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An FCCP-Sensitive Ca^{2+} Store in Bullfrog Sympathetic Neurons and Its Participation in Stimulus-evoked Changes in $[Ca^{2+}]_i$

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This study describes a Ca^{2+} store in fura-2–loaded bullfrog sympathetic neurons that modulates $[Ca^{2+}]_i$ responses elicited by either depolarization or Ca^{2+} release from a caffeine- and ryanodine-sensitive store. This store is insensitive to caffeine and ryanodine, but is sensitive to the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The FCCP-sensitive store slows both the rise in $[Ca^{2+}]_i$ during stimulation (apparently by accumulating Ca^{2+} from the cytosol) and the recovery following stimulation (by releasing the accumulated Ca^{2+} into the cytosol). For a fixed level of depolarization, recovery is slowed to an extent that depends on stimulus duration. $[Ca^{2+}]_i$ imaging shows that these effects are prominent in the soma but not in growth cones. Ca^{2+} uptake by the FCCP-sensitive store appears to be strongly $[Ca^{2+}]_i$ dependent, since it becomes influential only when $[Ca^{2+}]_i$ approaches ~500 nM. Therefore, this store may specifically influence $[Ca^{2+}]_i$ during moderate and strong stimulation. The effect of the store on responses to depolarization can be accounted for by a simple three-compartment scheme consisting of the extracellular medium, the cytosol, and a single internal store with a $[Ca^{2+}]_i$-dependent uptake mechanism resembling the mitochondrial Ca^{2+} uniporter. The store's effect on responses to caffeine-induced Ca^{2+} release can be accounted for by including a second internal compartment to represent the caffeine-sensitive store. While the identity of the FCCP-sensitive store is unknown, its sensitivity to FCCP is consistent with a mitochondrial pool. It is suggested that by modulating the temporal properties of $[Ca^{2+}]_i$ following stimulation, the FCCP-sensitive store may influence the degree of activation of intracellular $[Ca^{2+}]_i$-dependent processes.

[Key words: calcium, calcium stores, calcium channels, sympathetic neuron, FCCP, mitochondria]

The intracellular free calcium concentration ($[Ca^{2+}]_i$) in vertebrate neurons rises sharply upon membrane depolarization with elevated external K^+, but falls very slowly in the wake of a prolonged high $[K^+]_o$ challenge (Thayer et al., 1988; Duchen et al., 1990; Thayer and Miller, 1990). The slow recovery can appear as a pronounced plateau, whereby $[Ca^{2+}]_i$ remains elevated at a several hundred nanomolar concentration for several minutes. The delayed recovery of $[Ca^{2+}]_i$ is of considerable interest because of its presumed influence on the impact of a depolarizing stimulus on Ca^{2+}-dependent processes within the cell (Schulman, 1991; Greengard et al., 1993; Neher and Zucker, 1993).

The mechanism of the $[Ca^{2+}]_i$ plateau is not completely understood. Thayer et al. (1988) attributed it to saturation of an endogenous Ca^{2+} buffer that normally speeds the recovery of $[Ca^{2+}]_i$ following stimulation. On the other hand, Thayer and Miller (1990) suggested that the plateau is sustained by prolonged release of Ca^{2+} from mitochondria, having found that the plateau was abolished and the recovery speeded by a mitochondrial uncoupling agent, carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP). Their proposal was important, as it put forward the idea, as had others previously (e.g., Brinley et al., 1978; Duchen et al., 1990), that the mitochondria are a functionally important Ca^{2+} store. Subsequently, it has been pointed out that compounds like CCCP might also be expected to discharge nonmitochondrial Ca^{2+} stores (Jensen and Rehder, 1991; Marrion and Adams, 1992).

A number of questions arising from the work of Thayer and Miller (1990) have remained unanswered. Does the slow recovery of $[Ca^{2+}]_i$ reflect a lingering effect of high K^+ on surface membrane transport, or must one consider Ca^{2+} release from an internal store? Do other stimuli share the ability of high K^+ to produce prolonged elevations in $[Ca^{2+}]_i$? If an internal store is involved, what is its relationship to other Ca^{2+} stores known to exist within the cell? What types of Ca^{2+} transport precesses are responsible for the complex time course of recovery? We have approached these questions by studying the effects of various stimuli on $[Ca^{2+}]_i$ in bullfrog sympathetic neurons. Here we present new experimental evidence about the nature of the slow $[Ca^{2+}]_i$ recovery and its dependence on a Ca^{2+} store that is distinct from the well-accepted ryanodine-sensitive and inositol trisphosphate–sensitive Ca^{2+} stores (Berridge, 1993), and is presumably mitochondrial. The interplay between effects of this store and other mechanisms of $[Ca^{2+}]_i$ homeostasis is described in a quantitative model that accounts for key features of the slow Ca^{2+} recovery.

A preliminary account of this work has appeared in abstract form (Friel and Tsien, 1990).

Materials and Methods

Cell dissociation and primary culture conditions. The methods used to prepare cells have been described elsewhere (Friel and Tsien, 1992a) but will be summarized here. Sympathetic chains were obtained from bullfrogs (*Rana catesbeiana*) under aseptic conditions. After removing the surrounding connective tissue, the chains were incubated for 40 min at 35°C in amphibian Ringer's solution (see below) containing colla-

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genase (3 mg/ml; Worthington). After washing, the chains were transferred to Ringer's containing trypsin (1.5 mg/ml; Sigma, type III) and incubated for 10 min, again at 35°C. After washing, cells were dissociated by trituration, washed, and placed on poly-D-lysine–coated cover glasses covering 20 mm holes in the bottom of 60 mm culture dishes. After letting cells adhere to the substrate, a 1:1 mixture of normal Ringer's (+2 mM $CaCl_2$) and L-15 medium (GIBCO) was added. Cells were stored at room temperature (18–20°C) for up to 1 week. During this time, some cells extended processes. Unless stated otherwise (e.g., Fig. 5), recordings reflect $[Ca^{2+}]_i$ regulation in cells with little neurite outgrowth.

Solutions. Amphibian Ringer's solution contained (in mM) 128 NaCl, 2 KCl, 10 glucose, and 10 HEPES, with pH adjusted to 7.3 with NaOH. The control extracellular medium consisted of normal Ringer's + 2 mM $CaCl_2$. K^+ solutions (30 or 50 mM) were equivalent to the 2 mM Ca^{2+} Ringer's solution except that either 28 or 48 mM NaCl was replaced by KCl. "Ca-free" Ringer's was made by supplementing nominally Ca^{2+}-free Ringer's with 0.2 mM EGTA and adjusting the pH to 7.3 with NaOH. In the presence of this solution, depolarization had no detectable effect on $[Ca^{2+}]_i$ (Friel and Tsien, 1992a; Fig. 1*a*). "Na-free" and "Na-free, high K^+" Ringer's were made by replacing NaCl with *N*-methyl-D-glucamine (NMDG) and adjusting the pH to 7.3 with HCl. The internal solution used for whole-cell current clamp experiments contained (in mM) 115 KCl, 10 HEPES, 5 $MgCl_2$, 1 MgATP, 0.1 NaGTP, with or without 0.1 fura-2 pentapotassium salt, and pH adjusted to 7.3 with KOH.

Fura-2 loading. Cells were incubated for 40 min at room temperature in a solution containing 1 μl of fura-2 stock solution/ml 2 mM Ca Ringer's. The stock solution contained 1 mM fura-2 acetoxymethyl ester dissolved in dimethyl sulfoxide containing pluronic (25% w/w). Cells were then rinsed with 2 mM Ca Ringer's and placed on the stage of an inverted microscope (IM35, Zeiss). The recording chamber was then continuously superfused with 2 mM Ca^{2+} Ringer's solution. With this loading procedure, there is little compartmentalization of fura-2 based on the residual fluorescence observed after cells are dialyzed with fura-2 free internal solution under whole-cell current clamp (not shown).

$[Ca^{2+}]_i$ measurements. Two methods were used to measure $[Ca^{2+}]_i$. For relatively high time resolution, cells were illuminated with light from a 150 W xenon lamp (Muller Electronic-Optics) transmitted by a light guide to excitation filters (350 and 380 nm) mounted in a filter wheel that rotated at 20–40 Hz. Excitation light was reflected by a dichroic mirror and focused onto the cell being studied with a 40× objective (Nikon Fluor, NA 1.3). Emitted light was low-pass filtered (510 nm) and passed through an aperture positioned over the cell soma. Light intensity from this region was measured with a photomultiplier tube (PMT, Thorn, OL 30 series). The PMT signals associated with the two excitation wavelengths were separately integrated (Cairn Research Spectrophotometer) to give two analog signals (F_{350} and F_{380}), which were updated once per revolution of the filter wheel. Each channel was sampled every 200–1000 msec by digitizing these signals at 2.5 kHz over 12.8 msec intervals, computing the time average, and storing the results on a laboratory computer. $[Ca^{2+}]_i$ was calculated from F_{350} and F_{380} according to Grynkiewicz et al. (1985) after subtracting the light intensity measured in the absence of cells ($F_{350,bk}$ and $F_{380,bk}$) and computing the ratio $R = (F_{350} - F_{350,bk})/(F_{380} - F_{380,bk})$. The dissociation constant for fura-2 was taken to be 224 nM and multiple measurements of $F_{380,low\ Ca}/F_{380,high\ Ca}$ fell within the range of 11.6–12.1. R_{min} and R_{max} were determined as described in Friel and Tsien (1992a).

To resolve spatially heterogeneous changes in $[Ca^{2+}]_i$ in cells with processes, $[Ca^{2+}]_i$ imaging was used. Cells were illuminated with light from a 75 W xenon lamp (Zeiss) that passed through excitation filters (350 and 380 nm; Omega Optical) mounted on a computer-controlled filter wheel (Atlantex and Zieler Instrument Corporation) and followed the same optical path described above. Emitted light intensity was monitored with an SIT camera (Dage MTI, model 66), digitized (Gould FD5000 image processor) under computer control (PDP 11/73, Digital Equipment Corporation), and saved on an OMDR (Panasonic TQ-2028F). Sixteen to 32 images were averaged at each excitation wavelength and then corrected by subtracting averaged background images. For each pixel, $\log(R)$ was computed and coded by hue, while the mean intensity was coded by brightness, each with 6-bit resolution. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (1985) with $R_{min} = 1.5$, $R_{max} = 10.1$, and K_d ($F_{380,low\ Ca}/F_{380,high\ Ca}$) = 1.9 μM. These parameters were measured *in vitro* using buffered Ca^{2+} solutions (EGTA for $p[Ca^{2+}] = 6.5–8.5$, and HEDTA for $p[Ca^{2+}]_i = 4.0–6.0$) containing 10 μM fura-2 pentapotassium salt in Plexiglas wells sealed with cover glass. The design and operation of the image acquisition system was based on one developed by R. Y. Tsien. All experiments were carried out at room temperature.

Electrophysiology. Simultaneous measurements of $[Ca^{2+}]_i$ and membrane potential (V_m) were made either under whole-cell current clamp conditions with pipettes containing 100 μM fura-2 pentapotassium salt, or by impaling fura-2 AM–loaded cells with high-resistance microelectrodes (R ≅ 120 MΩ) filled with 3 M KCl. In most experiments, microelectrodes were used, since it was found that internal dialysis influenced $[Ca^{2+}]_i$ responses elicited by both high K^+ and caffeine. Under whole-cell conditions, $[Ca^{2+}]_i$ responses were smaller in magnitude and did not exhibit the slow secondary recovery that is the subject of this report; a possible explanation for this is given in the Results. Effects of high K^+ on V_m were measured with a List EPC-7 patch clamp, and the effects of repetitive current injection were studied using an Axoclamp-2A.

Solution changes. Solution changes were made with a system of microcapillaries (Drummond Microcaps) as in Friel and Tsien (1992a). Changes were complete within about 100–200 msec, based on measurements of the change in V_m observed when cells were exposed to external solutions with elevated $[K^+]_o$. The internal diameter of the capillaries was approximately five times the typical cell diameter. While cells were locally superfused with test solutions, the recording chamber was superfused with Ringer's at ~5 ml/min.

Drugs. Sources of chemicals were Sigma (salts, caffeine, FCCP, DMSO), Aldrich (salts for fura-2 calibration and whole-cell pipette solutions), Molecular Probes (fura-2, acetoxymethyl ester, and pentapotassium salt), Research Biochemicals Incorporated (ryanodine), and BASF Wyandotte (pluronic). All solution were made with Nanopure water (Barnstead).

Parameter estimation for the compartmental simulations. There are eight lumped parameters in the three-compartment scheme (see Fig. 9) and six additional parameters each for the four- and five-compartment schemes (see Figs. 10, 11). The parameters describing linear transport by the three-compartment system that includes the caffeine-sensitive store (κ_{l1}, κ_{p1}, $\kappa_{l,caff}^{(0)}$, $\kappa_{p,caff}$, γ_{caff}) were estimated from the properties of $[Ca^{2+}]_i$ relaxations following small perturbations near resting $[Ca^{2+}]_i$ (Friel and Tsien, 1992c). The remaining parameters describing Ca^{2+}-dependent transport by the caffeine-sensitive store ($\kappa_{l,caff}^{(1)}$, K_{caff}, n_{caff}) and transport by the FCCP-sensitive store ($\kappa_{l,fccp}$, $\kappa_{p,fccp}^{(0)}$, $\kappa_{p,fccp}^{(1)}$, γ_{fccp}, K_{fccp}, and n_{fccp}), and the high-capacity compartment within the FCCP-sensitive store ($\kappa_{l,fccp*}$, $\kappa_{p,fccp*}^{(0)}$, $\kappa_{l,fccp*}^{(1)}$, γ_{fccp*}, K_{fccp*}, and n_{fccp*}) were adjusted so that simulated changes in $[Ca^{2+}]_i$ following step changes in either κ_{l1} or K_{caff} resembled the observed effects of membrane depolarization and caffeine exposure, respectively. Parameter values were not optimized to fit the experimental records, since the main point of the kinetic analysis was to determine how complex a scheme must be to account for the qualitative properties of $[Ca^{2+}]_i$ responses elicited by stimulation. For simplicity, it was assumed that for the high-capacity compartment $\kappa_{l,fccp*} = 0$ (no passive leak) and $\kappa_{p,fccp*}^{(0)} = 0$ (no linear uptake). Numerical integrations of the dynamical equations were carried out with the fourth-order Runge-Kutta method (Boyce and DiPrima, 1969) on a PDP 11/23 computer using step size 0.1 sec. Further reduction in step size did not change the qualitative properties of the solutions.

