction obtained for the removal-corrected change in $[Ca^{2+}]$, once it stabilized, was relatively constant averaging 0.20 (*dashed line*). When removal was not considered, the fraction stabilized briefly at around 0.1 and then declined (*continuous line*). The time required for f to rise to a stable value reflects a delay imposed by a 41 point least square fit used to calculate the slope from part *A* rather than a real delay to achieve a stable value for the removal-corrected data.

Method of Analysis: Buffer Capacity

One of the key parameters that goes into the calculation of f, the fraction of the caffeine activated current carried by Ca^{2+}, is B, the Ca^{2+} buffer capacity of the cell. This is calculated from the relationship between the current obtained in the first 100 ms after depolarization and the resulting increase in $[Ca^{2+}]_i$ as measured with Fura-2. A key assumption in this calculation is that all of the measured current is carried by Ca^{2+}. We have previously shown (Walsh and Singer, 1987) that the voltage gated inward current is carried mainly by Ca^{2+} in these cells and that K^+ currents, which

are the only other major ionic current that is induced by depolarization is virtually abolished by TEA^+ in the media and Cs^+ in the patch pipette during these experiments (Clapp, Vivaudou, Walsh, and Singer, 1987). While it is possible that Cs^+ might also flow through the voltage-gated Ca channels thereby complicating interpretation of the current records we believe this does not have a major effect as replacing extracellular Na^+ with Cs^+ thereby abolishing any driving force on Cs^+ at 0 mV had no effect on estimates of the cell's Ca^{2+} buffer capacity using our method. Along these lines an additional assumption is that in the presence of ryanodine there is no amplification of Ca^{2+} entry through voltage-gated Ca^{2+} channels by Ca^{2+} induced Ca^{2+} release. This assumption is supported by studies on the properties of the purified ryanodine receptor from these smooth muscle cells in bilayers that reveals that ryanodine at micromolar concentrations causes these channels to go into a persistently open subconductance state where changes in Ca^{2+} can not cause them to open further (Xu, Lai, Cohn, Etter, Guerrero, Fay, and Meissner, 1994). Certainly the observation reported in the previous paper that ryanodine fully blocks all effects of caffeine on $[Ca^{2+}]_i$ (when Ca^{2+} influx across the membrane is also inhibited by Gd^{3+} or by depolarization to +60 mV) further substantiates the assumption that Ca^{2+}-induced Ca^{2+} release is absent in the presence of ryanodine.

An additional assumption inherent in our estimate of the buffer capacity, B, which, if incorrect, would cause us to overestimate B, is that Ca^{2+} extrusion from the cytoplasm by removal mechanisms has an inconsequential effect on the $[Ca^{2+}]_i$ change brought about by the Ca^{2+} current. As discussed previously the measured rate of $[Ca^{2+}]_i$ decline after a depolarization/repolarization stimulus was sufficiently slow that it would not be expected to significantly change the $[Ca^{2+}]_i$ rise observed during the first 100 ms after depolarization where influx is very high. It is of course possible that Ca^{2+} removal is in reality much higher in the first 100 ms after depolarization than 3 s later when the cell has been repolarized and removal is characterized. To test for this we also characterized Ca^{2+} removal using a brief 100-ms depolarization and found that the rate of Ca^{2+} removal after the 100-ms depolarization was not faster but in fact slightly slower than that seen at the end of a 3-s depolarization typically used to characterize Ca^{2+} removal (data not shown). Furthermore, we find that if we apply 10 mM BAPTA from a pipette onto a cell $\sim$1 s after onset of depolarization, Ca^{2+} falls in a manner predicted by the relationship between Ca^{2+} removal and $[Ca^{2+}]_i$ after cessation of the 1-s depolarization stimulus. Hence, we have no evidence to support the notion that an unsuspected high rate of Ca^{2+} removal is seriously causing us to overestimate the cells' Ca^{2+} buffer power, B. The calculation of f assumes that B, the buffer capacity of the cell is constant over the range of $[Ca^{2+}]_i$ induced by both the depolarization and caffeine applications. While the relationship between the numerical integral of the current and $[Ca^{2+}]_i$ was not perfectly linear (see Fig. 1), the data were well fit by a straight line in the range of 80 to 300 nM as indicated by a regression coefficient of 0.981.

