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The Role of Caffeine-Sensitive Calcium Stores in the Regulation of the Intracellular Free Calcium Concentration in Rat Sympathetic Neurons *In Vitro*

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SUMMARY

Intracellular Ca^{2+} stores were studied in sympathetic neurons grown in primary culture from the superior cervical ganglion of the rat. The $[Ca^{2+}]_i$ was measured in single cells using the fluorescent Ca^{2+} indicator fura-2 and a sensitive microfluorimeter. Superfusion of the cells with 10 mM caffeine elicited a rapid and transient increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, indicating the presence of a caffeine-sensitive intracellular Ca^{2+} storage site. After depletion of the store by mobilization of Ca^{2+} with caffeine, it could be refilled by elevating $[Ca^{2+}]_i$, allowing multiple caffeine-induced $[Ca^{2+}]_i$ transients to be elicited from a single neuron. Ryanodine (1 μM), an alkaloid that promotes Ca^{2+} release from the sarcoplasmic reticulum, was an effective inhibitor of the caffeine-induced $[Ca^{2+}]_i$ transients in sympathetic neurons. Exposure to ryanodine in the presence of caffeine was required to produce a subsequent inhibition of the caffeine-induced response, suggesting a "use-dependent" inhibition that may result from depletion of the Ca^{2+} stores. In contrast, dantrolene Na (10 μM), an agent known to interfere with Ca^{2+} release from the sarcoplasmic reticulum, also blocked the caffeine-induced $[Ca^{2+}]_i$ transients, but in a time-dependent rather than a use-dependent manner. Electrophysiological measurements using the whole cell version of the patch-clamp technique were made simultaneously with $[Ca^{2+}]_i$ microfluorimetric recordings. The magnitude of the $[Ca^{2+}]_i$ transients elicited by step depolarizations closely paralleled the magnitude of Ca^{2+} influx via voltage-sensitive Ca^{2+} channels, regardless of whether the magnitude of the Ca^{2+} current was modified by varying the test pulse duration or potential. The relationship between the magnitude of Ca^{2+} influx and the resulting increase in $[Ca^{2+}]_i$ saturated at large Ca^{2+} influxes resulting from long depolarizations, consistent with the activation of a large capacity, low affinity $[Ca^{2+}]_i$ buffering mechanism. Caffeine (10 mM) and ryanodine (10 μM), applied singly or together, produced a small and variable decrease in the $[Ca^{2+}]_i$ transient resulting from cell depolarization using the whole-cell patch-clamp technique. We conclude that mammalian sympathetic neurons possess intracellular Ca^{2+} stores with pharmacological characteristics that closely resemble those found in muscle but that these are relatively small and produce little amplification of $[Ca^{2+}]_i$ transients resulting from Ca^{2+} influx through voltage-sensitive Ca^{2+} channels.

The regulation of Ca^{2+} signalling in neurons is important for the control of a number of processes including cell excitability (1), cytoskeletal changes (2), synaptic plasticity (3), and neurotransmitter release (4). Several mechanisms exist for the regulation of $[Ca^{2+}]_i$ in neurons. Cytosolic Ca^{2+} can be elevated by Ca^{2+} influx via voltage-sensitive (5–7) and receptor-operated (8–11) ion channels and by mobilization from inositol phosphate; (12) and methylxanthine (13–20)-sensitive intracellular stores. The $[Ca^{2+}]_i$ is buffered by efflux mechanisms, which include a Na_o/Ca_i-exchanger and a Ca^{2+}/Mg^{2+}-ATPase as well as sequestration into intracellular stores by means of ATP-dependent pump (21–23). Parallel anatomical studies have identified intracellular organelles capable of sequestering Ca^{2+}, which include mitochondria, smooth endoplasmic reticulum, and the recently described calciosomes (23–28). Organelles capable of accumulating Ca^{2+} have been studied in synaptosomes (29) and squid axoplasm (30). However, mobilization of Ca^{2+} from these stores has not been demonstrated.

Kuba and colleagues (13–14) have indirectly studied a caffeine-sensitive Ca^{2+} store in bullfrog sympathetic neurons by measuring the activation state of a $[Ca^{2+}]_i$-dependent K^+ conductance. This work has suggested the presence of an intracellular Ca^{2+} store in these neurons that could be mobilized by caffeine, that was sensitive to the muscle relaxant dantrolene Na, and that could be effectively refilled by elevating extracellular Ca^{2+} (13–14). In support of this hypothesis, Smith *et al.* (15) demonstrated that caffeine-induced $[Ca^{2+}]_i$ transients could indeed be measured in sympathetic neurons using arsen-

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ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxy benzoate hydrochloride.

azo III. Similarly, Neering and McBurney (16) have recorded caffeine-induced $[Ca^{2+}]_i$ transients in sensory neurons using aequorin.

Recently, the development of the Ca^{2+}-sensitive dye fura-2 (31) has allowed the demonstration of caffeine-sensitive Ca^{2+} stores in a variety of neurons, including frog sympathetic (17) and rat sensory (18) and central (19) neurons. However, these reports have not described the properties of these sites or their physiological role. In cardiac muscle for example, caffeine-sensitive Ca^{2+} stores are thought to amplify the rise in $[Ca^{2+}]_i$ produced by Ca^{2+} influx through a Ca^{2+}-induced Ca^{2+} release mechanism (32–36).

We have studied the pharmacological and physiological characteristics of caffeine-sensitive Ca^{2+} stores in rat sympathetic neurons grown in primary culture. The properties of these stores appear to be remarkably similar to those thought to contribute to Ca^{2+}-induced Ca^{2+} release in muscle. However, in contrast to muscle, we demonstrate, using simultaneous whole cell patch-clamp and fura-2 microfluorimetry experiments, that the majority of the $[Ca^{2+}]_i$ transient resulting from membrane depolarization of these neurons appears to result from Ca^{2+} influx rather than release from intracellular stores.

Methods

The instrumentation and experimental procedures used in these experiments have been described in detail elsewhere (19). For excitation of the fura-2, a collimated light beam from a 200 W Hg arc lamp was passed through a dual beam spectrophotometer (Phoenix Instruments), which alternated wavelengths from 340 to 380 nm by means of a chopper spinning at a frequency of 60 Hz. In place of the original sample chamber, a fused silica lens was positioned to focus the light onto the end of a liquid light guide (3 mm × 1 m; Oriel, Stratford, CT). On the other end of the liquid light guide a similar lens was positioned for directing light through the eipfluorescence illuminator of the microscope. The light was reflected off a dicroic mirror (Nikon DM 400) and focused through a 70 × phase contrast oil immersion objective (E. Leitz Inc., Rockleigh, NJ, numerical aperture, 1.15). The emission fluorescence was selected for wavelength with a 480 nm barrier filter and recordings were defined spatially with a rectangular diaphragm. The fluorescence emission was analyzed with a photomultiplier tube (bialkali) and discriminator (APED II; Thorn EMI Gencom Inc., Plainview, NY). The discriminator output was converted to pulses, which were then integrated by passing the signal through an eight-pole low pass Bessel filter at 500 Hz. The signal from the filter was fed into one channel of an analog to a digital converter LSI-11/73 computer system (Indec Systems, Sunnyvale, CA). The signals from two photodiodes, each placed in a small portion of the light beam directed toward the monochromators, were fed into two additional channels of the analog to digital converter. Sorting the fluorescence output into signals corresponding to excitation at each of the two wavelengths was performed entirely with software. The photomultiplier output was sorted into signals from 340 and 380 nm excitation by using the photodiode outputs as timing signals. For experiments run over long periods of time (>16 sec) 30 ratios were determined in 1.1 sec, the average ratio was displayed on-line, and the average intensity values for each wavelength were stored. After completion of a given experiment, the microscope stage was adjusted so that no cells or debris occupied the field of view defined by the diaphragm and background light levels were determined. Records were later corrected for this background and the ratios recalculated. Traces were digitally filtered by adding one half the value of each data point to one quarter the value of each neighbor; the data was cycled through this algorithm five times.