Results

$[Ca^{2+}]_i$ plateau following exposure to 50 mM K^+

When vertebrate neurons are depolarized, the characteristics of subsequent $[Ca^{2+}]_i$ response exhibit a striking dependence on the duration of the depolarization. Figure 1*a* shows a series of $[Ca^{2+}]_i$ responses elicited from a cell by increasing $[K^+]_o$ from 2 mM to 50 mM for increasing periods of time. During each K^+ exposure (horizontal bar), $[Ca^{2+}]_i$ rose to a peak, and then declined slightly to a steady elevated level. After restoring $[K^+]_o$ to 2 mM, $[Ca^{2+}]_i$ recovered biphasically, first dropping sharply to a plateau level, and then undergoing a slow secondary decline. The graded descent from the plateau is seen most clearly in Figure 1*b*, which superimposes recoveries from the first five responses in *a*. As the duration of the preceding K^+ application was lengthened, the subsequent plateau level remained about the same, but the recovery became increasingly prolonged. However, as the depolarization length was increased further, recov-

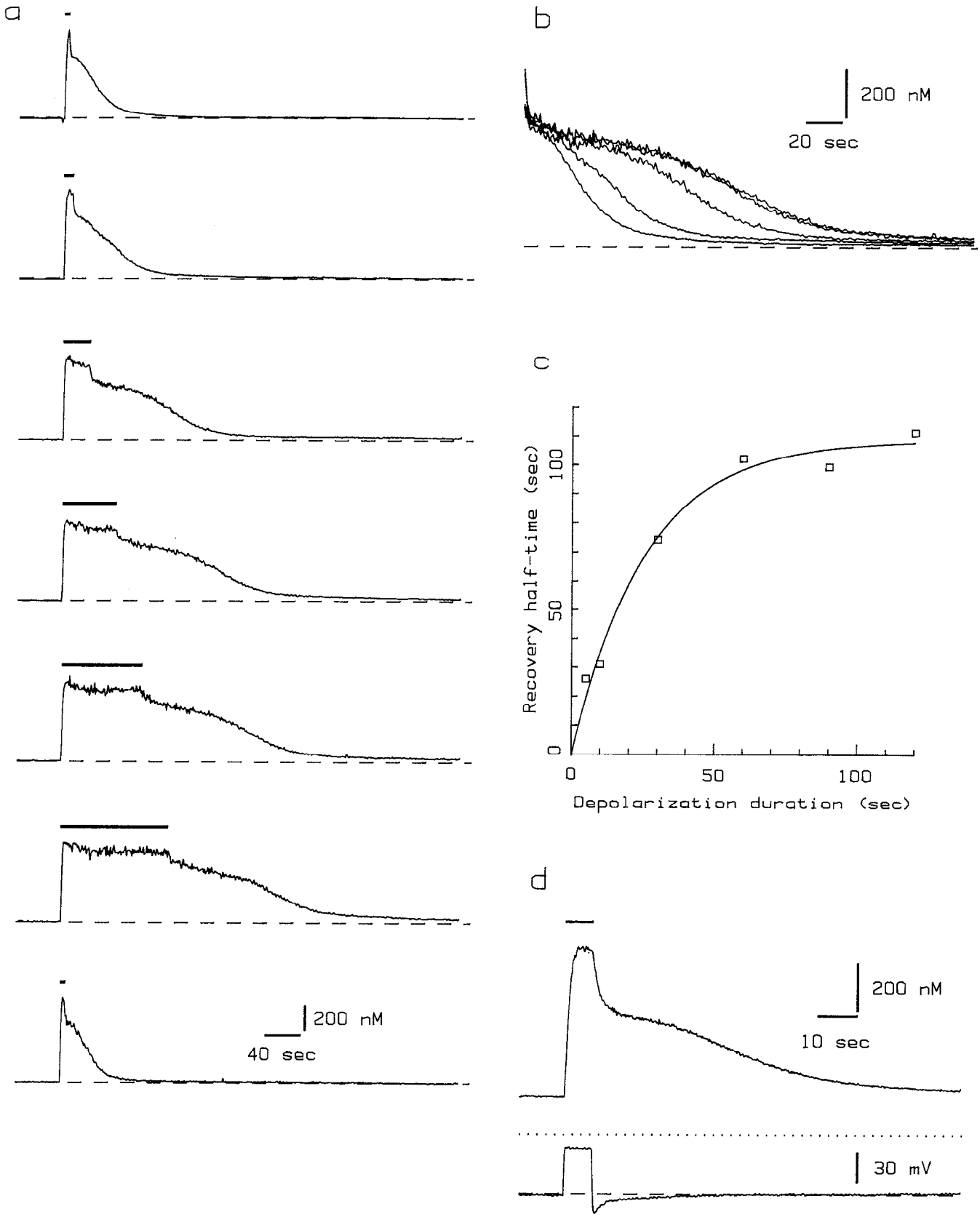


Figure 1. Characteristics of the slow $[Ca^{2+}]_i$ recovery following depolarization with high K^+. *a*, $[Ca^{2+}]_i$ responses elicited sequentially by exposure to 50 mM K^+ for various periods of time. *b*, Superposition of the recoveries from the first five responses in *a*. Prestimulation $[Ca^{2+}]_i$ (*dashed line*) was subtracted from each record in *b*. *c*, Dependence of recovery half time on high K^+ exposure duration. Recovery kinetics were robust: the half time of recovery following a 90 sec exposure to 50 mM K^+ in *a* was 99 sec; 65 min later in the experiment, it was 103 sec. Cell b07z. *d*, $[Ca^{2+}]_i$ (*upper trace*) and V_m (*lower trace*) during and after exposure to 50 mM K^+. *Dotted line* indicates the zero level for $[Ca^{2+}]_i$ and V_m, and *dashed line* gives the resting potential. Cell b10y.

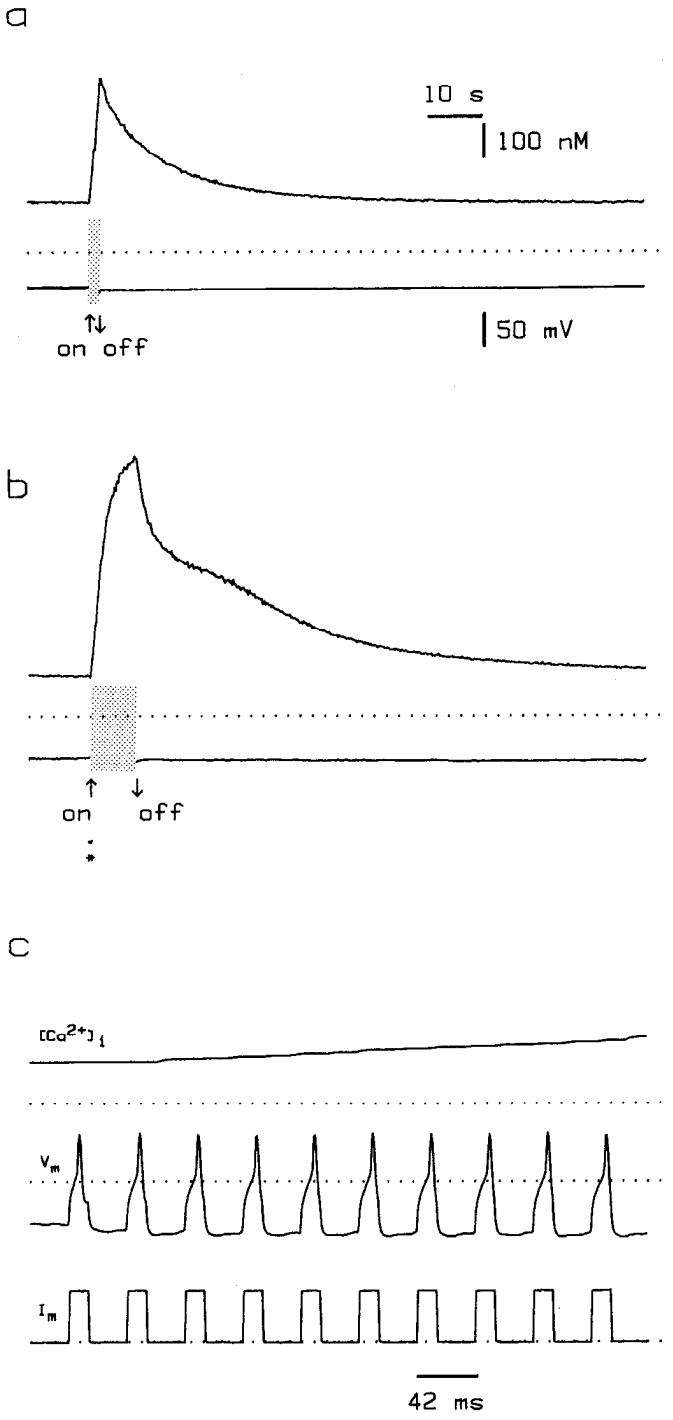


Figure 2. The slow $[Ca^{2+}]_i$ recovery is also observed following periodic electrical stimulation. In *a* and *b*, the *upper trace* is $[Ca^{2+}]_i$; *lower trace*, V_m. The period of stimulation is indicated by the *shaded region*. *c*, $[Ca^{2+}]_i$, V_m, and the injected current I_m on an expanded scale from the initial period of stimulation in *b* (see *bar marked by* *). Current was injected by a microelectrode at 23.8 Hz (450 pA pulses of 12 msec duration separated by 30 msec). F_{350}, F_{380}, V_m, and I_m were sampled during this time once every 960 μsec. *Dotted lines* indicate zero $[Ca^{2+}]_i$, V_m and, in *c*, I_m. Cell b11d.

eries approached a limiting time course (Fig. 1*b,c*). The plateau and subsequent slow recovery are not due to cell rundown, since the response to a brief high K^+ exposure near the end of the experiment (Fig. 1*a*, bottom trace) was very similar to the one elicited near the beginning (Fig. 1*a*, top trace). A similar dependence of recovery kinetics on stimulus duration was observed in each of seven cells where high K^+ exposure duration was varied systematically.

Microelectrode recordings illustrate the changes in membrane potential (V_m) that accompany such $[Ca^{2+}]_i$ transients (Fig. 1*d*). Exposure to 50 mM K^+ causes a sudden and maintained depolarization of V_m to about −15 to −20 mV (Friel and Tsien, 1992a), within the voltage range where Ca^{2+} channels activate in these neurons (Jones and Marks, 1989; D. D. Friel, unpublished observations). Returning $[K^+]_o$ to 2 mM led to a brisk repolarization that would be expected to deactivate these channels rapidly. Therefore, the slow recovery is not caused by prolonged membrane depolarization. In fact, V_m underwent a transient hyperpolarization, consistent with activation of a Ca^{2+}-dependent K^+ conductance (Adams et al., 1986). Evidently, the hyperpolarization is a consequence, rather than a cause, of the prolonged $[Ca^{2+}]_i$ elevation.

$[Ca^{2+}]_i$ plateau following trains of action potentials

To determine if K^+-evoked $[Ca^{2+}]_i$ responses are similar to those produced by other depolarizing stimuli, we examined responses to stimulated trains of action potentials (Fig. 2). A ~2 sec train (~24 Hz) produced a rise in $[Ca^{2+}]_i$ followed by a smooth recovery (Fig. 2*a*) while an ~8 sec train produced a larger $[Ca^{2+}]_i$ elevation followed by a slow recovery (Fig. 2*b*), much like that seen after prolonged exposure to 50 mM K^+. Thus, the $[Ca^{2+}]_i$ plateau does not result from elevations in $[K^+]_o$ per se, and follows intermittent as well as steady depolarizations. The most plausible interpretation is that the slow $[Ca^{2+}]_i$ recovery is an aftereffect of prolonged Ca^{2+} entry, regardless of how it is evoked.

What Ca^{2+} fluxes are responsible for the slow $[Ca^{2+}]_i$ recovery?

The rate at which $[Ca^{2+}]_i$ recovers following a stimulus is expected to reflect Ca^{2+} transport across the surface membrane and Ca^{2+} exchange between the cytosol and internal stores. Which type of transport is influenced by stimulus duration? Focusing first on surface membrane transport, the slow recovery could reflect either enhanced entry or impaired extrusion of Ca^{2+}. To test for enhanced Ca^{2+} entry, cells were exposed to 50 mM K^+ and then switched to a 2 mM K^+ solution that was Ca^{2+} free (Fig. 3*a,b*). External Ca^{2+} removal had little or no immediate effect on the plateau, indicating that the plateau does not reflect enhanced Ca^{2+} entry from the external medium. This was a consistent finding (e.g., Fig. 3*d,e*). To test for impaired Ca^{2+} extrusion, cells were exposed to 50 mM K^+, then to the Ca^{2+}-free solution, and after several seconds to 10 mM caffeine to liberate Ca^{2+} from an internal store (Fig. 3*d,e*). If, during the recovery, Ca^{2+} extrusion were impaired, then any stimulus that transiently delivers Ca^{2+} to the cytosol should produce an unusually prolonged $[Ca^{2+}]_i$ elevation, while any intervention that removes Ca^{2+} from the cytosol should overcome the impairment and speed recovery. When caffeine was applied steadily (Fig. 3*d,e*), $[Ca^{2+}]_i$ increased rapidly and transiently before falling to a somewhat lower plateau level. It was found that the decline in $[Ca^{2+}]_i$ following the spike was no slower than that observed when the same cell was exposed to caffeine under resting conditions (Fig. 3*f*, no depolarization; for comparison see Fig. 3*g*). A slower decline in $[Ca^{2+}]_i$ would be expected if plasma membrane Ca^{2+} extrusion were compromised.