Method of Analysis: Removal

The other key parameter that is used to obtain an estimate of f is R, the rate of Ca^{2+} removal by the cell's reuptake processes. As discussed above, while these processes do not significantly affect our estimate of the cells Ca^{2+} buffering capacity during the

rapid Ca^{2+} influx following depolarization they have a more pronounced effect on our estimate of the fraction of the slower caffeine activated current that is carried by Ca^{2+} (Fig. 10). What assumptions are involved in our estimate of the cells' Ca^{2+} removal characteristics? Perhaps the most important assumption is that the processes responsible for Ca^{2+} removal from the cell, characterized by analyzing the rate of $[Ca^{2+}]_i$ decline after cell depolarization, is not itself affected by caffeine. While there are some reports in the literature that caffeine induced emptying of Ca^{2+} stores does accelerate Ca^{2+} removal in some smooth muscles (Ganitkevich and Isenberg, 1992*a*; Baró, O'Neill, and Eisner, 1993) comparison of the rate of Ca^{2+} removal in the toad stomach smooth muscle cells after an increase in $[Ca^{2+}]_i$ induced in the same cell by depolarization before versus shortly after caffeine application failed to reveal any significant effect of caffeine on Ca^{2+} removal characteristics. For example $d[Ca^{2+}]_i/dt$ averaged 54.4 ± 6.2 vs 50.7 ± 6.5 nM/s when measured at 300 nM $[Ca^{2+}]_i$ before versus just after caffeine treatment in six cells in which the second depolarization pulse was applied ~30 s after the onset of caffeine application. Hence, the assumption that caffeine does not directly or indirectly affect Ca^{2+} removal mechanisms in these cells appears to be valid.

Another possible source of error in utilizing estimates of the cells Ca^{2+} removal characteristics to correct for Ca^{2+} removal during the response to caffeine results from the fact that Ca^{2+} removal in these cells is known to be persistently enhanced by prolonged large increases in $[Ca^{2+}]_i$ (Becker et al., 1989). While we attempted to match the magnitude and the duration of the $[Ca^{2+}]_i$ elevation used to characterize Ca^{2+} removal after depolarization for 3 s with that expected following caffeine application typically for 4 s, the match while quite good was not perfect. The peak $[Ca^{2+}]_i$ achieved following depolarization and that following caffeine application were statistically indistinguishable; this was true when responses to depolarization and caffeine were compared in cells in the presence or the absence of ryanodine. $[Ca^{2+}]_i$ was maintained near peak levels significantly longer during caffeine stimulation, however, than after a 3-s depolarization used to characterize Ca^{2+} removal in each cell. As the persistent up-regulation of the rate of $[Ca^{2+}]_i$ decline takes several seconds to develop (Becker, unpublished observation) the rate of Ca^{2+} removal calculated from the response to a 3-s depolarization may underestimate the actual rate of Ca^{2+} removal occurring during the response to caffeine. This may in turn explain the small occasional negative values for the rate of $[Ca^{2+}]_i$ rise due to release from stores in Figs. 5, 6, and 7 during the early parts of the response to caffeine.

Nonselective Cation Channels

The fraction of the current carried by Ca^{2+} for caffeine-activated channels in these smooth muscle cells appears to be considerably larger than that for nonselective cation channels in other preparations. Direct comparisons are not easily made because estimates of the fraction of the current carried by Ca^{2+} for these other cell types are obtained under different experimental conditions from either: (*a*) current-voltage relations under various ionic conditions, or; (*b*) measurements of changes in $[Ca^{2+}]_i$ by Fura-2 (without correcting for Ca^{2+} removal) and ionic currents. We have discussed above uncertainties associated with each of these methods. Keeping these uncertainties in mind it is nonetheless informative to compare our value for the

percentage of caffeine induced current carried by Ca^{2+} (19%) with that of other nonselective cation channels. For example, methods employing ion permeation models estimate the percentage as 2% for mouse skeletal muscle nicotinic acetylcholine receptors (Decker and Dani, 1990), 5.2% for neuronal nicotinic receptors in PC12 cells (Sands and Barish, 1991), 4% for the salamander retinal rod cyclic GMP-activated channels (Zimmerman and Baylor, 1992), and 12.2% for cultured mice neuron NMDA receptors (Mayer and Westbrook, 1987). Other studies employing Ca^{2+} indicators estimate the percentage to be 3.6% (and 20% in 10 mM external Ca^{2+}) for the 48/80 compound activated nonspecific cation channels in rat mast cells (Fasolato, Hoth, Matthews, and Penner, 1993) and 6.8% for rat brain NMDA receptors (Schneggenburger, Zhou, Konnerth, and Neher, 1993). For the ATP-activated channel in rabbit ear artery the values are 6% using ion permeation models (Benham and Tsien, 1987) and 10% using Ca^{2+} indicators (Benham, 1989). For ATP-activated channels in urinary bladder the values are 7 and 6% using both methods (Schneider, Hopp, and Isenberg, 1991). Hence, it would appear that the percentage of the current flowing through the nonselective cation channels carried by Ca^{2+} in response to caffeine is higher than reported for other non-selective cation channels using other methods. It remains to be seen if this reflects a real difference between channels or reflects biases introduced by the methods used to assess Ca^{2+} permeation characteristics.