Ratios were converted to free Ca^{2+} by using the equation $[Ca^{2+}] = K(R-R_{min})/(R_{max}-R)$ in which R is the 340/380 nm fluorescence ratio (31). The maximum ratio (R_{max}), the minimum ratio (R_{min}), and the constant K (K is the product of the dissociation constant for fura-2 and the ratio of the free and bound forms of the dye at 380 nm) were determined from a standard curve. The curve was fit to the data using the above equation with a nonlinear least squares fit computer program. The standard curve was determined for the fura-2 pentapotassium salt in calibration buffer (which contains, in mM: HEPES, 20; KCl, 120; NaCl, 5; $MgCl_2$,1; pH 7.1) containing 10 mM EGTA, $K_s = 3.969 \times 10^6$ M^{-1} (37), with calculated amounts of Ca^{2+} added to give free Ca^{2+} concentrations ranging from approximately 0 to 2000 nM.

The recording chamber routinely used consisted of a Plexiglas block machined to accommodate a 25-mm round coverglass as a bottom. The coverglass was held in place by a steel ring secured by four nuts threaded onto studs that protrude from the Plexiglas. Three reservoirs were cut into the block such that a thin sheet of buffer flowed out of the inlet reservoir, across the cells in the experimental chamber, and was drawn up across nylon mesh to the efflux reservoir for evacuation by suction. The solution exchange in the cell superfusion system was approximated by a step occurring over 10 sec. The tubing between the large media reservoirs and the inlet to the chamber delayed the onset of the solution exchange by an additional 10 sec. This chamber was mounted on an inverted microscope and cells or portions of cells were localized by standard phase contrast illumination.

Sympathetic neurons were cultured from the superior cervical ganglion of neonatal rats essentially as described by Thayer *et al.* (38). The cells were plated on glass coverglasses (no. 1, 25 mm round). Neurons were loaded with the dye by incubation in 2 μM fura-2 acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR), which is membrane permeant, for 1 hr at 37° in HEPES-buffered Hank's balanced salt solution, pH 7.45, containing 0.5% bovine serum albumin. The HEPES Hank's solution was composed of (in mM) HEPES, 20; NaCl, 137; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $MgCl_2$, 0.5; KCl, 5.0; KH_2PO_4, 0.4; $NaHPO_4$, 0.6; $NaHCO_3$, 3.0; and glucose, 5.6. After the loading incubation, during which time the dye ester is hydrolyzed by cytosolic esterases to the membrane-impermeant polycarboxylate anion that is fura-2, the cells were washed twice in the HEPES-Hank's solution and incubated for 30 min. The coverglasses containing the loaded and washed cells were then mounted in the recording chamber for viewing. Depolarization-induced Ca^{2+} influx was produced in the superfusion experiments by changing the perfusing solution from low K^+ (5 mM) to high K^+ (50 mM) with K^+ exchanged for Na^+ reciprocally. In Ca^{2+}-free solutions 20 μM EGTA was substituted for Ca^{2+}. Caffeine (10 mM) does not interfere with the fura-2 Ca^{2+} measurements described here.

The tight-seal whole-cell configuration of the patch-clamp technique (39) was used to record transmembrane Ca^{2+} currents from single cells while simultaneously measuring $[Ca^{2+}]_i$ transients. Cells were mounted in the recording chamber and thoroughly rinsed with a buffer composed of (in mM): NaCl, 143; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 10; pH adjusted to 7.4 with NaOH. A cell was then approached with a fire-polished pipette containing a solution composed of (in mM): fura-2 pentapotassium salt, 0.1; CsCl, 135; $MgCl_2$, 1; HEPES, 10; diTris phosphocreatine, 14; MgATP, 3.6; 50 units/ml creatinine phosphokinase; pH adjusted to 7.1 with CsOH. Background fluorescence was recorded after a gigaseal was formed, but before breaking into the cell. Thus, we could account for the fluorescence contributed by the fura-2 in the pipette (35). Because the pipette approached the cell from above, the objective was focused below the pipette nearer the middle of the cell to minimize the detection of pipette fluorescence. The perfusing solution was then changed to one chosen to isolate currents through Ca^{2+} channels from other currents (namely, Na and K currents) by replacing Na^+ with tetraethylammonium. The currents were recorded by a Yale Mark V amplifier with a 1.0 GΩ feedback resistor, filtered by an eight-pole low pass Bessel filter with a cutoff frequency of 200 Hz, and stored on the computer used for fluorescence data acquisition. Linear leak corrections were performed by first averaging 16 10-mV hyperpolarizing pulses from the holding potential. The DC component of the averaged leak current was then modelled so as to increase the signal to noise ratio. Digital summation of this leak template, after

appropriate scaling with the current obtained during depolarizing test pulses, provided the leak correction. $[Ca^{2+}]_i$ traces were digitally filtered by one cycle through an 11-point moving average algorithm. All experiments were performed at 20–22°.

Results

Superfusion of rat sympathetic neurons with 10 mM caffeine produced a rapid and transient increase in $[Ca^{2+}]_i$ (Fig. 1A) $[Ca^{2+}]_i$ rose to 357 ± 26 nM (70 experiments) above a basal level of 67 ± 4 nM (70 experiments). The production of such transients did not require the presence of extracellular Ca^{2+} inasmuch as they occurred after perfusion for several minutes in Ca^{2+}-free medium. Thus, consistent with other reports (13–20), the elevation in $[Ca^{2+}]_i$ appears to result from the mobilization of Ca^{2+} from intracellular stores. The observation that $[Ca^{2+}]_i$ rapidly returned to basal levels in the maintained presence of caffeine is consistent with the idea than an internal Ca^{2+} storage site was being depleted of Ca^{2+}. Furthermore, a second application of caffeine failed to elicit a response when applied in the continued absence of extracellular Ca^{2+}. However, if the cell was superfused with 50 mM K^+ in the presence of extracellular Ca^{2+}, the resulting depolarization and subsequent Ca^{2+} influx via voltage-sensitive Ca^{2+} channels allowed the rapid refilling of the intracellular Ca^{2+} store. After such treatment, reapplication of caffeine once again elicited a full response (Fig. 1A). The mobilization of intracellular Ca^{2+} produced by caffeine was not a function of its inhibitory actions on cAMP phosphodiesterase, as indicated by the absence any effect of 1 mM dibutyryl-cAMP on resting $[Ca^{2+}]_i$ (five experiments).