Additional information was provided by the response to caffeine removal (Fig. 3*e*). After caffeine was removed, $[Ca^{2+}]_i$ fell

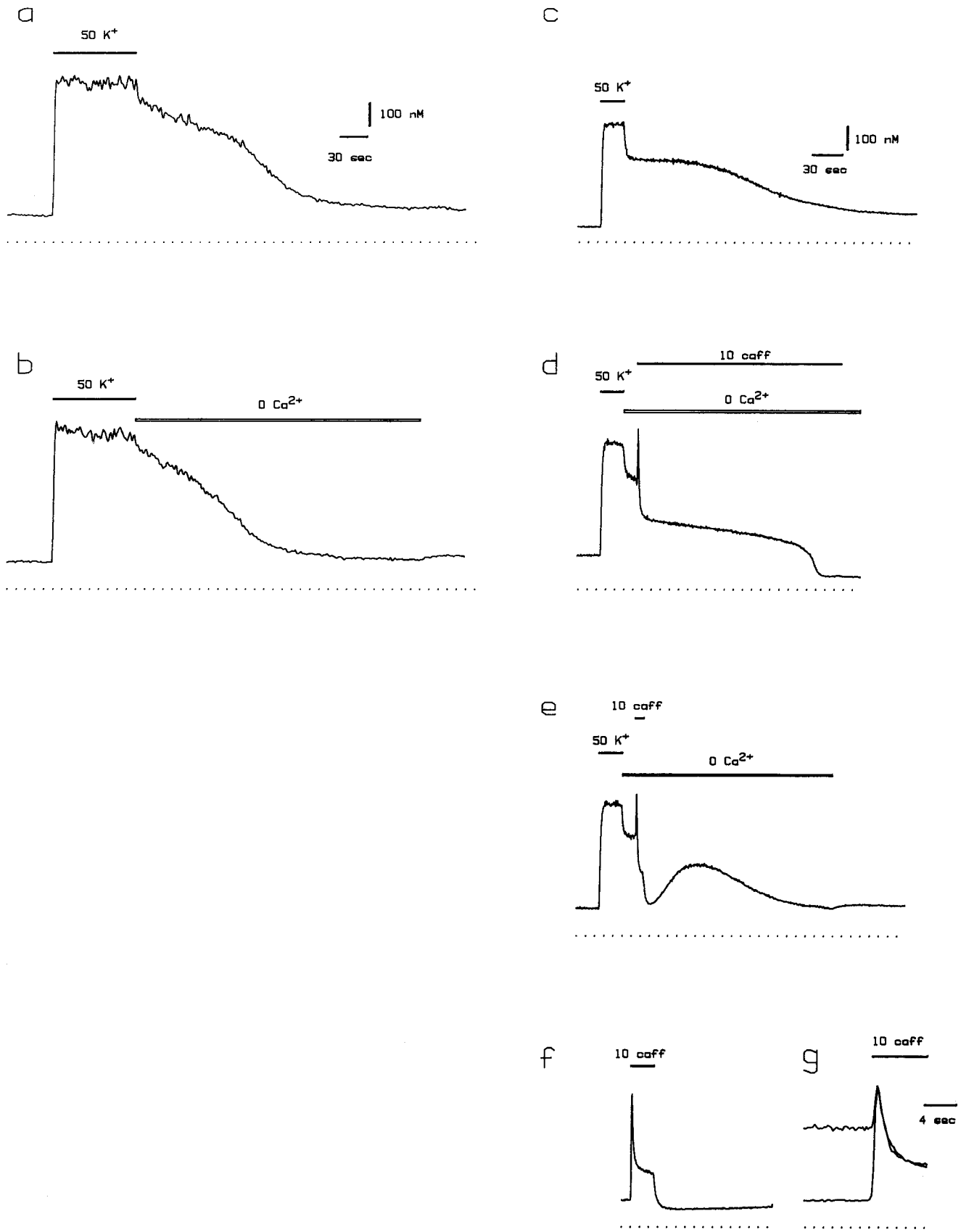


Figure 3. The slow $[Ca^{2+}]_i$ recovery reflects Ca^{2+} release from an internal store. *a* and *b*, Removal of external Ca^{2+} does not abolish the plateau, indicating that it does not arise from Ca^{2+} entry from the extracellular medium. Cell b07z. *c–g*, Impaired Ca^{2+} extrusion is also not responsible since caffeine-induced Ca^{2+} release during the slow recovery (*d, e*) elicits a normal $[Ca^{2+}]_i$ transient whose declining phase is indistinguishable from the one elicited under resting conditions (*f*; initial portion of caffeine responses from *e* and *f* are superimposed in *g*). *e*, Caffeine removal during the slow recovery unmasks Ca^{2+} release from an internal store. Cell b08w.

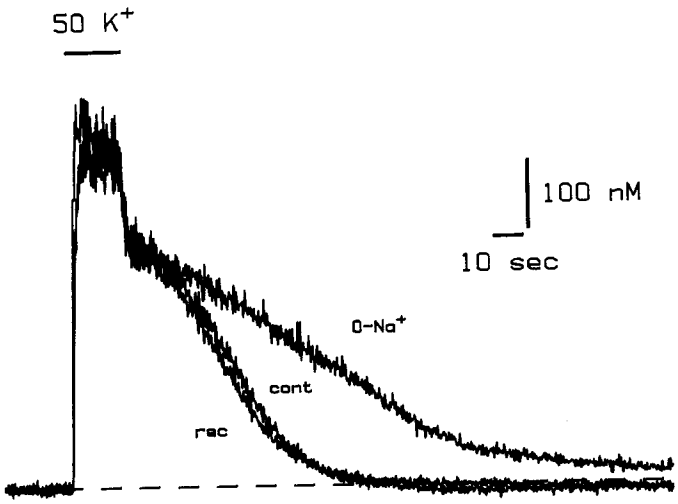


Figure 4. Assessment of the role of Na^+ on $[Ca^{2+}]_i$ response kinetics. $[Ca^{2+}]_i$ responses were elicited by exposure to 50 mM K^+ under control conditions (*cont*), in the absence of external Na^+ (*0-Na*$^+$), and after restoring external Na^+ (*rec*). Cell b08k.

rapidly, much as it does following caffeine removal in the absence of a K^+-evoked plateau (Fig. 3*f*), presumably reflecting powerful Ca^{2+} uptake by the caffeine-sensitive store (Friel and Tsien, 1992a,b). Following this drop, $[Ca^{2+}]_i$ did not remain low but increased transiently with a limiting time course that closely resembled the control recovery (seven of seven cells). This $[Ca^{2+}]_i$ rise took place in the absence of external Ca^{2+}, indicating that it reflects release of Ca^{2+} from an internal store. We propose that the secondary $[Ca^{2+}]_i$ rise following caffeine removal (Fig. 3*e*) reflects Ca^{2+} release from the same store that is responsible for the plateau and slow recovery. Ca^{2+} release would reverse the store's accumulation of Ca^{2+} during the preceding depolarization and reset its Ca^{2+} content to the initial, resting level (see also Thayer and Miller, 1990; cf. Thayer et al., 1988).

Assessment of the importance of external Na^+

In rat DRG neurons, Thayer and Miller (1990) showed that the slow $[Ca^{2+}]_i$ recovery following exposure to 50 mM K^+ is strongly influenced by removal of external Na^+. In the absence of Na^+, the post-K^+ recovery was slower with a distinctly lower plateau. These observations were interpreted in terms of the Na^+ dependence of mitochondrial Ca^{2+} efflux (Gunter and Pfeiffer, 1990). We found somewhat different effects of Na^+ removal on $[Ca^{2+}]_i$ responses in bullfrog sympathetic neurons. Na^+ removal did not detectably change the $[Ca^{2+}]_i$ plateau level attained after removing high K^+ and only moderately slowed the recovery (Fig. 4) (two cells). This slowing might be attributed to reduced Ca^{2+} efflux by a Na^+-dependent plasma membrane extrusion mechanism such as Na^+/Ca^{2+} exchange (Sanchez-Armass and Blaustein, 1987).

Regional differences in recovery kinetics

Previous studies have shown that in some peripheral neurons caffeine elevates $[Ca^{2+}]_i$ in the soma but to a much smaller extent in growth cones (Lipscombe et al., 1988; Thayer et al., 1988), suggesting that Ca^{2+} transport systems are not distributed uniformly in these cells. Therefore, we looked for possible regional differences in $[Ca^{2+}]_i$ recovery kinetics by means of $[Ca^{2+}]_i$ imaging. Figure 5*a* compares the effects of 50 mM K^+ on $[Ca^{2+}]_i$ in the cell body and growth cone of a sympathetic neuron. During the K^+ depolarization, $[Ca^{2+}]_i$ increased throughout the cell, although to different levels. Following removal of high K^+, the recovery of $[Ca^{2+}]_i$ displayed a prominent plateau in the soma but not in the growth cone. There was a comparably striking difference in the responsiveness to an earlier caffeine challenge at the same two locations. The caffeine-induced $[Ca^{2+}]_i$ transient was large in the soma but very small in the growth cone.

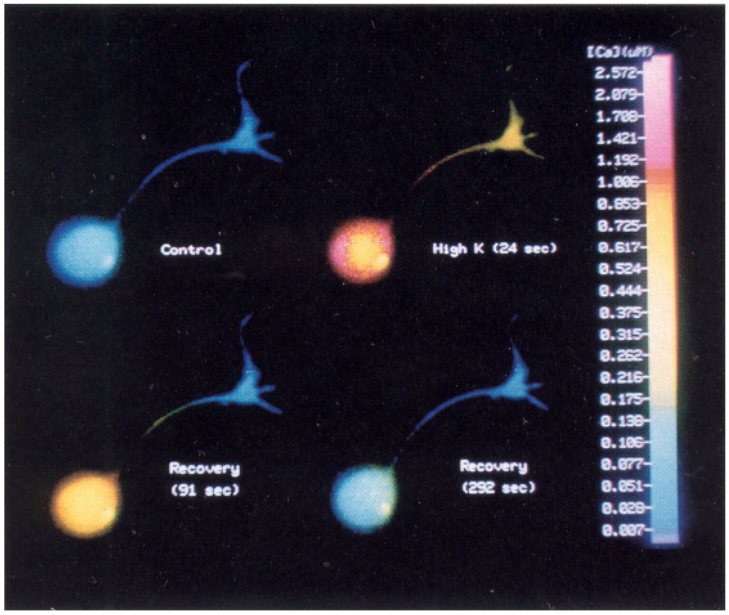
Is Ca^{2+} release from the caffeine-sensitive store responsible for the slow recovery?

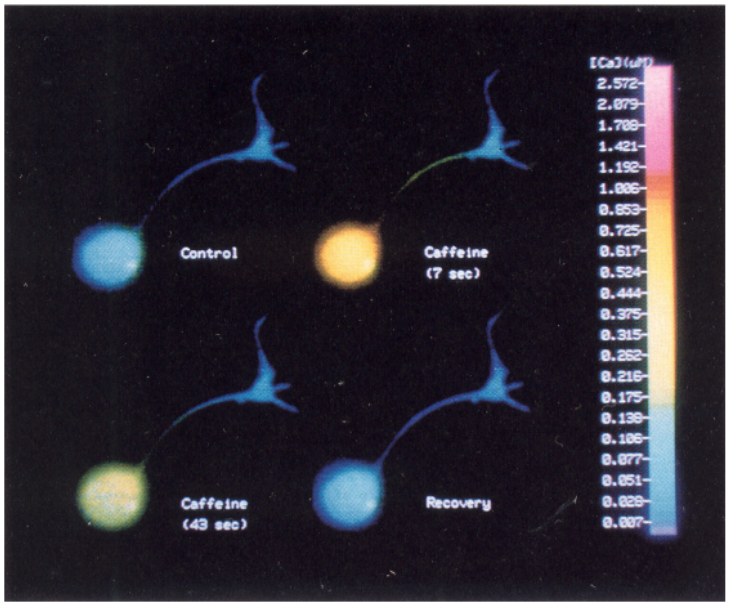
Since the slow recovery that follows depolarization is prominent in caffeine-responsive regions of the cell, but not in regions that lack responsiveness to caffeine, we considered the possibility that the slow recovery reflects Ca^{2+} release from the caffeine-sensitive store. One test was to see if exposure to caffeine altered the response to a 50 mM K^+ challenge. Figure 6*a* illustrates the effects of such a challenge, administered at similar initial $[Ca^{2+}]_i$ levels in the absence or continued presence of 10 mM caffeine (the $[Ca^{2+}]_i$ having stabilized near the resting level after the usual caffeine-induced $[Ca^{2+}]_i$ transient; see Fig. 7*b*). Upon lowering $[K^+]_o$ to 2 mM, $[Ca^{2+}]_i$ declined with very much the same time course in the presence of caffeine as in its absence. These results cannot be explained by a loss of sensitivity to caffeine during the plateau since rapid caffeine removal during the plateau leads to a sudden change in the rate at which $[Ca^{2+}]_i$ declines (Fig. 6*b*). The rapid decline in $[Ca^{2+}]_i$ induced by caffeine removal presumably reflects a sharp reduction of an ongoing caffeine-sensitive Ca^{2+} leak from the caffeine-sensitive store and a shift from net Ca^{2+} release to uptake (Friel and Tsien, 1992a,b).

Another approach to assessing the involvement of the caffeine-sensitive store in the slow recovery makes use of ryanodine, a well-known inhibitor of caffeine-induced Ca^{2+} release. Ryanodine (1 μM) abolishes caffeine-induced Ca^{2+} release in these cells (Friel and Tsien, 1992a) and slows the rising phase of the response to 50 mM K^+ (Fig. 6*c*), consistent with the idea that it prevents Ca^{2+}-induced Ca^{2+} release (Friel and Tsien, 1992a). In spite of these effects, ryanodine did not appreciably change the time course of $[Ca^{2+}]_i$ recovery following a high K^+ challenge. As a control, we confirmed that ryanodine remained effective during the plateau by showing that it was able even at this stage to prevent a caffeine-induced release of Ca^{2+} (as in Fig. 3*d,e*; not shown). Taken together, these results provide strong evidence that the store responsible for the slow recovery is distinct from the caffeine-sensitive store.

