Ca\(^{2+}\)-Dependent Ca\(^{2+}\) Clearance Via Mitochondrial Uptake and Plasmalemmal Extrusion in Frog Motor Nerve Terminals

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Suzuki, S., M. Osanai, N. Mitsumoto, T. Akita, K. Narita, H. Kijima, and K. Kuba. Ca\(^{2+}\)-dependent Ca\(^{2+}\) clearance via mitochondrial uptake and plasmalemmal extrusion in frog motor nerve terminals. J Neurophysiol 87: 1816–1823, 2002; 10.1152/jn.00456.2001. Ca\(^{2+}\) clearance in frog motor nerve terminals was studied by fluorometry of Ca\(^{2+}\) indicators. Rises in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in nerve terminals induced by tetanic nerve stimulation (100 Hz, 100 or 200 stimuli: Ca\(^{2+}\) transient) reached a peak or plateau within 6–20 stimuli and decayed at least in three phases with the time constants of 82–87 ms (81–85%), a few seconds (11–12%), and several tens of seconds (less than a few percentage). Blocking both Na/Ca exchangers and Ca\(^{2+}\) pumps at the cell membrane by external Li\(^+\) and high external pH (9.0), respectively, increased the time constants of the initial and second decay components with no change in their magnitudes. By contrast, similar effects by Li\(^+\) alone, but not by high alkaline alone, were seen only on 200 stimuli-induced Ca\(^{2+}\) transients. Blocking Ca\(^{2+}\) pumps at Ca\(^{2+}\) stores by thapsigargin did not affect 100 stimuli-induced Ca\(^{2+}\) transients but increased the initial decay time constant of 200 stimuli-induced Ca\(^{2+}\) transients with no change in other parameters. Inhibiting mitochondrial Ca\(^{2+}\) uptake by carbonyl cyanide m-chloronaphthaldehyde markedly increased the initial and second decay time constants of 100 stimuli-induced Ca\(^{2+}\) transients and the amplitudes of the second and the slowest components. Plotting the slopes of the decay of 100 stimuli-induced Ca\(^{2+}\) transients against [Ca\(^{2+}\)]\(_i\), yielded the supralinear [Ca\(^{2+}\)]\(_i\) dependence of Ca\(^{2+}\) efflux out of the cytosol. Blocking Ca\(^{2+}\) extrusion or mitochondrial Ca\(^{2+}\) uptake significantly reduced this [Ca\(^{2+}\)]\(_i\)-dependent Ca\(^{2+}\) efflux. Thus Ca\(^{2+}\)-dependent mitochondrial Ca\(^{2+}\) uptake and plasmalemmal Ca\(^{2+}\) extrusion clear out a small Ca\(^{2+}\) load in frog motor nerve terminals, while thapsigargin-sensitive Ca\(^{2+}\) uptake boosts the clearance of a heavy Ca\(^{2+}\) load. Furthermore, the activity of plasmalemmal Ca\(^{2+}\) pump and Na/Ca exchanger is complementary to each other with the slight predominance of the latter.

INTRODUCTION

Ca\(^{2+}\) in presynaptic terminals activates the exocytosis of neurotransmitters, its plasticity, recycling of the synaptic vesicles and other functions (see Katz 1969; Schweitzer et al. 1995; Zucker 1996). For these Ca\(^{2+}\) actions, the basal level of the intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) must be maintained far below that of external Ca\(^{2+}\), while rises in [Ca\(^{2+}\)]\(_i\) produced by physiological stimuli must be cleared out for the next stimulus. The low basal level of [Ca\(^{2+}\)]\(_i\) is maintained by active Ca\(^{2+}\) extrusion via the activity of Ca\(^{2+}\) pumps and Na/Ca exchanger, which is in equilibrium with passive Ca\(^{2+}\) entry at the cell membrane (see Carafoli 1987). Rises in [Ca\(^{2+}\)]\(_i\), by external stimuli or spontaneous cell activity are quickly buffered by binding to Ca\(^{2+}\)-binding proteins (see Kasai 1993) and cleared out by extrusion at the cell membrane and uptake via Ca\(^{2+}\) pumps into the endoplasmic reticulum and/or other organelles and via Ca\(^{2+}\) uniporter into mitochondria (see Miller 1991; Pozzan et al. 1994).