While we do not yet know what the natural transmitter is that activates these channels, the properties of these channels indicate that their activity is likely to have a profound influence on the contractile activity of these smooth muscle cells in vivo. Like other nonselective cation channels in smooth muscle (Amédée, Benham, Bolton, Byrne, and Large, 1990), the caffeine activated channel does not appear to be activated by membrane depolarization. Hence, channel opening appears to be determined only by the presence of the agonist that activates it, and the membrane potential will be expected to affect the response simply through its effect on the driving force for the ions that pass through this channel. The $[Ca^{2+}]_i$ changes resulting from the opening of these channels in a typical smooth muscle cell are several hundred nanomolar, a change that would be expected to have a marked effect on the contractile state of the smooth muscle cell (Yagi, Becker, and Fay, 1988).

Caffeine-activated Ca^{2+} Entry into Cytoplasm: Two Pathways

As pointed out in this and the preceding paper, caffeine causes an increase in Ca^{2+} entry into the cytoplasm by opening channels on both the plasma membrane as well as on the sarcoplasmic reticulum. By knowing the $[Ca^{2+}]_i$ change that can be attributed to the opening of the plasma membrane activated channels we can calculate the time course and magnitude of the $[Ca^{2+}]_i$ change that is due to opening of Ca^{2+} channels on the sarcoplasmic reticulum. There was considerable variability in the relative contribution of Ca^{2+} entry from these two pathways among cells with some cells having virtually their entire $[Ca^{2+}]_i$ change attributable to Ca^{2+} release from internal stores and others having virtually all of their Ca^{2+} entry induced by caffeine attributable to activation of plasma membrane channels. In most cells Ca^{2+} entry due to both pathways contributed almost equally to the $[Ca^{2+}]_i$ rise resulting from caffeine. Regardless of the relative roles played by these two pathways the time

course of Ca^{2+} release from internal stores was extremely similar in all cells where it occurred. That release was always transient in nature.

The transient nature of release of Ca^{2+} from internal stores has often been interpreted as resulting from depletion of Ca^{2+} from those stores (Leitjen and van Breemen, 1984). Knowing the time course of Ca^{2+} entry into the cytoplasm, correcting for Ca^{2+} loss from the cytoplasm due to removal processes and accounting for Ca^{2+} buffering, we calculate that the total amount of Ca^{2+} released from stores in response to caffeine averaged 50.6 ± 5.4 ($n = 4$) μmol/liter cell H_2O. As the sarcoplasmic reticulum in these smooth muscle cells is relatively sparse, the sarcoplasmic reticulum may occupy no more than 2% of the cell volume as it does in other visceral smooth muscles with a sparse complement of SR (Somlyo, 1985; Moriya and Miyazaki, 1979). Thus, the total Ca^{2+} released from the internal stores in response to caffeine might have an equivalent concentration within the sarcoplasmic reticulum of 2.5 ± 0.2 mM ($n = 4$). This is very close to estimates of the concentration of the total Ca^{2+} stored in the sarcoplasmic reticulum of smooth muscle as determined by electron probe microanalysis (5.0 mM in junctional SR and 3.0 mM in central SR (Bond et al., 1984) assuming that 80% of the smooth muscle cell is water (Scheid and Fay, 1980). Given that caffeine releases only about 50% of the total stored Ca^{2+} in skinned smooth muscle cells, (Iino et al., 1988) the estimates of the amount of Ca^{2+} released in response to caffeine in these cells are very close to the total amount of Ca^{2+} present within these stores. Hence, it is likely that the transient nature of Ca^{2+} release from stores by caffeine is due to depletion of those stores of Ca^{2+}. Of course it is also possible that the transient nature of caffeine's action to release stored Ca^{2+} is due to desensitization of the receptor to caffeine. While recent results indicate that the ryanodine receptor does desensitize at least partially when activated by Ca^{2+} (Gyorke and Fill, 1933), no desensitization has been noted in response to caffeine when the ryanodine receptor from cardiac muscle is incorporated into planar bilayers (Rousseau and Meissner, 1989). Thus, the transient nature of the release of Ca^{2+} from internal stores most likely reflects depletion of these stores.