The store responsible for the slow recovery is sensitive to FCCP

The postdepolarization recovery in rat DRG neurons is sensitive to the mitochondrial uncoupler FCCP (Thayer and Miller, 1990). This protonophore (Heytler and Pritchard, 1962) is expected to collapse the differences in pH and electrical potential across the inner mitochondrial membrane upon which passive mitochondrial Ca^{2+} uptake depends. When applied to bullfrog sympathetic neurons, FCCP (1 μM) produced a small transient rise in $[Ca^{2+}]_i$ (Fig. 7*a*, center). Subsequent exposure to high K^+ in the presence of FCCP produced a $[Ca^{2+}]_i$ response that was very different from the control. While $[Ca^{2+}]_i$ initially increased with a normal time course, it continued to rise beyond the steady level reached in the control (Fig. 7*a*, compare left and center). After lowering $[K^+]_o$, $[Ca^{2+}]_i$ declined rapidly without any indication of a plateau or a slow phase of recovery. These effects





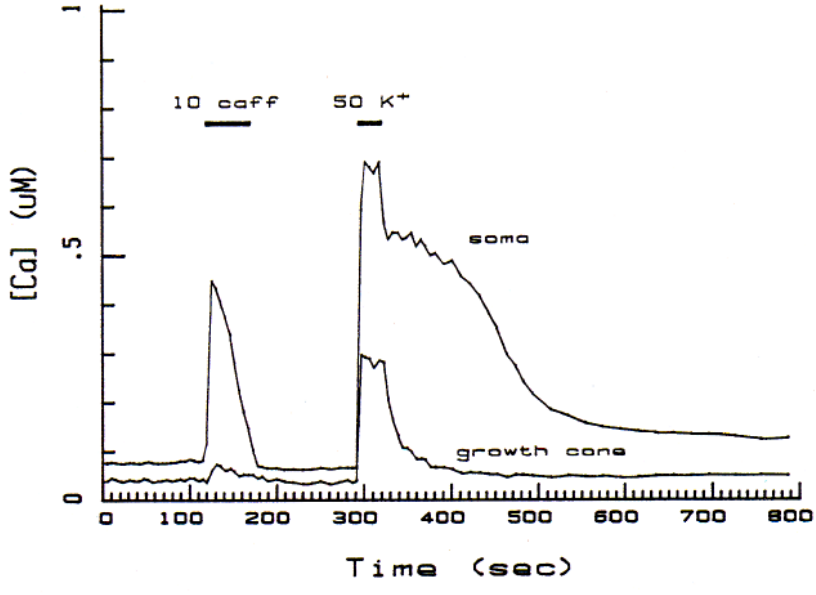


Figure 5. The slow recovery following K^+ depolarization is observed in the soma but not in growth cones. Pseudocolor images show spatial nonuniformities in $[Ca^{2+}]_i$ during (*high K*) and after (*recovery*) exposure to 50 mM K^+ (*a*) and during steady exposure to 10 mM caffeine (*b*). *c*, Comparison between the time course of responses to high K^+ and caffeine from the same cell. Traces represent $[Ca^{2+}]_i$ averaged within circular regions containing the soma and the growth cone. Cell b05x.

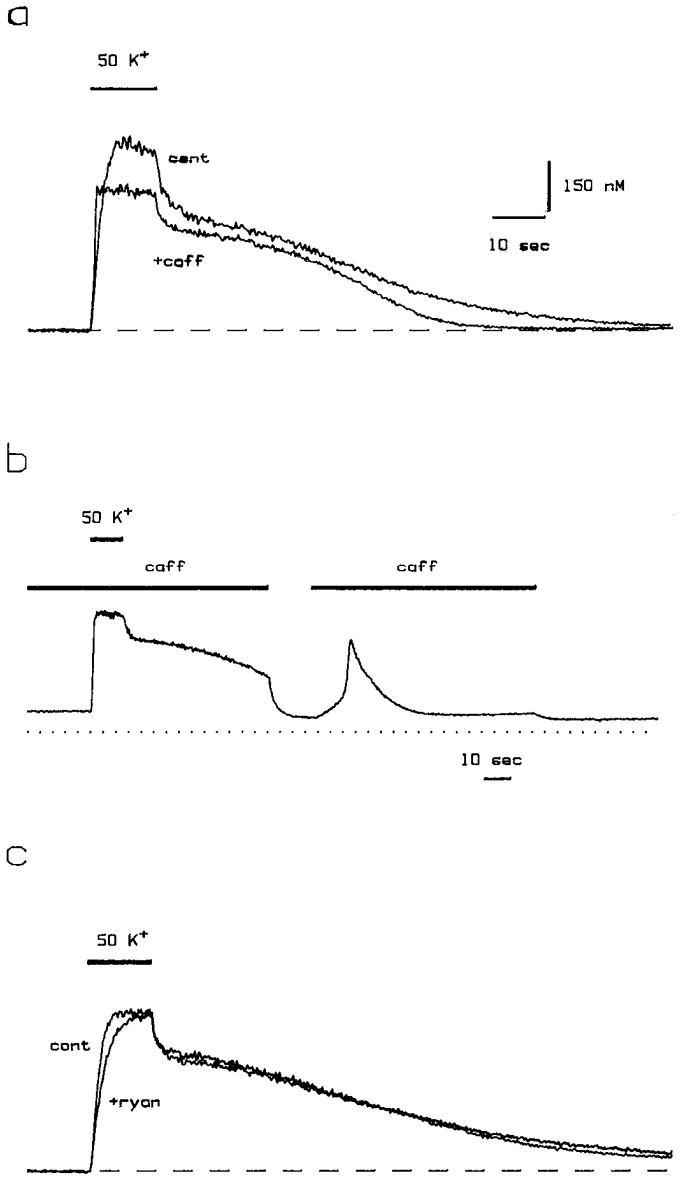


Figure 6. The caffeine-sensitive store is not responsible for the slow recovery following depolarization. *a*, $[Ca^{2+}]_i$ responses elicited by 50 mM K^+ before (*cont*) and during exposure to 10 mM caffeine (+*caff*). The smaller $[Ca^{2+}]_i$ elevation produced by 50 mM K^+ in the presence of 10 mM caffeine presumably reflects partial inhibition of voltage-sensitive Ca^{2+} entry by caffeine (Lipscombe et al., 1988). *b*, Test for loss of sensitivity to caffeine during the slow recovery. Cell b08o. *c*, Ryanodine (1 μM) did not influence the time course of recovery appreciably, although it completely inhibited caffeine-induced Ca^{2+} release (not shown). Cell b08r.

were reversed following washout of FCCP (Fig. 7*a*, right). FCCP had similar effects on responses to 50 mM K^+ in each of five cells tested, and the effects were reversible in four of these cells.

The acute effects of FCCP cannot be easily explained by depletion of cytosolic ATP. FCCP is expected to impair mitochondrial ATP synthesis (Heytler and Pritchard, 1962), but it seems unlikely that the availability of ATP would be significantly reduced during a 1 min period. In any case, ATP depletion would be expected to retard Ca^{2+} removal from the cytosol via ATP-dependent pathways and thus impair the recovery of $[Ca^{2+}]_i$ following repolarization, in contrast to the faster reestablishment of resting $[Ca^{2+}]_i$ that was observed. The action of FCCP is also difficult to explain as an induction of a surface membrane Ca^{2+} leak. If FCCP simply increased the Ca^{2+} permeability of the plasma membrane, it would steadily elevate $[Ca^{2+}]_i$, in contrast to the transient elevation that was observed. Further evidence against an FCCP-induced surface membrane Ca^{2+} leak is presented below (Fig. 8).

The time course of responses to 50 mM K^+ and their modification by FCCP can be explained in terms of Ca^{2+} uptake and release by a store that is distinct from the caffeine- and ryanodine-sensitive store (Fig. 7*a*). It is assumed that $[Ca^{2+}]_i$ changes under the influence of Ca^{2+} transport across the plasma membrane and uptake and release by two stores, one sensitive to FCCP, the other to caffeine and ryanodine. The direction and relative size of Ca^{2+} fluxes (arrows) are indicated during the onset and recovery phases for the control response (i, ii) and for the response elicited in the presence of FCCP (iii, iv). According to this scheme, depolarization promotes voltage-dependent Ca^{2+} entry and a rise in $[Ca^{2+}]_i$. Ca^{2+} uptake by the FCCP-sensitive store (Fig. 7*a*, i) opposes the effect of Ca^{2+} entry on $[Ca^{2+}]_i$, reducing the total Ca^{2+} flux into the cytosol and slowing the rise in $[Ca^{2+}]_i$. After repolarization, the Ca^{2+} concentration within each compartment relaxes toward its resting level. If the FCCP-sensitive store accumulates Ca^{2+} during the onset, it must release Ca^{2+} during the recovery to restore its initial Ca^{2+} load. Ca^{2+} release by an internal compartment opposes the effect of Ca^{2+} extrusion on $[Ca^{2+}]_i$, reducing the total Ca^{2+} flux from the cytosol and slowing the recovery (Fig. 7*a*, ii). Inhibition of Ca^{2+} uptake by the FCCP-sensitive store with FCCP increases the total inward Ca^{2+} flux during the onset (Fig. 7*a*, iii) and speeds the rise in $[Ca^{2+}]_i$. Failure to accumulate Ca^{2+} during the onset prevents Ca^{2+} release during the recovery, augmenting the outward flux and speeding the recovery (Fig. 7*a*, iv).

The caffeine-sensitive store also influences responses to depolarization. Ca^{2+} release during the onset (Fig. 7*a*, i) speeds the $[Ca^{2+}]_i$ elevation while Ca^{2+} accumulation during the recovery (Fig. 7*a*, ii) speeds the decline in $[Ca^{2+}]_i$ (Friel and Tsien, 1992a). Based on a comparison of the effects of ryanodine and FCCP on responses to depolarization (Figs. 6*c*, 7*a*), it appears that the FCCP-sensitive store is more influential in $[Ca^{2+}]_i$ regulation than the caffeine-sensitive store, at least at the high levels of $[Ca^{2+}]_i$ encountered during exposure to 50 mM K^+.

The FCCP-sensitive store also influences responses to caffeine

If the caffeine and FCCP-sensitive stores are distinct, then FCCP should also influence responses to caffeine-induced Ca^{2+} release. Caffeine produces a transient $[Ca^{2+}]_i$ elevation whose time course depends on caffeine concentration. At a low concentration (1 mM), caffeine elicits a small transient rise in $[Ca^{2+}]_i$ (Friel and Tsien, 1992a; Fig. 6*a*), while at higher concentrations (e.g., 10 mM), $[Ca^{2+}]_i$ rises more rapidly to a higher level and declines biphasically (Fig. 7*b*, left). The recovery consists of an initial rapid descent to a plateau followed by a slow secondary decline. In the presence of FCCP (1 μM), caffeine elicits a larger $[Ca^{2+}]_i$ rise (*b*, right; see also Marrion and Adams, 1992) followed by a rapid recovery (three cells).

The effects of FCCP on responses to caffeine can be accounted for by the ideas presented above. When $[Ca^{2+}]_i$ rises due to Ca^{2+} release from the caffeine-sensitive store, the FCCP-sensitive store accumulates Ca^{2+} from the cytosol (Fig. 7*b*, i). This abbreviates the rise in $[Ca^{2+}]_i$ and causes the rapid decline that follows the peak. If the Ca^{2+} content of the FCCP-sensitive store under

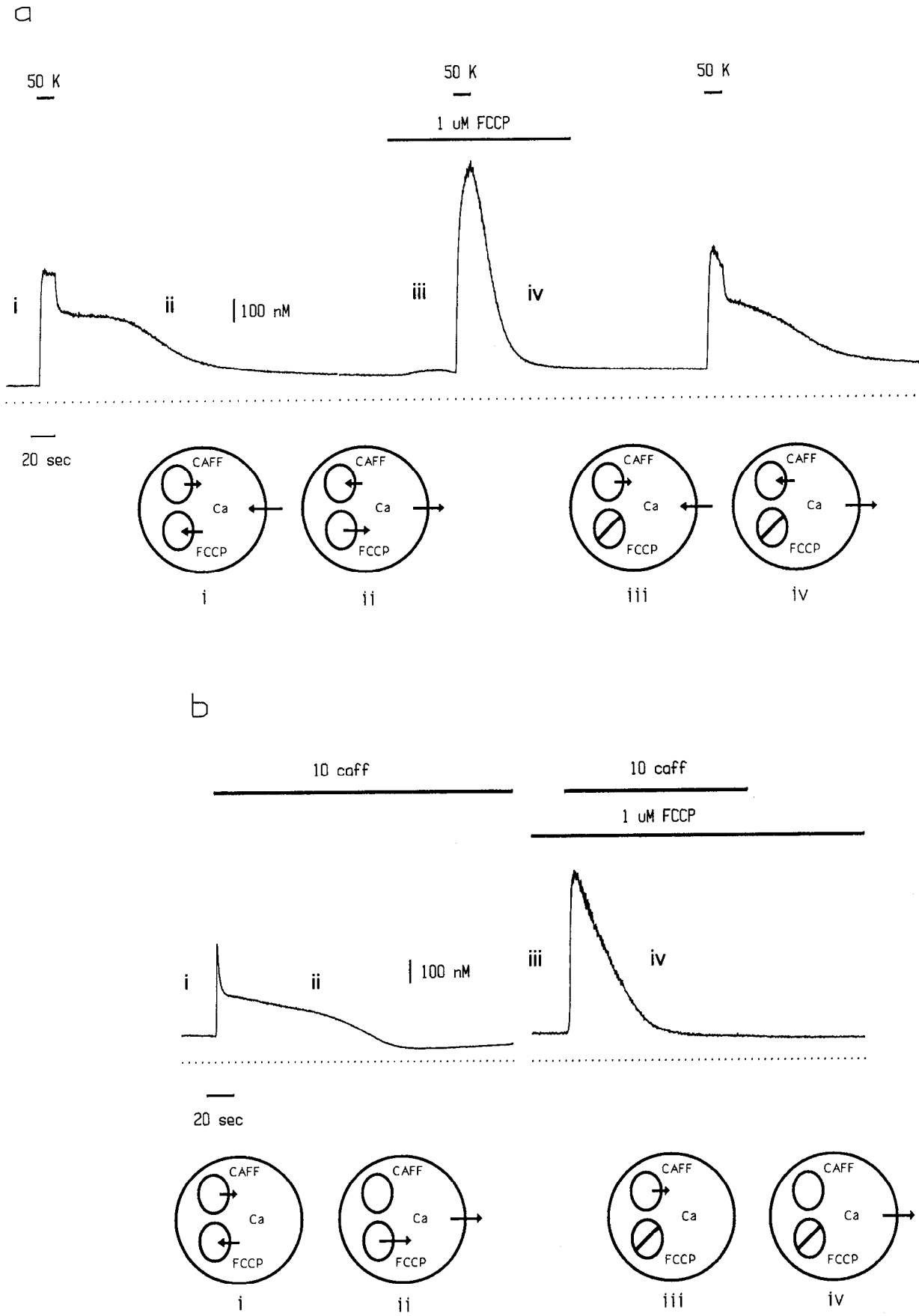


Figure 7. The store responsible for the slow $[Ca^{2+}]_i$ recovery is sensitive to FCCP. *a*, $[Ca^{2+}]_i$ responses elicited by 50 mM K^+ before, during, and after exposure to 1 μM FCCP. Cell b09c. *b*, $[Ca^{2+}]_i$ responses elicited by 10 mM caffeine before and during exposure to 1 μM FCCP. Cell b09h. See *illustrations i–iv* for an interpretation of response kinetics in terms of the underlying Ca^{2+} fluxes.