It is not precisely known, however, how these Ca\(^{2+}\)-buffering mechanisms operate in presynaptic terminals. Na/Ca exchange was reported to play a predominant role in the clearance of impulse-induced Ca\(^{2+}\) entry in presynaptic terminals of hippocampal neurons (Reuter and Poerz 1995), while mitochondrial Ca\(^{2+}\) uptake was emphasized for Ca\(^{2+}\) clearance in lizard motor nerve terminals (David et al. 1998). We have suggested that Ca\(^{2+}\) clearance in frog motor nerve terminals occurs in a cytosolic Ca\(^{2+}\)-dependent manner with the fastest component as fast as free diffusion (Suzuki et al. 2000). We report here that the not only mitochondrial Ca\(^{2+}\) uptake but also plasmalemmal Ca\(^{2+}\) extrusion clear out tetanus-induced rises in [Ca\(^{2+}\)]\(_i\), in a Ca\(^{2+}\)-dependent manner in frog motor nerve terminals, while thapsigargin-sensitive Ca\(^{2+}\) uptake boosts the clearance of a heavy Ca\(^{2+}\) load and that Ca\(^{2+}\) pump and Na/Ca exchanger at the cell membrane are complementary in operation to each other with the slight predominance of the latter.

METHODS

Preparations and Ca\(^{2+}\)-imaging techniques are essentially similar to those of the previous study (Suzuki et al. 2000). Briefly, frogs (Rana nigromaculata) were decapitated, and cutaneous pectoris muscles were isolated with the nerve attached. The composition of normal Ringer solution was (in mM) 113 NaCl, 2.0 KCl, 1.76 CaCl\(_2\), 2.3 NaHCO\(_3\), 5 glucose, and 5 HEPES-Na (pH 7.4). To avoid muscle contraction, 5–10 μM d-tubocurarine (Sigma) was added to perfusate throughout experiments. All the experiments were performed at 22 ± 1°C (mean ± SE). The motor nerve terminals were loaded with Ca\(^{2+}\) indicators [Indo-1/K, 19 mM, or Oregon green 488 BAPTA-1/K (OGB-1), 30 mM] from the cut end of the nerve bundle. Fluorescence of Indo-1 loaded in the nerve terminals was measured by line-scanning at 250 Hz with a UV confocal laser-scanning microscope (UV-

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CA CLEARANCE IN NERVE TERMINALS

CLSM; BioRad MRC-500 attached to Nikon TMD-300 with an objective, Nikon, Fluor \times 40 water, NA 1.15; Argon laser, 351 nm (Kuba et al. 1994) or by horizontal scanning at 60 Hz with a fast-scanning UV-CLSM (Noran Odyssey System; Argon laser, 363 nm) with the same objective. Indo-1 fluorescence was used to observe effects of a mitochondrial uncoupler on tetanus-induced rises in \( [Ca^{2+}] \), and changes in the basal \( [Ca^{2+}] \). Indo-1 fluorescence was separated into two wavelength ranges peaked at 406 and 475 nm, their ratio \( (F_{406}/F_{475}) \) was taken and converted to \( [Ca^{2+}] \), values as described previously (Suzuki et al. 2000). In other experiments, the fluorescence of OGB-1 was recorded with a cooled CCD camera (ARGUS/HiSca, Hamamatsu Photonics, Hamamatsu, Japan) with an image intensifier (Stardancer 2, Videoscope International, Sterling, VA; at 33 Hz) or an intensified CCD camera (Argus 50, Hamamatsu Photonics; at 30 Hz). The ratios of fluorescence changes during and after tetanic nerve stimulation to that before the tetanus were taken and converted to \( [Ca^{2+}] \), values as described previously (Suzuki et al. 2000).