The activation of channels on the surface membrane and those in the sarcoplasmic reticulum by caffeine occur with a similar delay. Is this because caffeine reaches the receptor channels on both the plasma membrane and sarcoplasmic reticulum virtually simultaneously or is it because the activities of these two Ca^{2+} entry pathways are linked in some manner? While we do not have a complete answer to this question, the results presented in this and the preceding paper (Guerrero et al., 1994) provide some constraints on our thinking about this. It has been suggested that the action of agonists in other cells where release of Ca^{2+} from internal stores and enhanced Ca^{2+} entry is coupled results from a signal dependent on the emptying of internal Ca^{2+} stores (Putney, 1990; Hallam, Jacob, and Merritt, 1989). This does not appear to be the mechanism linking the dual effects of caffeine in these smooth muscle cells. This follows from the observation that the effect of caffeine on the plasma membrane channels is observed even in cells in which release from stores does not take place because of treatment of the cells with ryanodine. Furthermore, the time course and magnitude of currents activated by caffeine were neither enhanced nor diminished when caffeine was applied at intervals separated by no more than 30 s, whereas release of Ca^{2+} from internal stores under these circumstances was markedly reduced

at the time of the second application presumably because Ca^{2+} stores were depleted. Finally, release of Ca^{2+} from internal stores in response to acetylcholine, which was also transient and of similar magnitude to that induced by caffeine (Guerrero, et al., 1994) did not cause activation of non-selective cation channels as was the case for caffeine. These observations are not consistent with the notion that the opening of surface membrane channels is triggered by a signal resulting from the degree to which the internal Ca^{2+} stores have been depleted. Nor is the activation of Ca^{2+} entry across both the plasma membrane and SR membrane linked by a rise in $[Ca^{2+}]_i$ as has been suggested in other systems (Pacaud and Bolton, 1991). The observations just reviewed above support this conclusion as does the observation that even in cells where the rise in cytoplasmic $[Ca^{2+}]$ is blocked by intracellular BAPTA, activation of membrane currents by caffeine is still observed.

One is left with really only two possibilities to explain the dual nature of the action of caffeine on these cells. For one, opening of the plasma membrane channels may be linked to activation of ryanodine receptors in SR membranes by a direct physical linkage in a manner analogous to the linkage now believed to couple voltage activation of the dihydropyridine (DHP) receptors in the *t*-tubules of skeletal muscle with ryanodine receptors in the terminal cisternae of the SR in skeletal muscle. Ultrastructurally, smooth muscle is known to contain feet-like structures linking the surface membrane to the junctional SR (Somlyo, 1985), an ultrastructural characteristic believed to be responsible for physically linking the SR ryanodine receptor and membrane DHP receptors in skeletal muscle. Activation of plasma membrane channels by caffeine might thus come about because caffeine binding to the ryanodine receptor causes a conformational change that is propagated to the plasma membrane channels via a physical linkage. Perhaps the only piece of data which at first glance would seem inconsistent with this hypothesis is that caffeine is still capable of triggering an increase in membrane currents in the presence of 100 μM ryanodine where release of Ca^{2+} from internal stores is blocked presumably because ryanodine has locked the SR channels in an open subconductance state. As caffeine and ryanodine interact with different sites in affecting the ryanodine receptor (Pessah, Stambuk, and Casida, 1987), caffeine might well cause a change in the conformation of the ryanodine receptor that could be propagated to plasma membrane nonselective cation channels even in the presence of ryanodine. While this is certainly plausible it is also possible that the similarity in the onset of effects of caffeine on channels on the surface and SR membranes merely reflect the fact that these channels are situated very close to another. Thus caffeine permeates the surface membrane very rapidly thereby reaching virtually simultaneously receptors on the SR and on the plasma membrane which respond to caffeine or to as yet unknown second messengers generated in response to caffeine. The ease of caffeine movement across the plasma membrane presumably explains why with caffeine in the pipette but none in the much larger bath there is no apparent effect on $[Ca^{2+}]_i$, membrane current, or the response to caffeine when applied from a puffer pipette. While ryanodine receptors have not been detected on the plasma membrane, there is both structural and functional precedents for receptors of the sarcoplasmic reticulum like the IP_3 receptor that are also present on the plasma membrane (McDonald, Premack, and

Gardner, 1993; Fujimoto, Nakade, Miyawaki, Mikoshiba, and Ogawa, 1992). Further work will be required to discern between these two possibilities.

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