resting conditions depends only on $[Ca^{2+}]_i$ and is not influenced by caffeine, then during steady exposure to caffeine, the store must ultimately release Ca^{2+} to restore its initial load. Ca^{2+} release slows the recovery (Fig. 7*b*, ii) much as it does in the aftermath of a high K^+ challenge. Indeed, the $[Ca^{2+}]_i$ relaxations that follow repolarization and steady caffeine exposure are qualitatively quite similar (compare Fig. 7*a,b*, left). Inhibition of Ca^{2+} uptake by the FCCP-sensitive store would increase the total inward Ca^{2+} flux during the onset (Fig. 7*b*, iii) and speed the rise in $[Ca^{2+}]_i$ (it appears that, as with responses to high K^+, this effect is most pronounced when $[Ca^{2+}]_i$ is high; see next section). Since the store does not accumulate Ca^{2+} when $[Ca^{2+}]_i$ is elevated, it does not release Ca^{2+} as $[Ca^{2+}]_i$ declines and, as a result, the recovery is fast and monophasic (Fig. 7*b*, iv).

The slow, FCCP-sensitive recovery follows large but not small elevations in $[Ca^{2+}]_i$

An important aspect of the slow recovery that has not yet been accounted for is that it occurs following moderate but not weak depolarization. This is illustrated by comparing responses to 30 mM and 50 mM K^+ (Fig. 8*a*). One possible explanation is that Ca^{2+} uptake by the FCCP-sensitive store is $[Ca^{2+}]_i$ dependent, and does not come into play over the lower range of $[Ca^{2+}]_i$ encountered during weak depolarizations. This would make sense if, for example, the FCCP-sensitive store were mitochondrial, since these organelles accumulate Ca^{2+} in a steeply $[Ca^{2+}]_i$-dependent manner, reflecting activation by cytosolic Ca^{2+} of a mitochondrial uniporter (Nicholls, 1985). If Ca^{2+} uptake by the FCCP-sensitive store occurs at high but not low $[Ca^{2+}]_i$, then FCCP should influence $[Ca^{2+}]_i$ responses to strong but not weak depolarization. To test this prediction (Fig. 8), exposures to 30 and 50 mM K^+ were repeated during application of 1 μM FCCP (*b*) and after washout of the protonophore (Fig. 8*c*). While FCCP strongly influences responses to 50 mM K^+, speeding both the response onset and recovery (right), it has no systematic effect on responses to 30 mM K^+ (Fig. 8*a–c*, left). Similar observations were made in each of three cells tested. Note that this provides additional evidence that FCCP does not simply induce a surface membrane Ca^{2+} leak. The simplest interpretation of these results is that the FCCP-sensitive store accumulates Ca^{2+} only when $[Ca^{2+}]_i$ exceeds a critical level (~400–500 nM in this case).

A steep $[Ca^{2+}]_i$ dependence of Ca^{2+} uptake by the FCCP-sensitive store might also explain several other puzzling observations mentioned earlier: (1) FCCP has little effect during the early portion of responses to depolarization and caffeine (Fig. 7*a,b*); (2) following stimulation with 50 mM K^+, $[Ca^{2+}]_i$ recovers much more rapidly in growth cones than in cell bodies (Fig. 5); (3) the slow biphasic recovery is not seen following stimulation with 50 mM K^+ when bullfrog sympathetic neurons are dialyzed under whole-cell recording conditions. The peak $[Ca^{2+}]_i$ level reached was much lower in dialyzed cells (208 ± 51 nM, N = 4) than in intact cells (643.6 ± 21.5 nM, N = 139), even though the depolarization was similar in dialyzed cells (V_m = −16.8 ± 0.5 mV vs 21.0 ± 1.5 mV in 21 nondialyzed cells). In each of these cases, the common factor is that $[Ca^{2+}]_i$ was below the level where strong uptake by the FCCP-sensitive store is expected to occur.

The kinetics of $[Ca^{2+}]_i$ during and after stimulation can be accounted for by a simple compartmental model

Behavior of a three-compartment scheme with a single internal compartment. The results presented above implicate a Ca^{2+} store that accumulates Ca^{2+} in a $[Ca^{2+}]_i$- and FCCP-dependent manner. Can such a store account for the $[Ca^{2+}]_i$ plateau and slow recovery that occurs following depolarization? Although there is evidence for two distinct Ca^{2+} stores, we will begin by considering a single internal compartment representing the FCCP-sensitive store (Fig. 9). In this scheme, there are three compartments: the external medium, the cytosol, and an internal store. The concentration of Ca^{2+} within each compartment (c_o, c_i, and c_{fccp}, respectively) is assumed to be spatially uniform. This is a reasonable approximation if Ca^{2+} diffusion within compartments is fast compared to transport between compartments. $[Ca^{2+}]_i$ imaging experiments show that this holds during the recovery following repolarization and during exposure to caffeine (Fig. 5). It is also assumed that Ca^{2+} exchange between compartments is accomplished by pumps and passive leaks and that c_o is constant. Finally, it is assumed that the FCCP-sensitive store accumulates Ca^{2+} at a rate that increases sigmoidally with c_i, but that all other pump and leak fluxes are proportional to Ca^{2+} concentration (see Appendix). Therefore, the scheme can be regarded as a linear system modified to include a single nonlinear transport system.

Figure 9*a* shows simulated changes in c_i (middle trace) and c_{fccp} (bottom trace) following reversible changes in Ca^{2+} entry, controlled by the plasma membrane Ca^{2+} permeability (κ_{l1}, upper trace) to model the effects of membrane depolarization. Responses to three different perturbations are illustrated: a small step increase in κ_{l1}, to mimic weak depolarization, and two larger steps in κ_{l1} of different duration to mimic stronger depolarization. Figure 9*a* illustrates simulated responses under control conditions, while panel b shows responses to the same perturbations after imposing the condition $\kappa_{p,fccp} = 0$ to model the effect of FCCP.

The simulations agree qualitatively with the following observations: (1) $[Ca^{2+}]_i$ recovers smoothly after weak depolarization but biphasically after stronger depolarization (Figs. 8*a*, 9*a*); (2) for stronger depolarizations, the recovery duration increases with depolarization length (Figs. 1*a*, 9*a*); (3) FCCP, which by hypothesis inhibits $[Ca^{2+}]_i$-dependent uptake by the store, speeds responses to moderate depolarizations, but not responses to weak depolarizations (Figs. 8*a,b*; 9*a,b*). Note that modeling the effects of high K^+ depolarization by a step increase in κ_{l1} is only an approximation, since time-dependent changes in surface membrane Ca^{2+} permeability during steady depolarization are not taken into consideration. However, modeling the effects of high K^+ removal by a rapid and steady reduction in κ_{l1} is realistic, since Ca^{2+} channels close rapidly after repolarization (Jones and Marks, 1989). Therefore, this should be a reasonable representation of the change in plasma membrane Ca^{2+} permeability that results from lowering $[K^+]_o$ from 50 mM to 2 mM.

Behavior of a four-compartment scheme that includes both FCCP- and the caffeine-sensitive stores. Another set of simulations was carried out to investigate whether $[Ca^{2+}]_i$-dependent uptake by an internal compartment can account for the characteristic time course of caffeine-induced changes in $[Ca^{2+}]_i$. A second internal compartment was added to represent the caffeine-sensitive store, with Ca^{2+} concentration c_{caff} (Fig. 10). The pump flux $J_{p,caff}$ regulating net Ca^{2+} uptake by this store is assumed to be proportional to c_i, while the leak flux, $J_{l,caff}$, is assumed to increase sigmoidally with c_i (see Appendix) to model $[Ca^{2+}]_i$-dependent gating of Ca^{2+} release channels ($[Ca^{2+}]_i$ < 100 μM; Bezprozvanny et al., 1991).

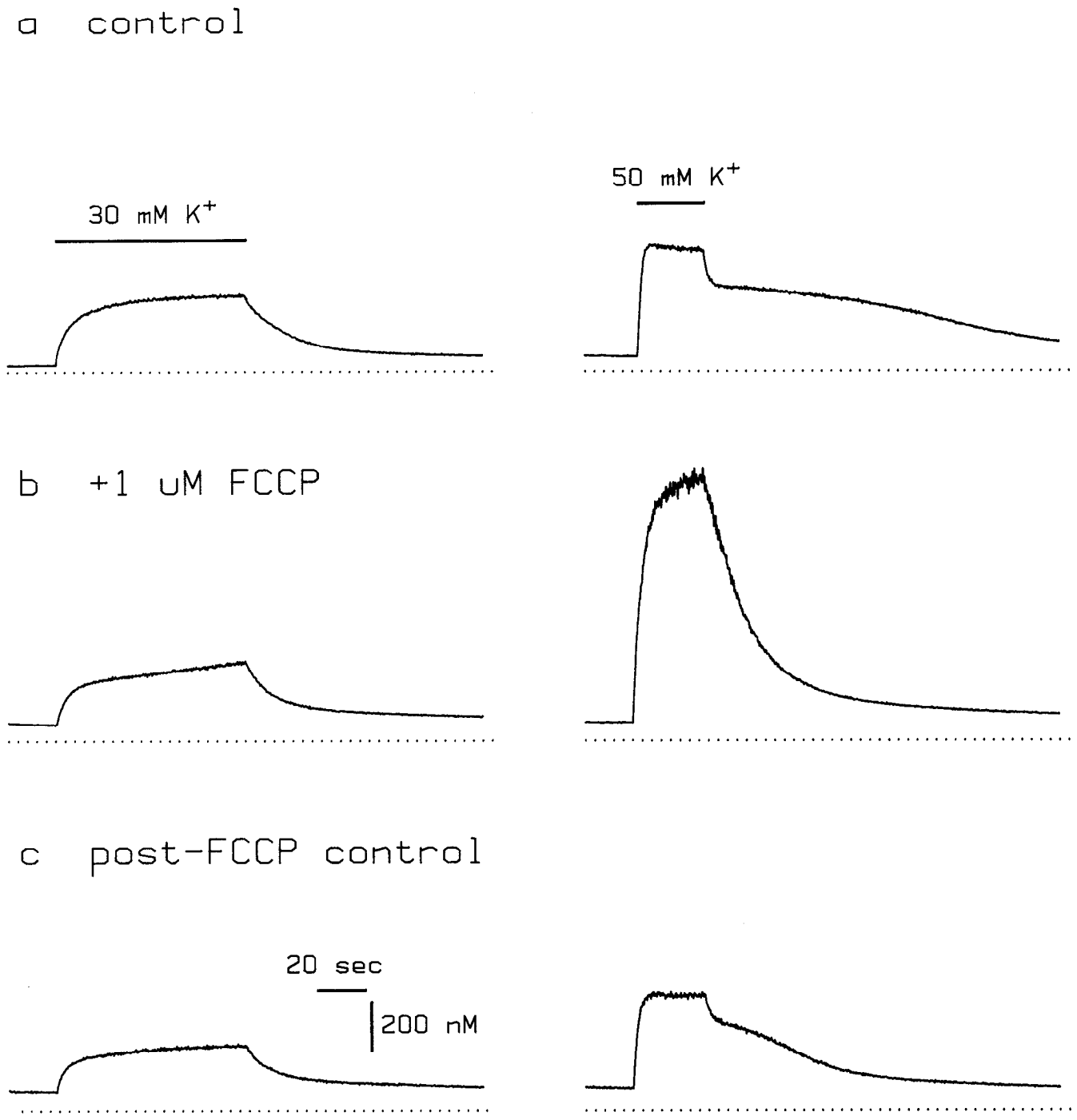


Figure 8. The FCCP-sensitive store influences responses to strong but not weak depolarization. Responses were elicited by 30 mM K^+ (*left column*) and 50 mM K^+ (*right column*) before (*a*), during (*b*), and after (*c*) exposure to FCCP (1 μM). Cell b13h.