The decay time course of an increase in \( [Ca^{2+}] \), produced by tetanic stimulation was fitted \( \pm 5 \) s after the beginning of decay with a double-exponential function plus a constant value, which represents the slowest decay component with the time constants of several tens of second (Suzuki et al. 2000). The relationship between the rate of the decay of \( [Ca^{2+}] \), and different levels of \( [Ca^{2+}] \), was measured as follows. The digitized decay phases of tetanus-induced rises in \( [Ca^{2+}] \), obtained from different experiments, which showed the \( [Ca^{2+}] \), value of plateau between 1 and 2 \( \mu M \), were averaged. The slopes of the decline of \( [Ca^{2+}] \), at individual \( [Ca^{2+}] \), values were then measured between two digitized points and plotted to each \( [Ca^{2+}] \), values. The rate of \( [Ca^{2+}] \), decay indicates the sum of \( Ca^{2+} \) effluxes out of the cytosol, which include both \( Ca^{2+} \) efflux at the cell membrane and \( Ca^{2+} \) uptake into mitochondria and \( Ca^{2+} \) storing organelles. Thus the rate of \( [Ca^{2+}] \), decay is simply defined as \( Ca^{2+} \) efflux out of the cytosol \( (J_{Ca}) \) (see Colegrove et al. 2000). The relationships between \( Ca^{2+} \) efflux and \( [Ca^{2+}] \), were fitted by the equation

\[
J_{Ca} = J_{max}/(1 + (K/[Ca^{2+}]^2))
\]  

where \( J_{max} \) and \( K \) are the maximum \( Ca^{2+} \) efflux and the apparent dissociation constant, respectively (see Gunter and Gunter 1994). Equation 1 is based on the assumption that a \( Ca^{2+} \) transporter translocates \( Ca^{2+} \) across the membrane after the cooperative binding of two \( Ca^{2+} \) to the translocation sites. The subtraction of the component of \( Ca^{2+} \) efflux via mitochondrial \( Ca^{2+} \) uptake or \( Ca^{2+} \) extrusion was made by first fitting the control data points and those after the blockade of \( Ca^{2+} \) clearance by Eq. 1 and then taking the differences between the two asymptotic curves at each \( [Ca^{2+}] \), value.

Data for each parameter of tetanus-induced \( Ca^{2+} \) transient in various conditions were expressed as means \( \pm SE \). Their statistical significance was examined by Student’s \( t \)-test. Statistical significance for differences between the relationships of \( Ca^{2+} \) efflux to \( [Ca^{2+}] \), in different conditions were examined as follows. Equation 1 was linearized by ignoring the first term of the denominator in the right side and taking the logarithm because all the \( K_{d} \), values (>7 \( \mu M \)) in fitting the data to the equation (Figs. 1D and 2, C and D) were greater than the range of \( [Ca^{2+}] \), fitted (<1.2 \( \mu M \)). Thus

\[
Y = \beta_1 + \beta_2X.
\]

where \( Y \) is log \( (J_{Ca}) \) at each \( [Ca^{2+}] \), \( X_1 = \log ( [Ca^{2+}] ) \), \( \beta_1 = \log ( J_{max}/K) \), and \( \beta_2 = 2 \), which is an assumed value. The null hypothesis was applied to pairs of the relationships of \( J_{Ca} \), to \( [Ca^{2+}] \), obtained before and after the blockade of \( Ca^{2+} \) extrusion or mitochondrial \( Ca^{2+} \) uptake. For instance, it was assumed that the value for \( \beta_1 \), before the application of carbonyl cyanide \( m \)-chlorophenylhydrazone (CCCP) was not different from that after the application and vice versa. \( t \)-test was made as to the sample regression coefficient (\( \beta_1^* \)) for \( \beta_1 \) by calculating the standard error of \( \beta_1^* \) and then a \( t \)-value for \( \beta_1^* \). Indo-1/K and OGB-1/K were obtained from Molecular Probes (Eugene, OR). Thapsigargin, CCCP, and dinitrophenol (DNP) were from Sigma.