The ability to refill caffeine-sensitive Ca^{2+} stores allowed us to use the paradigm displayed in Fig. 1B. Alternate application of 50 mM K^+ (with external Ca^{2+}) and 10 mM caffeine (in Ca^{2+}-free medium) resulted in the generation of multiple caffeine-induced $[Ca^{2+}]_i$ transients of similar magnitude. The average caffeine-induced $[Ca^{2+}]_i$ transient decreased slightly with repetitive stimulation. It is interesting to note, however, that the magnitude of the depolarization-induced $[Ca^{2+}]_i$ transient was attenuated somewhat after depletion of the caffeine-sensitive Ca^{2+} store (Fig. 1B). The inhibition of this depolarization-induced transient produced by prior caffeine exposure was variable but showed a significant correlation ($r = 0.50$) with the magnitude of the caffeine-induced $[Ca^{2+}]_i$ transient (Fig. 1C). One possible explanation for this finding is that the release of caffeine-sensitive Ca^{2+} stores are normally triggered by a rise in$[Ca^{2+}]_i$ resulting from Ca^{2+} influx. Thus, as in muscle, these stores may participate in Ca^{2+}-induced Ca^{2+} release to amplify the transient triggered by Ca^{2+} influx. Caffeine was not present during the exposure to high K^+ and, indeed, caffeine itself will produce a slight inhibition of Ca^{2+} influx via voltage-sensitive Ca^{2+} channels (Ref. 20 and see below). We considered that this inhibition of Ca^{2+} influx could result from a caffeine-induced elevation of cAMP levels inasmuch as caffeine is a known phosphodiesterase inhibitor and because dibutyryl cAMP was found to produce a slight inhibition of the 50 mM K^+-induced Ca^{2+} transient (23 ± 11% inhibition, five experiments). However, it is doubtful whether this was the basis for the caffeine-produced inhibition of the 50 mM K^+-induced $[Ca^{2+}]_i$ transient because, if this were the case, the inhibition would not be expected to correlate with the size of the intracellular Ca^{2+} stores. These questions are further addressed below.

We next investigated the pharmacology of the caffeine response. The effectiveness of millimolar concentrations of caffeine in inducing Ca^{2+} release from the sarcoplasmic reticulum (40, 41) suggested that the caffeine-sensitive Ca^{2+} stores in sympathetic neurons might share a pharmacological profile similar to that found in muscle. We tested the effects of several agents that are thought to modulate $[Ca^{2+}]_i$ in muscle. For example, the alkaloidal insecticide ryanodine (42) has been found to modulate the release of Ca^{2+} from the sarcoplasmic reticulum[1] (43–47). In sympathetic neurons 1 μM ryanodine completely blocked the caffeine-induced $[Ca^{2+}]_i$ transient (Fig. 2 A and B). It should be noted that ryanodine did not block

[1] M. Fill and R. Coronado. Ryanodine receptor channel of sarcoplasmic reticulum. Submitted for publication.

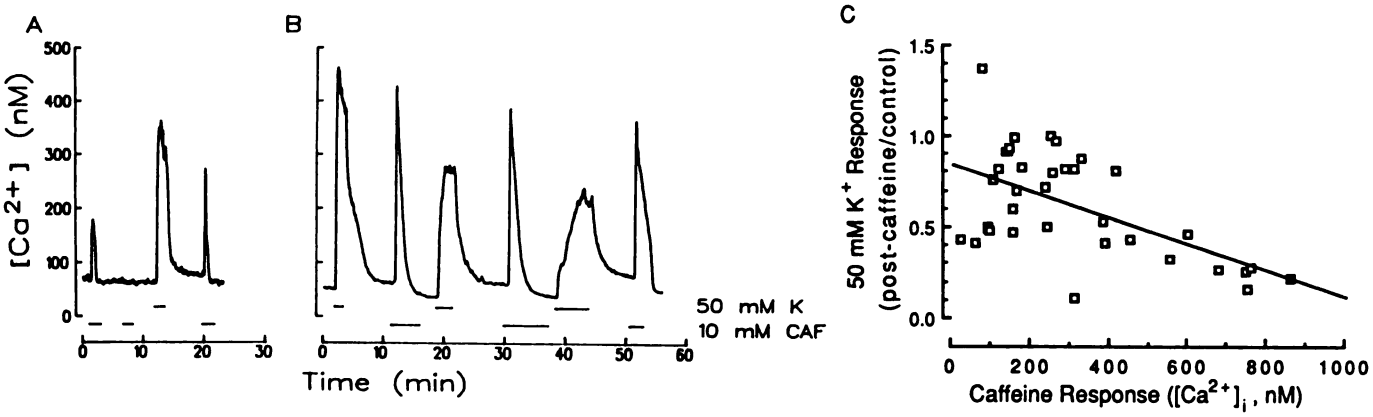

Fig. 1. Caffeine-induced $[Ca^{2+}]_i$ transients in sympathetic neurons. A and B $[Ca^{2+}]_i$ recordings were made from single superior cervical ganglion neurons continuously perfused with Ca^{2+}-free (20 μM EGTA) media that contained 10 mM caffeine (*CAF*) during the time indicated by the *horizontal bars*. *Horizontal bars* also indicate perfusion with 50 mM K^+ in normal Ca^{2+}-containing media (1.3 mM). Thus, extracellular Ca^{2+} was present in the depolarizing medium only. $[Ca^{2+}]_i$ was measured as described in Methods. The second application of caffeine in A failed to elicit a response, yet elevating $[Ca^{2+}]_i$ by perfusion with 50 mM K^+ enabled another response to be generated. In B, multiple caffeine-induced $[Ca^{2+}]_i$ transients were elicited by alternating caffeine with 50 mM K^+ exposures. Note the decreased size of the second 50 mM K^+-induced $[Ca^{2+}]_i$ transient. The data from 33 similar experiments are displayed in C, in which the size of the caffeine-induced response is plotted against the ratio of the high K^+ response after caffeine exposure relative to the initial control response. In untreated cells this ratio was not significantly different from 1 (three experiments) (see also Ref. 38). The curve was fit by linear regression ($r = 0.50$) and analysis of variance indicated a significant trend ($p = 0.002$).

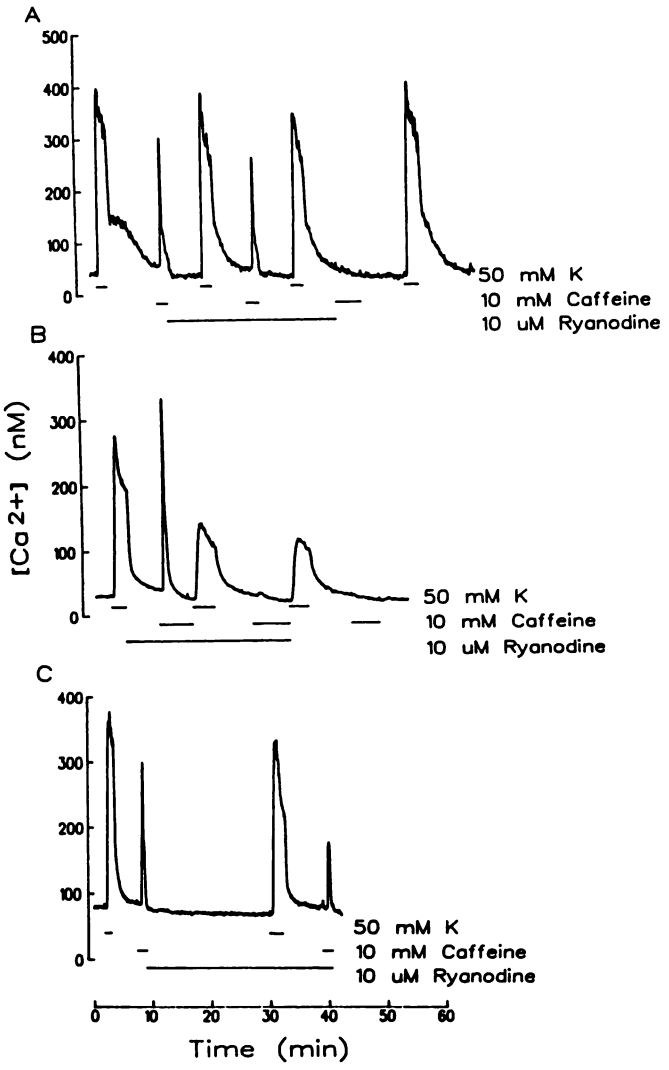

Fig. 2. Ryanodine inhibition of caffeine-induced $[Ca^{2+}]_i$ transients. A and B, Multiple caffeine-induced $[Ca^{2+}]_i$ transients were elicited as described in Fig. 1B. Ryanodine (1 μM) had no significant effect on either the 50 mM K^+-induced $[Ca^{2+}]_i$ transient or the first transient elicited by caffeine in the presence of ryanodine. The second caffeine-induced response in the presence of ryanodine was completely blocked. Ryanodine (1 μM) produced only a slight inhibition of the first caffeine exposure (C) if the cells were incubated with ryanodine for 30 min. Recordings were made as described in Methods and additions to the media are indicated by the *horizontal bars* as labeled.