Figure 10 shows how c_i, c_{fccp}, and c_{caff} change with time in response to a step increase in the Ca^{2+} sensitivity of CICR (modeled by a step reduction of K_{caff}, the c_i at which the c_i-sensitive leak pathway is half-maximally activated) to model the effects of caffeine. All parameter values are the same as in Figure 9 except that $\kappa_{l,fccp}$ has been increased to compensate for Ca^{2+} uptake by the caffeine-sensitive store. After lowering K_{caff}, c_i first increases and then falls sharply before undergoing a slow secondary decline, much like the observed response to caffeine (Fig. 7*b*, left). This is caused by Ca^{2+} release from the caffeine-sensitive store of sufficient strength to raise $[Ca^{2+}]_i$ above the threshold for uptake by the FCCP-sensitive store. After $[Ca^{2+}]_i$ falls under the influence of extrusion across the plasma membrane, Ca^{2+} uptake by the FCCP-sensitive store gives way to release, which prolongs the c_i recovery. After eliminating Ca^{2+} uptake by the FCCP-sensitive store (Fig. 7*b*), c_i rises to a higher level and recovers more rapidly than the control. This is very similar to the observed response to caffeine in the presence of FCCP (Fig. 7*b*, right).

Behavior of a five-compartment scheme with a high-capacity compartment within the FCCP-sensitive store. The two-store model accounts for broad features of responses to high K^+ and

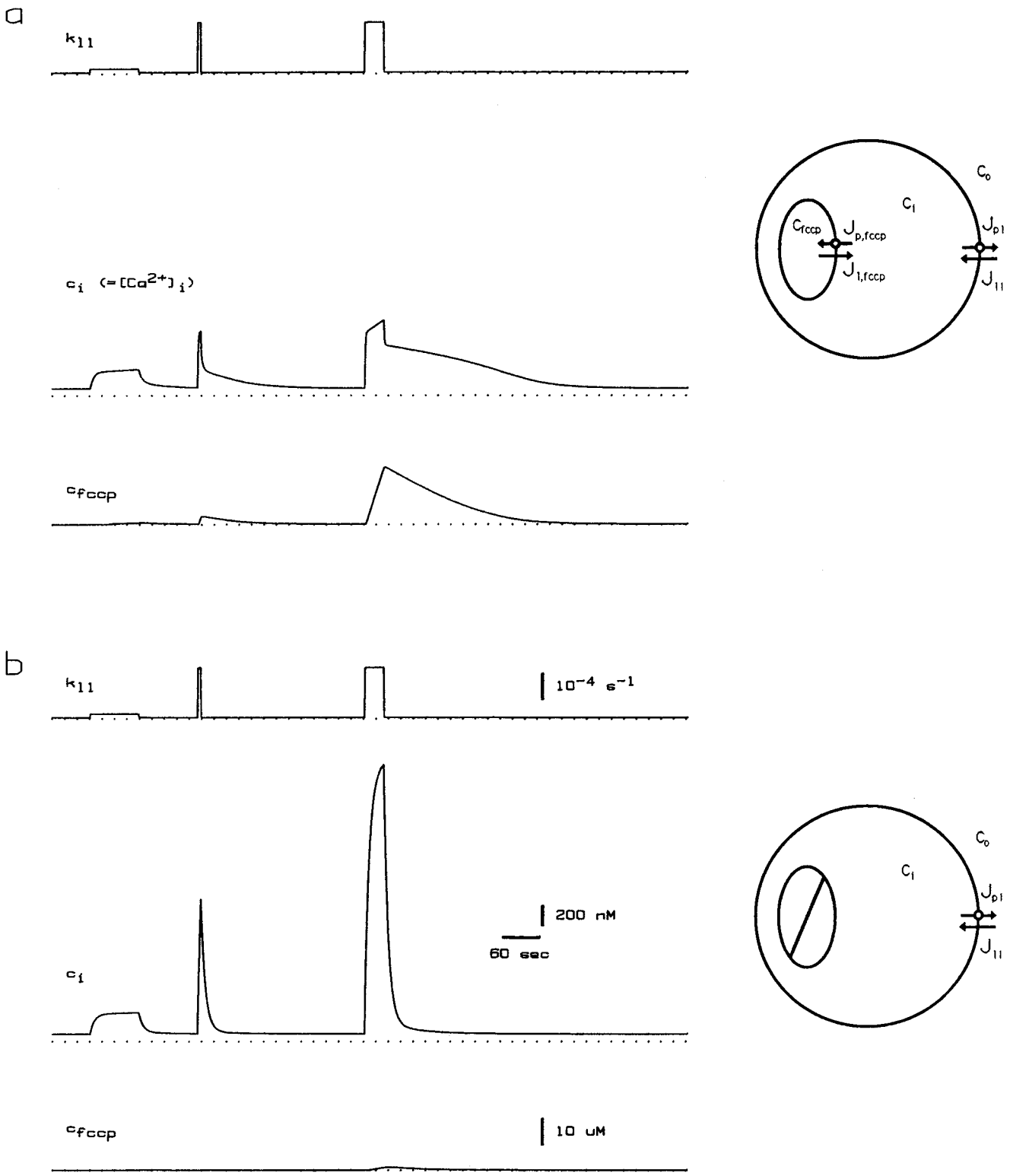


Figure 9. Simulated $[Ca^{2+}]_i$ responses to depolarization based on the three-compartment scheme before (*a*) and after (*b*) inhibiting c_i-dependent Ca^{2+} uptake by the internal compartment. *a* and *b*, *Top trace*, surface membrane Ca^{2+} permeability (κ_{11}); *middle trace*, c_i (=$[Ca^{2+}]_i$); *bottom trace*, c_{fccp}, the Ca^{2+} concentration inside the FCCP-sensitive compartment. *Dotted lines* indicate zero levels for κ_{11}, c_i, and c_{fccp}. Rate parameters had the following values (in sec^{-1}): $\kappa_{11} = 5 \times 10^{-6}$, $\kappa_{p1} = 0.132$, $\kappa_{i,fccp} = 2.25 \times 10^{-2}$, $\kappa_{p,fccp}^{(0)} = 0$, $\kappa_{p,fccp}^{(1)} = 9$. The remaining parameter values were $K_{fccp} = 6.5 \times 10^{-7}$ M, $n_{fccp} = 4$, $\gamma_{fccp} = 0.4$, and $c_o = 2 \times 10^{-3}$ M. Initial values of c_i and c_{fccp} represent the (steady-state) solutions of the system $dc_i/dt = dc_{fccp}/dt = 0$. Weak and strong depolarizations were modeled by increasing κ_{11} (in sec^{-1}) from 5×10^{-6} to 2×10^{-5} and 2×10^{-4}.

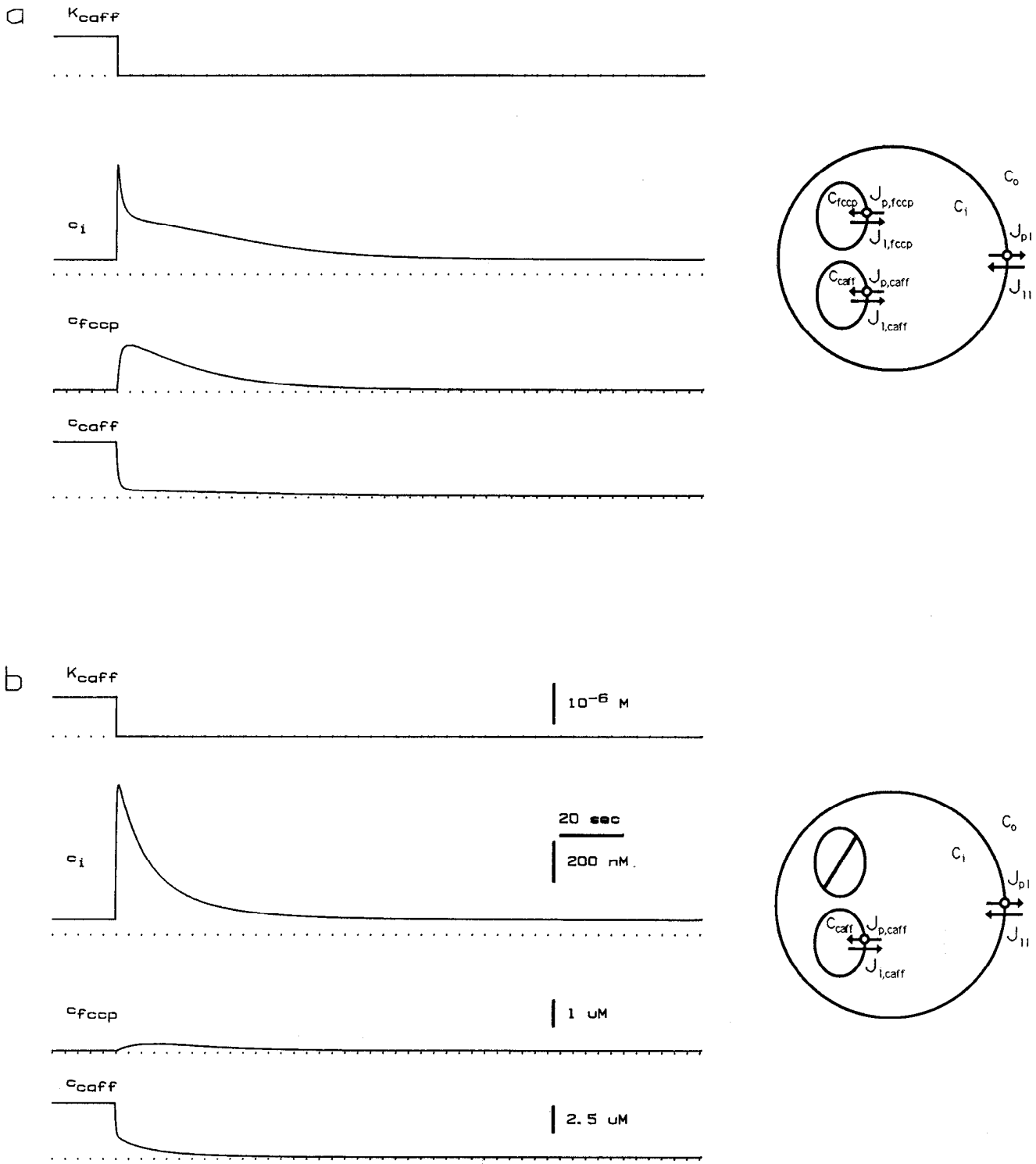


Figure 10. Simulated $[Ca^{2+}]_i$ responses to caffeine based on the four-compartment scheme before (*a*) and after (*b*) inhibiting c_i-dependent Ca^{2+} uptake into the FCCP-sensitive store. *a* and *b*, *Top trace*, K_{caff}, the c_i sensitivity of Ca^{2+}-induced Ca^{2+} release; *second trace*, c_i; *third trace*, c_{fccp}; *fourth trace*, c_{caff}, the Ca^{2+} concentration inside the caffeine-sensitive compartment. *Dotted lines* indicate zero levels for K_{caff}, c_i, c_{fccp}, and c_{caff}. All parameters in common with the three-compartment scheme were unchanged except for $\kappa_{l,fccp}$, which was increased to 0.09 sec^{-1} (see Results). Rate constants describing Ca^{2+} transport by the caffeine-sensitive store were (in sec^{-1}) $\kappa_{l,caff}^{(0)} = 0.054$, $\kappa_{l,caff}^{(1)} = 2.4$, $\kappa_{p,caff} = 3.78$. The remaining parameter values were $K_{caff} = 10^{-6}$ M, $n_{caff} = 3.04$, and $\gamma_{caff} = 0.24$. Initial values of c_i, c_{fccp}, and c_{caff} represent the solution of the system $dc_i/dt = dc_{fccp}/dt = dc_{caff}/dt = 0$. Exposure to caffeine was modeled by lowering K_{caff} from 10^{-6} M to 10^{-9} M.

caffeine but fails to account for the following paradox: during depolarization with 50 mM K^+, $[Ca^{2+}]_i$ appears to stabilize at a level (~500 nM; Fig. 1*a*) that is well below that approached in the presence of FCCP (>1 μM; Fig. 8). An identity between these two levels might be expected from the following argument. If an agent such as FCCP only influences Ca^{2+} transport between the cytosol and an internal compartment that does not directly influence Ca^{2+} transport across the plasma membrane, then it

should not influence the steady-state $[Ca^{2+}]_i$ level reached during depolarization (Friel and Tsien, 1992a). This is observed for caffeine and ryanodine at low concentrations (Friel and Tsien, 1992a), but it is far from true for FCCP.

Initially, the lower $[Ca^{2+}]_i$ level reached during prolonged exposure to high K^+ (e.g., Fig. 1*a*) seems consistent with a steady-state distribution of Ca^{2+} for two reasons: (1) it appears stable, and (2) once it is reached, repolarization is followed by a slow recovery with a limiting time course (Fig. 1*b,c*). This would make sense in terms of Ca^{2+} release by an internal compartment with finite capacity, since recovery should be slowed to an extent that depends on the store's Ca^{2+} content at the instant of repolarization. Thus, depolarizations that are long enough to establish a steady-state distribution of Ca^{2+} should be followed by the same limiting time course of recovery, irrespective of duration.

One possible resolution to this paradox is that the $[Ca^{2+}]_i$ level reached during prolonged K^+ depolarizations does not reflect a true steady state at all, but a very slow approach to a steady state in which $[Ca^{2+}]_i$ attains a much higher level. For example, this could occur if during long depolarizations, the FCCP-sensitive store accumulates Ca^{2+} at nearly the same rate at which it enters the cytosol across the plasma membrane. Of course, the accumulated Ca^{2+} must be accounted for. One possibility is that the FCCP-sensitive store contains a high-capacity compartment that accumulates Ca^{2+} whenever c_{fccp} approaches a critical level, say K_{fccp*}, and releases Ca^{2+} only very slowly as c_{fccp} declines during recovery. With such a compartment, $[Ca^{2+}]_i$ would recover following repolarization with a time course that depends on the amount of Ca^{2+} available for direct exchange with the cytosol at the instant of repolarization. If c_{fccp} is limited by K_{fccp*}, the recovery duration would also be limited.