RESULTS

Characteristics of tetanus-induced rises in \( [Ca^{2+}] \)

As in the previous study (Suzuki et al. 2000), a high-frequency tetanus (100 Hz, 100 or 200 stimuli) caused a rise in \( [Ca^{2+}] \), in the nerve terminals (\( Ca^{2+} \) transient; measured by OGB-1 fluorescence), which rose quickly for the initial 6–20 stimuli, reached a plateau, and decayed in three phases after the end of tetanus (Figs. 1 and 2). The time constant and fraction of the initial, fast and second decay components of 100 stimuli-induced \( Ca^{2+} \) transients were 87 ms and 85.2% and 2.2 s and 11.0%, respectively (Fig. 3A). The slowest component had the time constant much longer than several second and therefore handled as a constant value (3.8% of the peak) in analysis (Fig. 3A; see METHODS). The time constant and fraction of the initial and second decay components of 200 stimuli-induced \( Ca^{2+} \) transient (OGB-1 fluorescence) were 82 ms and 80.5 \( \pm 1.1% \) (\( n = 20 \)) and 1.14 s and 12.1 \( \pm 1.3\% \), respectively, with the slowest component of 4.9 \( \pm 0.5\% \) (Fig. 3B for the time constants). In the previous study (Suzuki et al. 2000), the time constant of the initial decay component was found to decrease with the elevation of the plateau phase of tetanus-induced \( Ca^{2+} \) transients produced by tetani of different frequencies, indicating the \( [Ca^{2+}] \), dependence of the initial component. The \( [Ca^{2+}] \), dependence of \( Ca^{2+} \) clearance can be more clearly demonstrated by measuring the slope of the decay phase at different levels of \( [Ca^{2+}] \); along the course of the decay and plotting them against \( [Ca^{2+}] \), (see METHODS). The rate of the decay of the increased \( [Ca^{2+}] \), reflects the magnitude of \( Ca^{2+} \) efflux out of the cytoplasmic space at each \( [Ca^{2+}] \), value (see Colegrove et al. 2000). \( Ca^{2+} \) efflux out of the cytosol after the end of a 100-stimuli-induced \( Ca^{2+} \) transient clearly decreased with the reduction of \( [Ca^{2+}] \), level (Figs. 1D and 2C). A similar \( [Ca^{2+}] \),-dependence of \( Ca^{2+} \) efflux was seen after a 200-stimuli-induced \( Ca^{2+} \) transient (not shown).

Effects of blocking \( Ca^{2+} \) extrusion at the plasma membrane

We first tested the effect of inhibition of \( Ca^{2+} \) pumps at the cell membrane on tetanus-induced \( Ca^{2+} \) transients by raising external pH (Benham et al. 1992; Milianick 1990; Nigglì et al. 1982). Increasing external pH to 9.0 affected neither the resting \( [Ca^{2+}] \), nor the amplitude and decay phases of \( Ca^{2+} \) transients induced by a tetanus (100 Hz) of 100 stimuli with a tendency of an increase in the amplitude of the second decay component (but, not significant: Figs. 1A and 3A). The initial and second decay time constant of \( Ca^{2+} \) transients induced by 200 stimuli, however, tended to increase with no change in other parameters. Although the increases in the initial and second decay constants averaged over all the terminals examined were not statistically significant (Fig. 3B), some of the terminals showed significant increases to 130% (\( n = 3 \)) and 165% (\( n = 5 \)), respectively, with no change in other parameters.