the first caffeine-induced $[Ca^{2+}]_i$ transient elicited in its presence yet completely blocked the second caffeine-induced $[Ca^{2+}]_i$ transient after the application of ryanodine. This "use dependence" of the ryanodine blockade of the caffeine response is consistent with the mechanism of action of this drug in muscle where it stabilizes an open state of the channel responsible for Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum[1] (43–47). Thus, a possible mechanism of action for ryanodine inhibition of the caffeine-induced $[Ca^{2+}]_i$ transients described here may be as follows. First, ryanodine binds to the caffeine-activated channel, which depletes the intracellular store. Second, plasmalemma Ca^{2+} efflux mechanisms remove the elevated $[Ca^{2+}]_i$ from the cytoplasm. Third, removal of the caffeine does not close the channel as it is now locked in an open state by ryanodine; thus, the store cannot refill. Finally, reapplication of caffeine fails to elicit a response because the store has been depleted. This hypothesis is consistent with experiments such as the one described in Fig. 2C. Ryanodine was applied to a cell for over 30 min, after an initial control caffeine-induced $[Ca^{2+}]_i$ transient. After refilling the store, reapplication of caffeine still elicited a $[Ca^{2+}]_i$ transient (five experiments). The caffeine-induced $[Ca^{2+}]_i$ transient elicited after the long preincubation with ryanodine was slightly smaller than the control, suggesting that the ryanodine did have minor time-dependent inhibitory actions. However, this inhibition was clearly less dramatic than the complete block produced, in Fig. 2 A and B, by a much shorter incubation time after a combined ryanodine-caffeine exposure.

We also investigated other agents known to be effective on Ca^{2+} stores in muscle. Dantrolene Na is a muscle relaxant that blocks Ca^{2+} release from the sarcoplasmic reticulum (48, 49). We found that dantrolene (10 μM) completely blocked caffeine-induced $[Ca^{2+}]_i$ transients in sympathetic neurons as well (five experiments) (Fig. 3A). In contrast to the ryanodine inhibition, however, dantrolene showed a gradual onset of inhibition acting in a time-dependent rather than a use-dependent fashion. At a concentration of 10 μM, dantrolene also produced a significant inhibition of 50 mM K^+-induced $[Ca^{2+}]_i$ transients (51 ± 14% inhibition, three experiments). The putative inhibitor of Ca^{2+} release from intracellular stores, TMB-8, failed to produce an effect in our paradigm (four experiments) (Fig. 3B).

The presence of an intracellular Ca^{2+} store in neurons with a pharmacological profile similar to that seen in muscle suggested that these stores might play a physiological role similar to that seen in muscle. We therefore investigated the possibility that these stores amplified $[Ca^{2+}]_i$ transients elicited by depolarization-induced activation of voltage-gated Ca^{2+} channels. Inward Ca^{2+} currents were measured with the whole cell con-

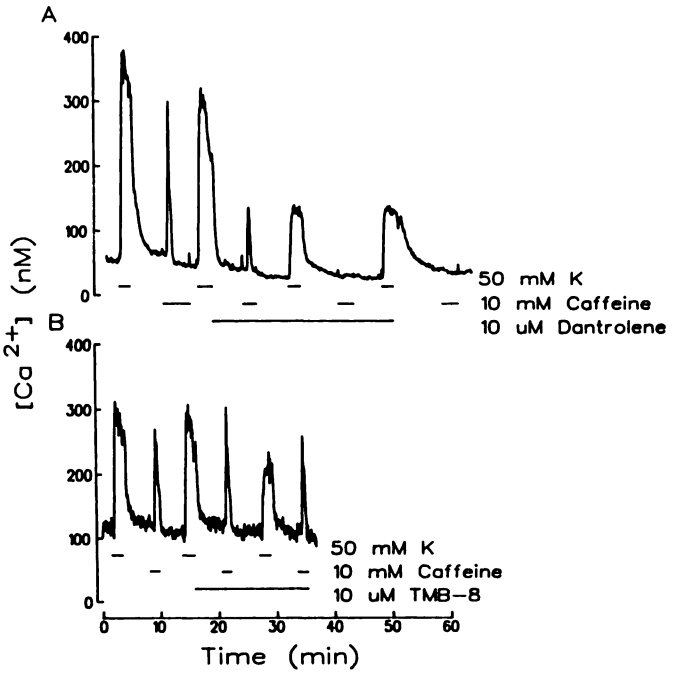

Fig. 3. The effect of dantrolene Na and TMB-8 on caffeine-induced $[Ca^{2+}]_i$ transients in sympathetic neurons. The paradigm described in Fig. 2A was used to test the effects of dantrolene (10 μM) (A) and TMB-8 (10 μM) (B) on the caffeine-induced $[Ca^{2+}]_i$ transients. Recordings were made as described in Methods and additions to the media are indicated by the *horizontal bars* as labeled.

figuration of the patch-clamp technique as described in Methods.

Fig. 4A illustrates a combined whole-cell patch-clamp and fura-2 microfluorimetry experiment in which a sympathetic neuron was voltage clamped at −80 mV and depolarized to 0 mV. The $[Ca^{2+}]_i$ rose rapidly during the test pulse, reaching a maximum at the end of the pulse. $[Ca^{2+}]_i$ then slowly returned to basal levels during the remainder of this 16-sec sweep. In Fig. 4B another cell was voltage clamped at −80 mV and depolarized to 0 mV for different test pulse durations. Seven sweeps with test pulse durations ranging from 10 to 640 msec are superimposed. Ca^{2+} currents were isolated by replacement of external Na^+ with tetraethylammonium$^+$ and internal K^+ with Cs^+. Thus, the downward traces on the current scale represent charge moving into the cell, carried by Ca^{2+}. The upward traces describe the $[Ca^{2+}]_i$. Aside from qualitatively describing the relationship between Ca^{2+} currents and $[Ca^{2+}]_i$ transients, two additional points are apparent from this experiment. First, it is clear that $[Ca^{2+}]_i$ remains elevated long after the Ca^{2+} influx has ceased and the cell has repolarized. $[Ca^{2+}]_i$ did actually return to basal levels before eliciting the next sweep, as shown for the very long sweep in Fig. 4A and the low resting $[Ca^{2+}]_i$ at the beginning of each sweep in Fig. 4B.

The second point of interest is that longer test pulse durations increased the peak rise in $[Ca^{2+}]_i$. The data in Fig. 4B that describe the relation between Ca^{2+} influx and $[Ca^{2+}]_i$ are illustrated in a more quantitative format in Fig. 4C. Ca^{2+} influx was determined by integrating the inward current during each test pulse, resulting in the net influx of Ca^{2+} in Coulombs. These data were then plotted against the peak of the $[Ca^{2+}]_i$ transient that resulted from that test pulse. The relationship is described by a saturation isotherm. It appears essentially linear for small influxes of Ca^{2+} (short test pulses) and begins to saturate at very large Ca^{2+} fluxes (long test pulses). Cytosolic buffering apparently increases at higher $[Ca^{2+}]_i$ values, causing the saturation that is observed at large influxes of Ca^{2+}.