To see if this kind of explanation is plausible, the two-store model was expanded to include another compartment within the FCCP-sensitive compartment, with Ca^{2+} concentration c_{fccp*} (Fig. 11). Simulated responses to step elevations in κ_{f1} of variable duration had the following characteristics: (1) following the step in κ_{f1}, c_i increased monotonically to a steady level below the steady-state level approached during the step after disabling c_i-dependent uptake by the store (compare *a* and *b*); (2) after restoring κ_{f1} to its initial value, c_i declined with a time course that became slower as steps in κ_{f1} were lengthened; (3) the c_i recovery approached a limiting time course as steps in κ_{f1} became sufficiently long for c_{fccp} to stabilize during the step. This agrees with the results in Figure 1*a*. The simulations did not reproduce the biphasic rise in $[Ca^{2+}]_i$ during depolarization in Figure 1*a*, but this feature was not observed consistently; in other cells, a monotonic rise was observed that more closely resembled the simulations (e.g., Figs. 2*b*, 6*c*). It is interesting to note that as c_i rises in response to the step in κ_{f1}, c_{caff} rises slightly and then declines. The decline in c_{caff} is an expression of Ca^{2+}-induced Ca^{2+} release as c_i approaches K_{caff}. As expected, the impact of CICR on c_i is strongly influenced by K_{caff}. For smaller values of K_{caff}, Ca^{2+} is released earlier and more powerfully during the onset, and for sufficiently small values, c_i transiently overshoots the steady level approached during the step, as observed in some cells (e.g., Fig. 1). It is possible that cell-to-cell differences in the $[Ca^{2+}]_i$ sensitivity of CICR contribute to the observed variability in onset kinetics. Overall, the kinetic scheme provides a very reasonable account of the complex kinetic properties of both high K^+ and caffeine-induced $[Ca^{2+}]_i$ responses and the way they are influenced by FCCP.

A physical example of a high-capacity compartment that would behave in this way is a Ca^{2+} complex that forms rapidly whenever c_{fccp} approaches K_{ccp*} but dissociates very slowly after c_{fccp} falls. It is interesting to note that under certain conditions liver mitochondria contain a Ca^{2+}-phosphate complex that greatly increases their capacity for Ca^{2+} (Lehninger, 1970; Zoccarato and Nicholls, 1982).

Discussion

This study presents a number of new observations that help explain why $[Ca^{2+}]_i$ recovers slowly following moderate or strong depolarization in bullfrog sympathetic neurons. Similar $[Ca^{2+}]_i$ dynamics occur in a variety of neurons, including rat sensory, sympathetic, and central neurons (Thayer et al., 1988) so it is possible that the conclusions are generally applicable. While the time course of recovery depends on stimulus history, we found that $[Ca^{2+}]_i$ responses with similar kinetics could be elicited by different depolarization protocols, suggesting that the kinetics of recovery are determined by the time course of $[Ca^{2+}]_i$ during stimulation, rather than the time course of V_m. We demonstrated that the slow recovery does not reflect long-term changes in surface membrane Ca^{2+} transport: neither enhanced Ca^{2+} entry nor impaired extrusion across the plasma membrane was responsible, thereby implicating Ca^{2+} transport by an internal store. This store can be operationally defined by its sensitivity to the protonophore FCCP, and is distinct from the caffeine- and ryanodine-sensitive Ca^{2+} store that has also been described in sympathetic neurons. FCCP is known to inhibit mitochondrial Ca^{2+} transport, raising the possibility that the store is mitochondrial (see below).

The FCCP-sensitive store slows changes in $[Ca^{2+}]_i$ during and after stimulation. This appears to reflect Ca^{2+} uptake by the store while $[Ca^{2+}]_i$ rises, followed by Ca^{2+} release from the store while $[Ca^{2+}]_i$ declines. Thus, the store can be regarded as an inertial element in $[Ca^{2+}]_i$ regulation, slowing the rate at which $[Ca^{2+}]_i$ changes in response to perturbations of Ca^{2+} delivery. The FCCP-sensitive store modifies responses to stimuli irrespective of the Ca^{2+} delivery pathways they recruit, similarly influencing responses to high K^+-induced Ca^{2+} entry and caffeine-induced Ca^{2+} release. When participation of the store is inhibited by FCCP, $[Ca^{2+}]_i$ responses are greatly accelerated. The FCCP-sensitive store is expected to have two major effects on stimulus-induced changes in $[Ca^{2+}]_i$: (1) reduction of the $[Ca^{2+}]_i$ level reached during stimulation, and (2) prolongation of recovery following stimulation. Both of these effects become much more pronounced as the magnitude of the $[Ca^{2+}]_i$ response is increased, so their prominence will rise sharply as stimulus strength is increased.

Comparison with previous studies: possible identity of the FCCP-sensitive store

There have been several other reports of FCCP-sensitive $[Ca^{2+}]_i$ regulation in neurons. Brinley et al. (1978) described a Ca^{2+} buffering system in squid axons that is inhibited by FCCP (and CN^-) and accumulates Ca^{2+} from the axoplasm during electrical stimulation when $[Ca^{2+}]_i$ exceeds 200–400 nM. In mouse sensory neurons, Duchen et al. (1990) characterized a store that sequesters Ca^{2+} during stimulation and is inhibited by FCCP, CN^- or removal of glucose. Duchen (1990) found that FCCP does not directly influence surface membrane permeability, but leads to membrane hyperpolarization due to changes in $[Ca^{2+}]_i$. In rat DRG neurons, Thayer and Miller (1990) described a ruthe-

nium red–sensitive system that buffers Ca^{2+} with increasing strength as $[Ca^{2+}]_i$ rises during depolarization. The most obvious candidate for the FCCP-sensitive store is the mitochondria. Isolated mitochondria are known to take up Ca^{2+} from the surrounding medium (Lehninger, 1970; Crompton, 1985) and net uptake is inhibited by protonophores such as FCCP (Scarpa and Azzone, 1970). However, it has been argued that this is not important under physiological conditions because vigorous Ca^{2+} uptake by isolated mitochondria occurs only when Ca^{2+} in the bathing medium reaches very high (μM) levels that may not be encountered under physiological conditions. This has led to the suggestion that mitochondrial Ca^{2+} accumulation is important only under conditions of Ca^{2+} overload (Somlyo et al., 1988; but see Miller, 1991). On the other hand, it seems possible that the threshold for Ca^{2+} uptake by mitochondria may vary with the cell type and may be lower in intact cells. In cultured endothelial cells, mitochondrial Ca^{2+} levels rise even during stimuli that elevate $[Ca^{2+}]_i$ to submicromolar levels (Rizzuto et al., 1992). This suggests that mitochondrial Ca^{2+} uptake may occur during physiological stimulation in some cells. However, there are other cell types (e.g., liver) where this is clearly not the case (Bond et al., 1987).

In neurons, Thayer and Miller (1990) identified the FCCP-sensitive store as the mitochondria on the basis of effects of CCCP, ruthenium red, and Na^+ removal. However, it seems possible that other stores may be involved. Jensen and Rehder (1991) described a Ca^{2+} store in *Helisoma* neurons that is sensitive to FCCP but not to a cocktail of mitochondrial inhibitors, implicating a nonmitochondrial store that is sensitive to FCCP. In addition, Marrion and Adams (1992) found that intracellularly applied FCCP failed to mimic the actions of extracellular FCCP and intracellular ruthenium red, and proposed that FCCP influences a nonmitochondrial store that is sensitive to ruthenium red. Until more information is available, we will simply refer to "the FCCP-sensitive store." Note that the compartmental scheme described below makes several specific predictions about how the Ca^{2+} concentration in the FCCP-sensitive store changes during stimulation, and could, therefore, be helpful in identifying the store.

A simple compartmental scheme that largely accounts for the experimental observations

To understand $[Ca^{2+}]_i$ dynamics during and after stimulation, one must consider the contributions of multiple transport systems that jointly regulate $[Ca^{2+}]_i$. We developed a kinetic scheme with a minimal set of features needed to describe the interplay between key Ca^{2+} transport systems in sympathetic neurons. The model depicts $[Ca^{2+}]_i$ changes in terms of Ca^{2+} fluxes between multiple compartments. According to the scheme, within a small neighborhood of the resting $[Ca^{2+}]_i$ level, Ca^{2+} is transported between compartments by pumps and passive leaks at a rate approximately proportional to Ca^{2+} concentration. This is consistent with the way $[Ca^{2+}]_i$ relaxes in sympathetic neurons following small perturbations (Friel and Tsien, 1992c). Departures from the expected behavior of this linear scheme that are observed during stronger stimulation are accounted for by $[Ca^{2+}]_i$-dependent transport by the two stores. The FCCP-sensitive store is assumed to accumulate Ca^{2+} from the cytosol in a steeply $[Ca^{2+}]_i$-dependent manner, as motivated by the $[Ca^{2+}]_i$ dependence of mitochondrial Ca^{2+} uptake. The caffeine-sensitive store is assumed to release Ca^{2+} via CICR (Friel and Tsien, 1992a). According to the scheme, these two $[Ca^{2+}]_i$-dependent transport systems play a minor role in Ca^{2+} regulation as long as $[Ca^{2+}]_i$ is near the resting level, but become increasingly important as $[Ca^{2+}]_i$ rises during stimulation. Based on the preceding results, the relative importance of the FCCP- and caffeine-sensitive stores in shaping $[Ca^{2+}]_i$ during stimulation depends on the range of $[Ca^{2+}]_i$. At low $[Ca^{2+}]_i$ during weak depolarization, the caffeine-sensitive store is more influential (Friel and Tsien, 1992a), while at the high $[Ca^{2+}]_i$ levels reached during moderate to strong depolarization, the FCCP-sensitive store is dominant.

In its simplest form, the model includes three compartments: the extracellular medium, the cytosol, and the FCCP-sensitive store. Despite its simplicity, this scheme reproduced the qualitative properties of responses to depolarization and their sensitivity to FCCP (Fig. 9). For example, it successfully accounts for the observation that the decline in $[Ca^{2+}]_i$ upon repolarization following a strong depolarization was neither exponential (cf. Ahmed and Connor, 1988; Thayer and Miller, 1990) nor biexponential (cf. Duchen et al., 1990). Including a second store that releases Ca^{2+} in a $[Ca^{2+}]_i$-dependent fashion (CICR) permitted simulation of the distinctive properties of caffeine responses and their sensitivity to FCCP (Fig. 10). Finally, introducing a high-capacity compartment within the FCCP-sensitive store provided one way of explaining the saturable increase in recovery half times following longer depolarizations that occurs despite steady $[Ca^{2+}]_i$ elevations during depolarization that are well below those reached during depolarization in the presence of FCCP (Fig. 11).

The scheme also provides an interesting interpretation of the caffeine-sensitive store's modulatory effects on responses to depolarization. It was proposed that this store speeds $[Ca^{2+}]_i$ responses by (1) releasing Ca^{2+} into the cytosol during the response onset and (2) accumulating Ca^{2+} from the cytosol during the recovery, both in a $[Ca^{2+}]_i$-dependent manner (Friel and Tsien, 1992a,b). This represents the simplest case in which CICR discharges the store following depolarization, and Ca^{2+} uptake during the recovery restores its initial load. However, if resting c_i is well below K_{caff}, then during the response onset c_{caff} will first rise and then fall as c_i approaches K_{caff} (see c_{caff} in Fig. 11*a*). This will first slow and then speed the rise in c_i. Similarly, as c_i declines after repolarization, the store will accumulate Ca^{2+} and speed recovery. However, if uptake is so strong that c_{caff} overshoots the prestimulation level, Ca^{2+} must eventually be released to restore its initial Ca^{2+} load, which will slow the c_i recovery. Slow Ca^{2+} release by the caffeine-sensitive store could be responsible for the slow $[Ca^{2+}]_i$ tails observed during recovery after depolarization (e.g., Fig. 1). Note that when c_i varies over a wide range (Fig. 11*b*), a sequence of three kinetically distinct phases are seen corresponding to ranges where $c_i \ll K_{caff}$, $c_i \sim K_{caff}$, and $c_i \gg K_{caff}$.

Further studies will be needed to establish the detailed properties of the Ca^{2+} transport systems operating in sympathetic neurons. However, the overall agreement between the model and the experiments suggests that stimulus-evoked $[Ca^{2+}]_i$ dynamics within the cell body can be described rather simply, at least for $[Ca^{2+}]_i$ within the range 100–600 nM on a time scale of ~0.1–100 sec.