We next examined the effect of blockade of Na/Ca ex-
FIG. 1. Effects of blocking Ca\(^{2+}\) pumps and/or Na/Ca exchangers at the cell membrane on tetanus-induced Ca\(^{2+}\) transients. A: effects of raising external pH to 9.0. B: effects of replacement of most external Na\(^{+}\) with Li\(^{+}\). C: effects of combination of treatments with Li\(^{+}\) and high external pH. Ca\(^{2+}\) transients were induced by a tetanus of 100 stimuli at 100 Hz to the nerve and recorded by measuring the fluorescence of OGB-1. Thin traces are the control records, while thick traces are those after treatment with high pH (A), Li\(^{+}\) (B), or both (C). Insets: the falling phase of Ca\(^{2+}\) transients on semi-log coordinates. D: the [Ca\(^{2+}\)]\(_i\) dependence of the rate of the decay of tetanus-induced Ca\(^{2+}\) transient and effects of blocking Ca\(^{2+}\) pumps and Na/Ca exchangers. The slope of the decline of [Ca\(^{2+}\)]\(_i\), Ca\(^{2+}\) efflux, along the decay time course of Ca\(^{2+}\) transient was plotted against the corresponding [Ca\(^{2+}\)]\(_i\), value. Open triangles, the control Ca\(^{2+}\) efflux; closed triangles, those after treatment with external Li\(^{+}\) at pH 9. Data points were the averages of the relationships obtained from 5 terminals and fitted by Eq. 1 (see METHODS), where values for K were 7.6 and 3.5 \(\mu\)M for the control relationship (a) and that in the presence of Li\(^{+}\) at pH 9 (b), respectively, and \(J_{\text{max}}\) were 514.0 and 84.3 \(\mu\)M/s, respectively. The difference between the control curve and that after blocking Ca\(^{2+}\) extrusion represents the component sensitive to Li\(^{+}\) and high pH and is shown by a thick curve (a – b: \(J_{\text{Ca(exm)}}\)). The difference is statistically significant with \(P < 0.05\) (see METHODS). It is to be noted that fitting the data points with Eq. 1 was only for approximation to take the difference between the 2 relationships.

This was achieved by replacing external Na\(^{+}\) with Li\(^{+}\), which is known to be incapable of substituting the role of Na\(^{+}\) in Na/Ca exchange (see Reuter and Porzig 1995). External Li\(^{+}\) slowly but only slightly elevated the basal level of [Ca\(^{2+}\)]\(_i\), over several minutes (Fig. 3A), which was measured with changes in the ratio of indo-1 fluorescence. Under this condition, the amplitude and decay phase of Ca\(^{2+}\) transients induced by 100 stimuli (100 Hz) remained unchanged except for a small increase in the time constant of the initial decay component (Figs. 1B, e, and 3A). Both the initial and second decay time constants of Ca\(^{2+}\) transients produced by 200 stimuli (100 Hz), however, were prolonged to 140 and 135%, respectively, with no change in other parameters (Fig. 3B). These effects are apparently stronger than those of high alkaline, which were not consistent among all the terminals.

In contrast to the effect of blocking Na/Ca exchangers or Ca\(^{2+}\) pumps alone, the combined application of high external pH and Li\(^{+}\) significantly increased the time constants of the initial and second decay components of Ca\(^{2+}\) transients induced even by 100 stimuli (100 Hz: Figs. 1C and 3A). Under this condition, there was little change in the magnitude of each component of the decay although the second component tended to increase.

The results shown in the preceding text indicate that both the initial and second decay components are caused by Ca\(^{2+}\) extrusion operating in different modes depending on [Ca\(^{2+}\)]. Effects of blocking Ca\(^{2+}\) extrusion on the [Ca\(^{2+}\)]\(_i\), dependence of Ca\(^{2+}\) clearance are more clearly shown by the effects on the [Ca\(^{2+}\)]\(_i\)-dependent and [Ca\(^{2+}\)]\(_i\)-independent clearance (see METHODS). The rates of the decay of 100 stimuli-induced Ca\(^{2+}\) transient at different [Ca\(^{2+}\)]\(_i\) values in the presence of external Li\(^{+}\) at high external pH were plotted against the corresponding [Ca\(^{2+}\)]\(_i\), value and the relationship was fitted by Eq. 1 (Fig. 1D, b). This asymptotic curve was subtracted from the asymptotic curve for the control relationship (curve a in Fig. 1D, a). The net difference (Fig. 1D, a – b) yielded the fraction of Ca\(^{2+}\) clearance achieved by Ca\(^{2+}\) extrusion at the cell membrane. The rate of Ca\(^{2+}\) extrusion was thus clearly [Ca\(^{2+}\)]\(_i\)-dependent and amounted to be ~30% of the total flux.
Effects of blockade of Ca$^{2+}$ uptake into thapsigargin-sensitive Ca$^{2+}$ stores