The precise mechanism for triggering the mobilization of Ca^{2+} from intracellular stores in muscle is not fully understood. Several possibilities have been suggested, however, including Ca^{2+}-induced Ca^{2+} release (32–36), release triggered by inositol trisphosphate (50), and a direct activation of a voltage sensor by membrane depolarization (51, 52). It is possible that different mechanisms are operative in different muscle types. We considered the possibility that Ca^{2+} could be mobilized from intracellular stores in a voltage-dependent fashion. The experiment in Fig. 5 was performed to determine the relationship between test potential, Ca^{2+} currents, and $[Ca^{2+}]_i$ transients. Again it is clear that, as in Fig. 4, the magnitude of the $[Ca^{2+}]_i$ transients closely parallels that of the inward Ca^{2+} currents elicited. Stepwise increases in the test potential recruited additional channels producing a peak inward current at 0 mV. Similarly, the $[Ca^{2+}]_i$ transient peaked at 0 mV. As the potential was stepped more positive than 0 mV and began to approach the Ca^{2+} reversal potential, the reduction in the driving force for Ca^{2+} influx resulted in a smaller inward current and a decreased $[Ca^{2+}]_i$ transient. At very positive potentials (positive to 40 mV) the apparent Ca^{2+} reversal potential was reached and no detectable inward current resulted, yet a small $[Ca^{2+}]_i$ transient remained. This apparent discrepancy can be explained by a technical problem in determining the true Ca^{2+} reversal potential, which experimentally is lower than the theoretical value. Contaminating outward currents are generally thought to be the cause of this effect. Because the measurement of $[Ca^{2+}]_i$ transients with fura-2 is not affected by such outward currents, the potential dependence of the peak of the $[Ca^{2+}]_i$ transient is in better agreement with the theoretical value and thus appears to reverse at a more positive potential. In contrast to the results displayed in Fig. 5, similar experiments performed on rat cardiac cells show a half maximal activation of the $[Ca^{2+}]_i$ at lower test potentials than the half maximal activation of the Ca^{2+} current (52). This finding was interpreted as indicating a contribution of voltage-dependent Ca^{2+} release from intracellular stores to the $[Ca^{2+}]_i$ transient. In the experiment shown in Fig. 5, it is clear that in rat sympathetic neurons both the Ca^{2+} current and the $[Ca^{2+}]_i$ transient are half maximally activated at approximately −10 mV. Thus, the similar dependence on membrane potential for the Ca^{2+} current and the $[Ca^{2+}]_i$ transient in these cells indicates that the rise in Ca^{2+} resulting from Ca^{2+} influx is not greatly amplified by voltage-activated Ca^{2+} release from intracellular stores.

We have also used a pharmacological approach to explore the possible contribution of the caffeine/ryanodine-sensitive Ca^{2+} store to the $[Ca^{2+}]_i$ transient resulting from depolarization. In combined whole-cell patch-clamp and fura-2 microfluorimetric experiments, ryanodine and caffeine were tested for their ability to depress the $[Ca^{2+}]_i$ transient resulting from depolarization from −80 mV to 0 mV. A 320 msec test pulse was applied to the cell every 20 sec and the inward Ca^{2+} current and the basal and peak of the $[Ca^{2+}]_i$ transients were determined. Ryanodine (10 μM) alone had no effect on any of these parameters (three experiments). Whether the lack of effect was due to the complicated use dependency of this drug or the small contribution of the stores to the Ca^{2+} transient is not clear. However, it should be pointed out that this paradigm has proved to be effective in demonstrating a Ca^{2+}-induced Ca^{2+} release component to the $[Ca^{2+}]_i$ transient in cardiac cells (35, 36). Application of 10 mM caffeine produced a transient rise in basal $[Ca^{2+}]_i$ with a magnitude and time course similar to the experiments shown in Figs. 1–3. In the maintained presence of caffeine, a slight inhibition of the inward current as well as a slight inhibition of the peak of the $[Ca^{2+}]_i$ transient resulted (three experiments). Interpretation of these results is ambiguous, however, because it is not clear whether the reduction in the size of the $[Ca^{2+}]_i$ transient is the result of depleted caffeine-sensitive Ca^{2+} stores or the result of the inhibited inward current.

Fig. 6 illustrates an experiment in which caffeine and ryanodine were applied together to a cell under whole-cell voltage clamp. To determine the effect of the caffeine/ryanodine combination on the relationship between Ca^{2+} influx and the $[Ca^{2+}]_i$ transient, Ca^{2+} influx versus $[Ca^{2+}]_i$ curves were generated in three experiments. The Ca^{2+} influx-$[Ca^{2+}]_i$ relationship was determined by applying test pulses of different duration in a paradigm identical to that described in Fig. 4 B and C. The control (drug-free) curve in Fig. 6A was generated before the first point plotted in Fig. 6B. As shown in Fig. 6B, basal and peak $[Ca^{2+}]_i$ as well as the inward current were stable before the addition of the drugs. Addition of 10 mM caffeine and 10 μM ryanodine produced a dramatic increase in the basal $[Ca^{2+}]_i$. The time course of this elevation in $[Ca^{2+}]_i$ is in good agreement with the duration of the caffeine-induced $[Ca^{2+}]_i$ transients described in Figs. 1–3, indicating that dialysis of the