Possible physiological roles of the FCCP-sensitive store

The experiments make it clear that the FCCP-sensitive store has considerable impact on both the level and duration of cellular $[Ca^{2+}]_i$ responses to stimulation. In exerting these effects,

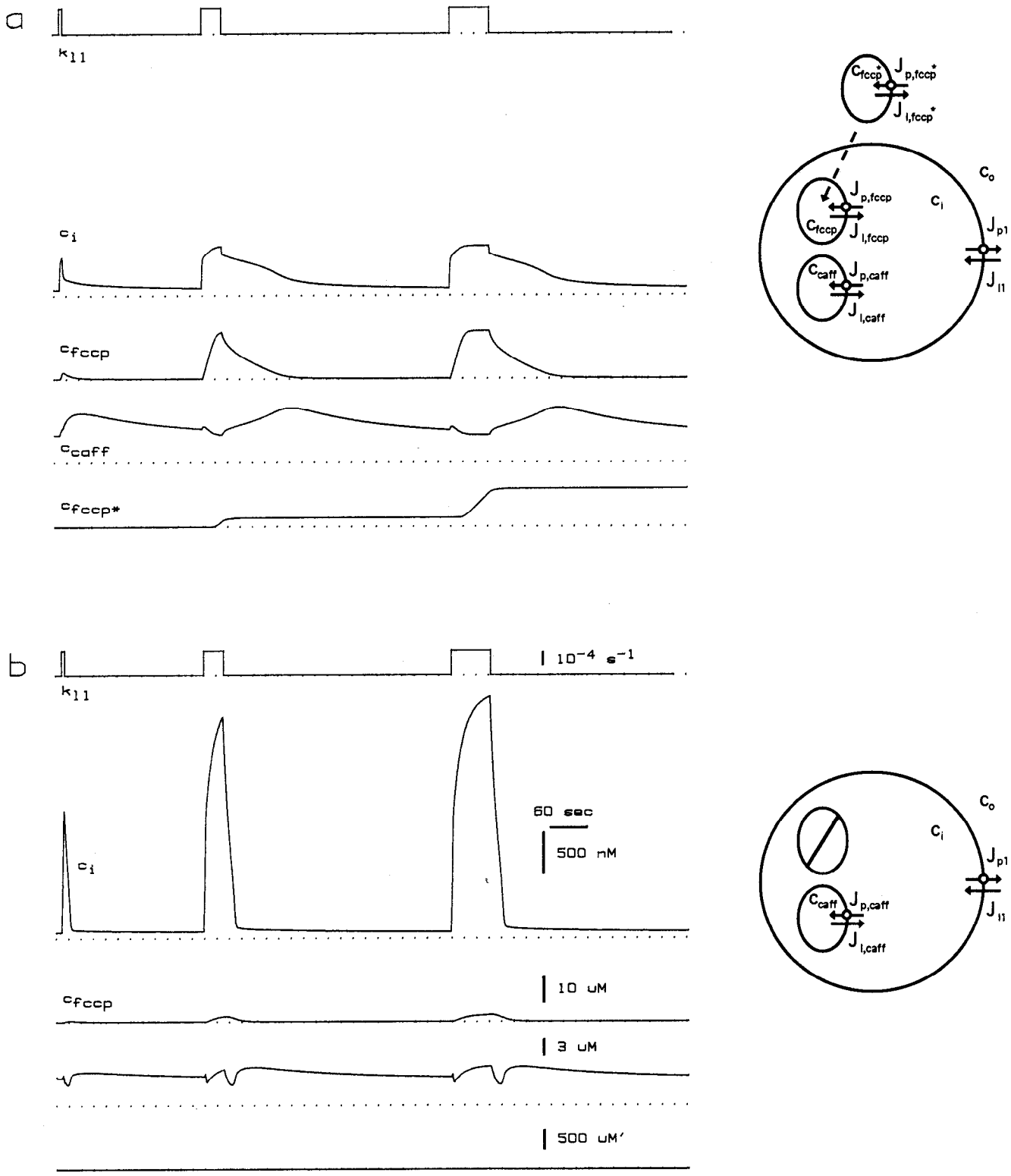


Figure 11. Simulated $[Ca^{2+}]_i$ responses to depolarization of different duration based on the five-compartment scheme before (*a*) and after (*b*) inhibiting c_i-dependent Ca^{2+} uptake into the FCCP-sensitive compartment. *a* and *b*, *Top trace*, κ_{11}; *second trace*, c_i; *third trace*, c_{fccp}; *fourth trace*, c_{caff}; *fifth trace*, c_{fccp^*}, the Ca^{2+} concentration inside the high-capacity compartment. *Dotted lines* indicate zero levels for K_{caff}, c_i, c_{fccp}, c_{caff}, c_{fccp^*}. For simplicity, it was assumed that the high-capacity compartment has no passive permeability to Ca^{2+} ($\kappa_{l,fccp^*} = 0$) so that Ca^{2+} accumulates and c_{fccp^*} rises irreversibly following successive stimuli. Essentially the same dynamics are observed for nonzero $\kappa_{l,fccp^*}$ as long as it is small relative to the other rate parameters (not shown). Note that even with $\kappa_{p,fccp} = 0$, c_{fccp} was weakly influenced by changes in c_i because $\kappa_{l,fccp}$ is nonzero. All parameters common with the four-compartment scheme had the same values as in Figure 10. Rate parameters describing the high-capacity compartment were (in sec^{-1}) $\kappa_{l,fccp^*} = 0$, $\kappa_{p,fccp^*}^{(0)} = 0$, $\kappa_{p,fccp^*}^{(1)} = 90$. The remaining parameter values were $K_{fccp^*} = 6.5 \times 10^{-5}$ M, $n_{fccp^*} = 4$, and $\gamma_{fccp^*} = 0.05$. Initial values of c_i, c_{fccp}, c_{caff}, and c_{fccp^*} represent the solution of the system $dc_i/dt = dc_{fccp}/dt = dc_{caff}/dt = dc_{fccp^*}/dt = 0$. Depolarization was modeled by elevating κ_{11} from 5×10^{-6} sec^{-1} to 2×10^{-4} sec^{-1}.

the store may participate in one or more physiologically beneficial functions.

Neuroprotection. Ca^{2+} at high concentrations is known to produce both short-term and long-term cytotoxic effects (Choi, 1990), and the FCCP-sensitive store strongly limits the peak cytosolic Ca^{2+} response. Thus, Ca^{2+} uptake by this compartment could play a significant neuroprotective role.

Prolongation of the cytosolic Ca^{2+} signal. Cytosolic Ca^{2+} signals are communicated to target systems by means of various Ca^{2+} binding proteins. In prolonging the $[Ca^{2+}]_i$ transient and delaying the deactivation of $[Ca^{2+}]_i$-dependent signaling molecules, the FCCP-sensitive store might amplify the effects of a given stimulus in a way that reflects stimulus history. The slow recovery, which can last for several minutes in bullfrog sympathetic neurons and perhaps longer in rat sensory neurons (Thayer and Miller, 1990), would be expected to greatly increase the activation of Ca^{2+}-dependent enzymes.

Metabolic signaling. A third possibility is that Ca^{2+} serves a useful function as it accumulates within the lumen of the FCCP-sensitive store itself. Ca^{2+} uptake by the store will increase the intracompartmental Ca^{2+} content, and this could serve as a metabolic signal that reflects the cell's recent history of $[Ca^{2+}]_i$. Indeed, such a role has been proposed for Ca^{2+} uptake by mitochondria (Denton and McCormack, 1990).

Appendix

Three-compartment scheme (Fig. 9)

The first scheme consists of the external medium, the cytosol, and a single internal store, where the concentrations of Ca^{2+} within compartments are designated c_o, c_i, and c_{fccp}, respectively. It is assumed that the plasma membrane pump and leak fluxes, J_{p1} and J_{l1}, are proportional to Ca^{2+} concentration in the following way:

$$J_{p1} = k_{p1} \times c_i,$$

$$J_{l1} = k_{l1} \times (c_i - c_o),$$

where k_{p1} and k_{l1} are constants. The fluxes $J_{p,fccp}$ and $J_{l,fccp}$, which describe Ca^{2+} exchange between the cytosol and the internal compartment, are assumed to obey

$$J_{p,fccp} = k_{p,fccp} \times c_i,$$

$$J_{l,fccp} = k_{l,fccp} \times (c_i - c_{fccp}),$$

where $k_{l,fccp}$ is a constant and $k_{p,fccp}$ increases with c_i sigmoidally:

$$k_{p,fccp} = k_{p,fccp}^{(0)} + k_{p,fccp}^{(1)}/[1 + (K_{fccp}/c_i)^{n,fccp}],$$

where $k_{p,fccp}^{(0)}$, $k_{p,fccp}^{(1)}$, K_{ccp}, and n_{fccp} are constant. $k_{p,fccp}^{(1)}$ describes the strength of c_i-dependent uptake by the store, K_{fccp} its c_i sensitivity, and n_{fccp} determines how sharply $k_{p,fccp}$ increases with c_i. This definition is motivated by the $[Ca^{2+}]_i$ dependence of Ca^{2+} uptake by the mitochondrial uniporter (Crompton, 1985). Note that $k_{p,fccp}$ is assumed to change instantaneously following changes in c_i. The dynamical equations that describe this system are

$$dc_i(t)/dt = -[J_{l1} + J_{p1} + J_{l,fccp} + J_{p,fccp}]/v_i,$$

$$dc_{fccp}(t)/dt = [J_{l,fccp} + J_{p,fccp}]/v_{fccp}.$$

Defining $\kappa_{l1} = k_{l1}/v_i$, $\kappa_{p1} = k_{p1}/v_i$, $\kappa_{l,fccp} = k_{l,fccp}/v_{fccp}$, $\kappa_{p,fccp} = k_{p,fccp}/v_{fccp}$, and $\gamma_{fccp} = v_{fccp}/v_i$ and substituting gives

$$dc_i(t)/dt = -[\kappa_{l1} + \kappa_{p1} + \gamma_{fccp}(\kappa_{l,fccp} + \kappa_{p,fccp})]c_i + \gamma_{fccp}\kappa_{l,fccp}c_{fccp} + \kappa_{l1}c_o,$$

$$dc_{fccp}(t)/dt = [\kappa_{l,fccp} + \kappa_{p,fccp}]c_i - \kappa_{l,fccp}c_{fccp}.$$

Note that Ca^{2+} fluxes into the cytosol are negative by convention.

Four-compartment scheme (Fig. 10)

To account for caffeine-induced Ca^{2+} release from a distinct store, a second internal compartment was included with internal Ca^{2+} concentration c_{caff}. Instead of accumulating Ca^{2+} in a c_i-dependent manner, this store releases Ca^{2+} in a c_i-dependent fashion. Ca^{2+} exchange with the cytosol is via the pump and leak fluxes:

$$J_{p,caff} = k_{p,caff} \times c_i,$$

$$J_{l,caff} = k_{l,caff} \times (c_i - c_{caff}),$$

where $k_{p,caff}$ is constant and $k_{l,caff}$ depends on c_i as follows:

$$k_{l,caff} = k_{l,caff}^0 + k_{l,caff}^1/[1 + (K_{caff}/c_i)^{n,caff}],$$

with $k_{l,caff}^0$, $k_{l,caff}^1$, K_{caff}, and n_{caff} constant. K_{caff} describes the c_i sensitivity of CICR and n_{caff} influences how rapidly $k_{l,caff}$ changes with c_i. For this four-compartment system,

$$dc_i(t)/dt = -[J_{l1} + J_{p1} + J_{l,fccp} + J_{p,fccp} + J_{l,caff} + J_{p,caff}]/v_i,$$

$$dc_{fccp}(t)/dt = [J_{l,fccp} + J_{p,fccp}]/v_{fccp},$$

$$dc_{caff}(t)/dt = [J_{l,caff} + J_{p,caff}]/v_{caff},$$

where v_{caff} is the volume of the caffeine-sensitive store. Defining $\kappa_{l,caff} = k_{l,caff}/v_{caff}$, $\kappa_{p,caff} = k_{p,caff}/v_{caff}$, and $\gamma_{caff} = v_{caff}/v_i$ and substituting gives

$$dc_i(t)/dt = -[\kappa_{l1} + k_{p1} + \gamma_{fccp}(\kappa_{l,fccp} + \kappa_{p,fccp}) + \gamma_{caff}(\kappa_{l,caff} + \kappa_{p,caff})]c_i + \gamma_{fccp}\kappa_{l,fccp}c_{fccp} + \gamma_{caff}\kappa_{l,caff}c_{caff} + \kappa_{l1}c_o,$$

$$dc_{fccp}(t)/dt = [\kappa_{l,fccp} + \kappa_{p,fccp}]c_i - \kappa_{l,fccp}c_{fccp},$$

$$dc_{caff}(t)/dt = [\kappa_{l,caff} + \kappa_{p,caff}]c_i - \kappa_{l,caff}c_{caff}.$$

Five-compartment scheme (Fig. 11)

The high-capacity compartment was assumed to transport Ca^{2+} according to the following rate equations:

$$J_{p,fccp^*} = k_{p,fccp^*} \times c_{fccp},$$

$$J_{l,fccp^*} = k_{l,fccp^*} \times (c_{fccp} - c_{fccp^*}),$$

where $k_{p,fccp^*}$ and $k_{l,fccp^*}$ describe Ca^{2+} uptake and a passive leak by the high-capacity compartment. To ensure that the rate of Ca^{2+} uptake by the high-capacity compartment increases with c_{fccp}, $k_{p,fccp^*}$ was defined as follows:

$$k_{p,fccp^*} = k_{p,fccp^*}^{(0)} + k_{p,fccp^*}^{(1)}/[1 + (K_{ccp^*}/c_{fccp})^{n,fccp^*}],$$

where $k_{p,fccp^*}^{(0)}$, $k_{p,fccp^*}^{(1)}$, K_{ccp^*}, and n_{fccp^*} are constants. The five-compartment system is described by

$$dc_i(t)/dt = -[J_{l1} + J_{p1} + J_{l,fccp} + J_{p,fccp} + J_{l,caff} + J_{p,caff}]/v_i,$$

$$dc_{fccp}(t)/dt = [J_{l,fccp} + J_{p,fccp} - (J_{l,fccp^*} + J_{p,fccp^*})]/v_{fccp},$$

$$dc_{caff}(t)/dt = [J_{l,caff} + J_{p,caff}]/v_{caff},$$

$$dc_{fccp^*}(t)/dt = [J_{l,fccp^*} + J_{p,fccp}]/v_{fccp^*},$$

where v_{fccp*} is the volume of the high-capacity compartment. Defining $\kappa_{l,fccp*} = k_{l,fccp*}/v_{fccp*}$, $\kappa_{p,fccp*} = k_{p,fccp*}/v_{fccp*}$, and $\gamma_{fccp*} = v_{fccp*}/v_{fccp}$ and substituting gives

$$\begin{aligned}
dc_i(t)/dt &= -[\kappa_{l1} + \kappa_{p1} + \gamma_{fccp}(\kappa_{l,fccp} + \kappa_{p,fccp}) \\
&\quad + \gamma_{caff}(\kappa_{l,caff} + \kappa_{p,caff})]c_i \\
&\quad + \gamma_{fccp}\kappa_{l,fccp}c_{fccp} + \gamma_{caff}\kappa_{l,caff}c_{caff} + \kappa_{l1}c_o, \\
dc_{fccp}(t)/dt &= [\kappa_{l,fccp} + \kappa_{p,fccp}]c_i \\
&\quad - [\kappa_{l,fccp} + \gamma_{fccp*}(\kappa_{l,fccp*} + \kappa_{p,fccp*})]c_{fccp} \\
&\quad + \kappa_{l,fccp*}c_{fccp*}, \\
dc_{caff}(t)/dt &= [\kappa_{l,caff} + \kappa_{p,caff}]c_i - \kappa_{l,caff}c_{caff}, \\
dc_{fccp*}(t)/dt &= [\kappa_{l,fccp*} + \kappa_{p,fccp*}]c_{fccp} - \kappa_{l,fccp*}c_{fccp*}.
\end{aligned}$$

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