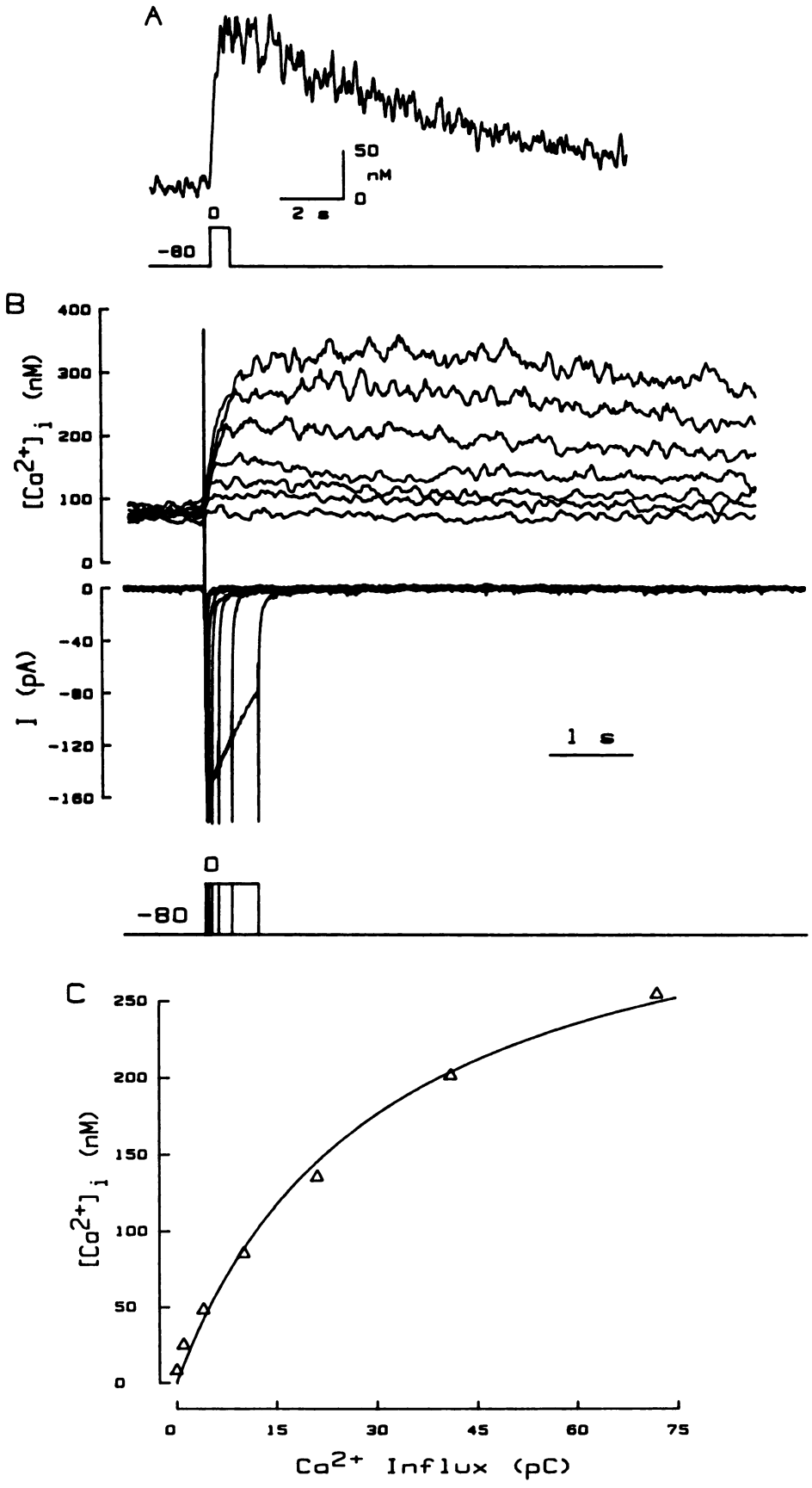

Fig. 4. Combined patch-clamp and microfluorimetric analysis of depolarization-induced Ca^{2+} currents and $[Ca^{2+}]_i$ transients in sympathetic neurons. Stepping from a holding potential of −80 mV to a test potential of 0 mV produced a large increase in $[Ca^{2+}]_i$ as shown in A. The voltage protocol displayed below the $[Ca^{2+}]_i$ trace indicates that the $[Ca^{2+}]_i$ transient lasted far longer than the 320–msec depolarization, taking the entire 16 sec sweep to recover to near basal levels. In B, seven sweeps with test pulse durations of 10, 20, 40, 80, 160, 320, and 640 msec are superimposed. All ionic conductances except Ca^{2+} currents were blocked by ionic substitution as described in Methods. Thus, the downward current traces represent charge carried by Ca^{2+}. This charge movement was integrated over the duration of each test pulse and plotted against the peak of the corresponding $[Ca^{2+}]_i$ transient in C. Peak $[Ca^{2+}]_i$ values plotted are net increases above the basal $[Ca^{2+}]_i$ determined during the 240 ms just prior to evoking the test pulse. The curve was fit to the experimental data by a nonlinear least squares computer program using the equation for a rectangular hyperbola, $y = ax/(b + x)$ where y is the $[Ca^{2+}]_i$, x is Ca^{2+} Influx in pC, a is the maximum theoretical $[Ca^{2+}]_i$ obtainable, and b is the inward charge movement resulting in a half maximal $[Ca^{2+}]_i$. Values for a and b were found to equal 352 nM and 30 pC, respectively.

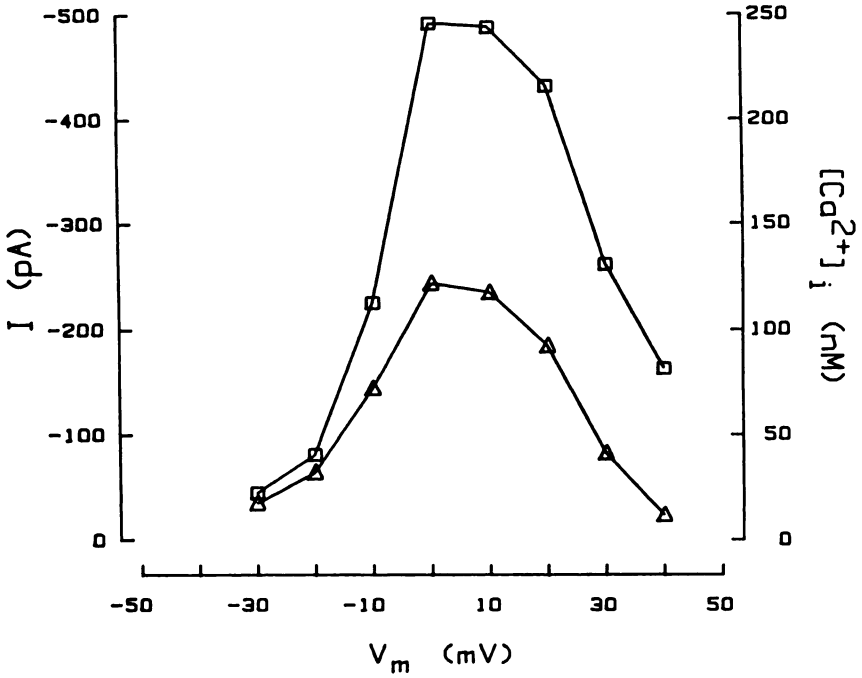

Fig. 5. The Ca^{2+} current and $[Ca^{2+}]_i$ versus membrane voltage relationship for a sympathetic neuron. The neuron was voltage clamped in the whole-cell configuration at −80 mV and 320-msec test pulses to the potentials indicated were applied to the cell to elicit Ca^{2+} currents (△). Leak was assumed linear and corrected as described in Methods. $[Ca^{2+}]_i$ values (□) are net increases above the basal $[Ca^{2+}]_i$ determined during the 240 msec just before the test pulse was evoked. For clarity, the axes were scaled to prevent superimposed points.

cytosol with the pipette solution did not disrupt the Ca^{2+} stores. The caffeine and ryanodine combination produced an inhibition of the inward current and a slight decrease in the peak of the $[Ca^{2+}]_i$ transient as described above for caffeine alone. Identical results were obtained in seven replicate experiments. The current and $[Ca^{2+}]_i$ again stabilized and another Ca^{2+} influx-$[Ca^{2+}]_i$ curve was generated. This curve, in the presence of caffeine and ryanodine, was offset from the control curve. Thus, in this experiment caffeine and ryanodine have apparently depleted the intracellular Ca^{2+} stores, preventing them from contributing to the $[Ca^{2+}]_i$ transient and resulting in a slightly depressed peak $[Ca^{2+}]_i$ obtained for a given amount of Ca^{2+} flowing across the plasmalemma. Fig. 6 illustrates the largest effect of ryanodine and caffeine on the $[Ca^{2+}]_i$ transient we observed; in the other two cells, the two curves were not significantly different. It is clear that, in contrast to cardiac muscle (35, 36) or even bullfrog sympathetic neurons (20), the contribution of Ca^{2+}-induced Ca^{2+} release from intracellular stores to the depolarization-induced $[Ca^{2+}]_i$ transient in rat sympathetic neurons is relatively modest compared with the contribution mediated by Ca^{2+} influx.

Discussion

We have described the presence of intracellular Ca^{2+} stores in rat sympathetic neurons with pharmacological characteristics similar to those described in the sarcoplasmic reticulum. As in the sarcoplasmic reticulum, these stores could be mobilized by millimolar concentrations of caffeine. Production of caffeine-induced $[Ca^{2+}]_i$ transients did not require the presence of extracellular Ca^{2+}, as would be expected for the release of Ca^{2+} from intracellular storage sites. The rapid and transient elevations in $[Ca^{2+}]_i$ produced by caffeine have a time course similar to the $[Ca^{2+}]_i$-dependent hyperpolarizations reported previously for bullfrog sympathetic neurons (13, 14). Elevating the $[Ca^{2+}]_i$ aided in refilling these stores, suggesting that they may contribute to Ca^{2+} buffering when $[Ca^{2+}]_i$ is elevated under physiological circumstances. Indeed, we have shown elsewhere that depletion of the caffeine-sensitive Ca^{2+} store in sensory neurons will produce a dramatic shortening of the depolarization-induced $[Ca^{2+}]_i$ transient (18). We suggested that the depleted stores were able to participate in buffering cytosolic Ca^{2+}.

In rat sympathetic neurons, the caffeine-induced $[Ca^{2+}]_i$ transients could be blocked by 1 μM ryanodine and 10 μM dantrolene. Both of these agents are known for their effectiveness in modulating Ca^{2+} release from the sarcoplasmic reticulum[1] (42–49). This suggests the presence in neurons of a Ca^{2+} release channel with properties similar to those of the channel recently isolated from muscle and reconstituted into lipid bilayers (43). Indeed, Henkart *et al.* (24) have provided anatomical evidence indicating that the endoplasmic reticulum in neurons can accumulate Ca^{2+} and that, at the interface of the terminal cisternae and the plasma membrane, an electron-dense material is present that is reminiscent of the "feet" structures located at the sarcoplasmic reticulum T tubule junction (53). The feet proteins are thought to represent the ryanodine binding site-Ca^{2+} release channel.[1] Furthermore, an antibody to the ryanodine binding site from sarcoplasmic reticulum has been found to cross-react with a microsomal fraction isolated from our rat sensory neuron cultures.[2]

The required exposure of ryanodine in the presence of caffeine for maximal inhibition of the caffeine-induced $[Ca^{2+}]_i$ transient is consistent with the "use-dependence" of ryanodine action in muscle tissues (47–54). In single channel recordings of the reconstituted sarcoplasmic reticulum Ca^{2+} release channel, ryanodine has been shown to stabilize a channel open state of intermediate conductance[1] (43–46). However, in contrast to the results from muscle, the elevation of $[Ca^{2+}]_i$ produced by membrane depolarization (Fig. 2A) was not sufficient to enhance the action of ryanodine. Dantrolene Na is a muscle relaxant that prevents the release of Ca^{2+} from the sarcoplasmic reticulum (48, 49). The time-dependent block of the caffeine-

[2] K. Campbell personal communication

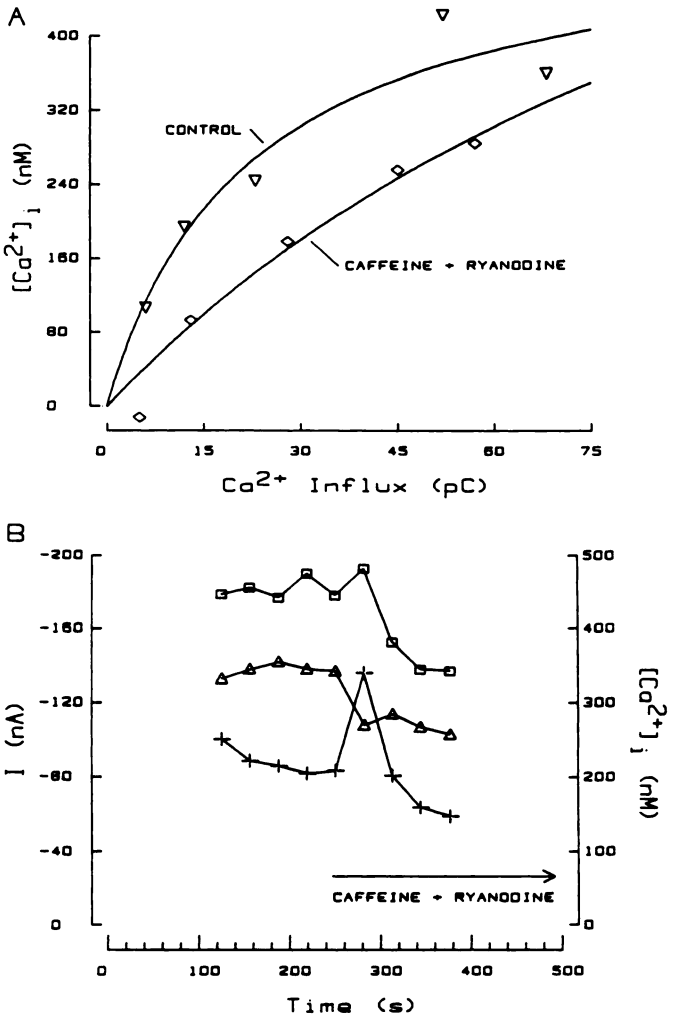

Fig. 6. Caffeine and ryanodine inhibition of the depolarization-induced $[Ca^{2+}]_i$ transient. Ca^{2+} current and $[Ca^{2+}]_i$ recordings were made using the combined electrophysiological and microfluorimetric technique described in Methods and applied in Figs. 4 and 5. A control (drug-free) Ca^{2+} influx-$[Ca^{2+}]_i$ relationship was determined in an experiment similar to the one described in Fig. 4, B and C. The resulting curve is plotted in A (▽). The experiment then continued as shown in B; basal $[Ca^{2+}]_i$ at the beginning of each sweep (+) peak $[Ca^{2+}]_i$ (□), and peak Ca^{2+} current (△) are plotted against time. All three parameters were stable until 10 mM caffeine and 10 μM ryanodine were added to the perfusing solution. The drugs produced an initial increase in basal $[Ca^{2+}]_i$. The peak of the $[Ca^{2+}]_i$ transient was relatively unchanged in spite of the slight decrease in Ca^{2+} current. The basal $[Ca^{2+}]_i$ then dropped to slightly below pre-drug levels and the peak $[Ca^{2+}]_i$ and Ca^{2+} current were slightly inhibited. At this time the Ca^{2+} influx-$[Ca^{2+}]_i$ relationship was determined again and is plotted in A (◇). *Curves* were fit as described in Fig. 4C with the following constants: control (▽), a = 525 nM, b = 22 pC; caffeine plus ryanodine (◇), a = 125 nM, b = 936 pC.

induced $[Ca^{2+}]_i$ transient we have observed is consistent with an inhibition of the Ca^{2+} release channel by dantrolene, although the mechanistic details of its action are not known. In similar experiments on the bullfrog sympathetic neuron, Kuba (13) found that dantrolene was an effective inhibitor of the increase in the action potential after hyperpolarization produced by caffeine. It is not known whether dantrolene acts on the same protein as ryanodine, although it appears that the caffeine-sensitive Ca^{2+} stores in neurons have the same pharmacological regulatory sites as the Ca^{2+} release mechanism in the sarcoplasmic reticulum. The absence of effects of TMB-8 on the caffeine response is not surprising, given the controversial effectiveness of this drug in other tissues (55).

Although caffeine-sensitive Ca^{2+} stores are clearly present in rat sympathetic neurons, we suggest that they play a relatively minor role in the transduction of membrane depolarization to an elevation in $[Ca^{2+}]_i$. In contrast, Lipscombe *et al.* (20) have demonstrated a more dramatic decrease in the magnitude of a depolarization-induced increase in Ca^{2+} after depletion of the caffeine-sensitive Ca^{2+} store in bullfrog sympathetic neurons. In rat sympathetic neurons, we used a similar paradigm (Fig. 1 B and C) but found a variable effect of the depletion of the Ca^{2+} store on the 50 mM K^+-induced $[Ca^{2+}]_i$ transient that followed. A quantitative comparison of the results from bullfrog neurons with those from rat suggests that the caffeine-sensitive Ca^{2+} stores in bullfrog may be considerably larger relative to the magnitude of the depolarization-induced $[Ca^{2+}]_i$ transients. We did, however, occasionally observe very large caffeine-induced $[Ca^{2+}]_i$ transient, indicating much larger caffeine-sensitive stores in some cells. These larger stores appeared to contribute to a greater proportion of the depolarization-induced Ca^{2+} transient, as indicated by the marked inhibition of the $[Ca^{2+}]_i$ transient elicited by 50 mM K^+ after depletion of the caffeine-sensitive Ca^{2+} store (Fig. 1C). However, the correlation between the size of the caffeine-induced $[Ca^{2+}]_i$ transient and the inhibition of a subsequent high K^+-induced response seen in this paradigm could be due to a number of factors, including the inhibition of Ca^{2+} channels by prior exposure to elevated $[Ca^{2+}]_i$.

Because of such problems, we employed the combined use of the whole-cell voltage-clamp technique and fura-2 microfluorimetry to measure $[Ca^{2+}]_i$ transients under conditions in which the Ca^{2+} load applied to the cell as well as the membrane potential could be more precisely controlled. Membrane depolarization activated voltage-sensitive Ca^{2+} channels producing a long lasting increase in $[Ca^{2+}]_i$ with a duration similar to that reported for similar experiments in spinal cord neurons (10). Increasing the Ca^{2+} load applied to the cell by increasing the test pulse duration (Fig. 4 B and C) caused the peak $[Ca^{2+}]_i$ to saturate. This effect is consistent with the recruitment of a low affinity high capacity Ca^{2+}-buffering process. Indeed, McBurney and Neering (56) have estimated that the powerful buffering mechanisms in neurons allow less than 1% of a Ca^{2+} influx to remain as free Ca^{2+} in the cytosol.

We found no evidence for a voltage-dependent release of Ca^{2+} from intracellular Ca^{2+} stores as has been suggested to occur in other cell types (51, 52). The relationship between the Ca^{2+} current and $[Ca^{2+}]_i$ transient remained constant over a wide range of test potentials (Fig. 5). A slight apparent discrepancy was noted at very positive test potentials but this was most likely the result of contaminating outward currents at these potentials. In contrast to our findings in rat sympathetic neurons, Cannel *et al.* (52), using cardiac cells, found a shift in the hyperpolarized direction for the $[Ca^{2+}]_i$ transient relative to the inward current. They interpreted this result as indicating the voltage-dependent mobilization of Ca^{2+} from intracellular stores. Unfortunately, they did not describe the relationship between Ca^{2+} influx and the resulting increase in $[Ca^{2+}]_i$. Thus, it is not clear whether the hyperpolarizing shift was the result of a saturation at the peak of the $[Ca^{2+}]_i$ versus membrane potential curve or was due to a genuine voltage-dependent release of intracellular Ca^{2+} from storage sites. The relationship

between Ca^{2+} current, $[Ca^{2+}]_i$, and membrane potential we describe in Fig. 5 was generated from Ca^{2+} influx data obtained below the saturation point of the cell and thus demonstrates the absence of voltage-dependent Ca^{2+} release from intracellular stores.

Pharmacological inhibition of the caffeine-sensitive Ca^{2+} store was used to determine the contribution of caffeine-sensitive Ca^{2+} stores to the $[Ca^{2+}]_i$ transient elicited by membrane depolarization. Ryanodine (10 μM) failed to decrease the $[Ca^{2+}]_i$ transient in sympathetic neurons, even though ryanodine has proven effective in inhibiting the $[Ca^{2+}]_i$ transient in cardiac cells using an identical paradigm (35, 36). Interpretation of this result is somewhat complicated, however, by the use dependency of ryanodine action (57). In cardiac cells, stimulation at a higher frequency can be used without producing the $[Ca^{2+}]_i$ overload that will result in neurons, because of their slow buffering rates (Fig. 4A). Thus, we additionally tried depleting the stores with caffeine. This approach was also problematic in that caffeine directly inhibited Ca^{2+} influx by producing a slight channel block (20). In the experiment described in Fig. 6 we determined the relationship between Ca^{2+} influx and the rise in $[Ca^{2+}]_i$ in the absence and presence of a combination of caffeine and ryanodine. The curve generated in the presence of these drugs is clearly inhibited relative to the curve generated before their application. This comparison of the two Ca^{2+} influx versus $[Ca^{2+}]_i$ curves provides a more compelling argument in support of some contribution of the caffeine-sensitive store to the depolarization-induced $[Ca^{2+}]_i$ transient. If the contribution of Ca^{2+}-induced Ca^{2+} release is small relative to the contribution from Ca^{2+} influx, then a proportionately larger effect might be seen at smaller Ca^{2+} influxes. This is the result seen in Fig. 6B; the curves actually get closer together at larger Ca^{2+} influxes, although the data points do not overlap at any point. Furthermore, presenting the data in this format removes the question of whether the experiment was performed at high Ca^{2+} influx levels at which any inhibition might be masked by the heavy Ca^{2+} buffering occurring at these elevated $[Ca^{2+}]_i$ levels. The result illustrated in Fig. 6 is the most robust of three similar experiments. The other two experiments produced data in which the drug-treated Ca^{2+} influx-$[Ca^{2+}]_i$ relationship was not convincingly different from the control. The technical difficulty in successfully completing this experiment limited replicates to three, in contrast to the experiment in Fig. 1C in which 33 trials were performed and that clearly demonstrated the variability of the system.

Our difficulty in demonstrating a significant contribution of Ca^{2+}-induced Ca^{2+} release from intracellular stores in neurons is not surprising when one considers the relative scarcity of Ca^{2+}-containing organelles in neurons, compared with the abundance of the sarcoplasmic reticulum in muscle (24–28, 53). Thus, it may be that the contribution to the depolarization-induced $[Ca^{2+}]_i$ transient in rat sympathetic neurons by the caffeine-sensitive stores may be too small to be consistently detected with the technology or paradigms employed in this study. Furthermore, these stores in rat sympathetic neurons may be more important for the sequestration of cytosolic Ca^{2+} than for its mobilization. The complex morphology and multiple functions of neurons, compared with the simple shape and unitary function of muscle cells, is another consideration. In neurons, the caffeine-sensitive stores may be localized to limit their contribution to specific functions. Indeed, in rat sensory neurons and bullfrog sympathetic neurons, these stores are preferentially localized to the soma rather than the processes, in contrast to the inositol trisphosphate-sensitive stores, which are homogeneously distributed (17, 18). A further localization within the soma might enable the caffeine-sensitive stores to produce a significant amplification of $[Ca^{2+}]_i$ transients in a small part of the cell, such as the area just beneath the plasma membrane. This could be very important for the activation of Ca^{2+}-dependent ion channels. Our methods of recording from the cell body would integrate the signal over the entire soma and thus diminish the magnitude of responses localized to a small portion of the cytosol. Additional studies using imaging techniques for measuring $[Ca^{2+}]_i$ should provide the spatial resolution for testing this hypothesis.

It is not surprising, from an evolutionary perspective, that neurons and muscle cells both contain intracellular Ca^{2+} storage organelles with similar Ca^{2+} release channels. However, in light of the relatively small contribution caffeine-sensitive Ca^{2+} stores make to depolarization-induced $[Ca^{2+}]_i$ transients in rat sympathetic neurons, it is an intriguing and as yet unanswered question as to what physiological role these stores play in the function of these cells.

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