Next, we tested the effect of blocking Ca$^{2+}$ uptake into Ca$^{2+}$-storing organelles. Thapsigargin (2 μM), a blocker of Ca$^{2+}$ pump at Ca$^{2+}$-storing organelles, did not affect Ca$^{2+}$ transients caused by 100 stimuli (100 Hz), although there was a tendency of a slight increase in the time constant of the initial component of the decay (Figs. 2A and 3A). The initial decay time constant of Ca$^{2+}$ transients induced by 200 stimuli (100 Hz), however, was significantly increased to 134% by thapsigargin (2 μM; Fig. 3B) with no change in other parameters. This suggests that Ca$^{2+}$ uptake into thapsigargin-sensitive Ca$^{2+}$ stores plays minor roles in Ca$^{2+}$ clearance of a small Ca$^{2+}$ load but operates as a Ca$^{2+}$ sink only for a greater Ca$^{2+}$ load. This may conform to the previous suggestion that this Ca$^{2+}$ store involved in Ca$^{2+}$-induced Ca$^{2+}$ release is normally filled with Ca$^{2+}$ (Narita et al. 1998).

Effects of blockade of mitochondrial Ca$^{2+}$ uptake

Blocking Ca$^{2+}$ uptake into mitochondria had drastic effects on Ca$^{2+}$ clearance in the nerve terminals. CCCP (1 μM), a mitochondrial uncoupler, markedly increased the plateau of tetanus-induced Ca$^{2+}$ transients, the initial and second components of the decay phase, and the magnitude of the slowest component (Figs. 2B and 3). Under this condition, the basal level of [Ca$^{2+}$]$_i$, was tended to increase (Fig. 3A). In two terminals, a high concentration of CCCP (5 μM) caused an increase in the basal level of [Ca$^{2+}$]$_i$ by several tens of nM (unpublished observations; see also David 1999; Tsang et al. 2000; see Narita et al. 1998 for the action of CN, another
mitochondrial poison). The increase in plateau must be mainly
due to the decrease in the rate of Ca\(^{2+}\) clearance seen as
increases in the time constants of the initial and second com-
ponents of [Ca\(^{2+}\)]\(_i\) decay and the magnitude of the latter
because the plateau is determined by the apparent equilibrium
of Ca\(^{2+}\) entry and clearance (see DISCUSSION).

The effect of blocking mitochondrial Ca\(^{2+}\) uptake on the
decay phase of Ca\(^{2+}\) transient can be shown more relevantly by
the effect on the [Ca\(^{2+}\)]\(_i\) dependence of the decay rate of the
increased [Ca\(^{2+}\)]\(_i\). The [Ca\(^{2+}\)]\(_i\)-dependent Ca\(^{2+}\) efflux from
the cytosol was considerably decreased under the block-
ade of mitochondrial Ca\(^{2+}\) uptake (Fig. 2C). The CCCP-
sensitive component of Ca\(^{2+}\) efflux obtained by subtraction
[Fig. 2C, thick curve (a–b)] was also [Ca\(^{2+}\)]\(_i\) dependent and
amounted to be \(~70\%\) of the total Ca\(^{2+}\) efflux. This [Ca\(^{2+}\)]\(_i\)-
dependence of the CCCP-sensitive Ca\(^{2+}\) efflux (\(\approx J_{\text{Ca}\(\text{pm}\)}\)
replotted in Fig. 2D), reflecting that of Ca\(^{2+}\) efflux via mitochon-
drial Ca\(^{2+}\) uniporter (see DISCUSSION), is very similar to that in
the presence of Li\(^+\) at high pH (thin curve with triangles
replotted in Fig. 2D). On the other hand, the relationship
between the Ca\(^{2+}\) efflux remaining in the presence of CCCP
and [Ca\(^{2+}\)]\(_i\) (thin curve with circles replotted in Fig. 2D) fairly
resembles that of the Ca\(^{2+}\) efflux sensitive to Li\(^+\) at pH 9
(\(\approx J_{\text{Ca}\(\text{pm}\)}\)) replotted in Fig. 2D). This remaining component
must be caused by Ca\(^{2+}\) extrusion at the cell membrane.

It is to be added that DNP (20 or 50 \(\mu M\)), an uncoupler, and
NaCN (2 mM), a blocker of an electron transfer system, had
effects similar to those of CCCP on the decay phases of
tetanus-induced Ca\(^{2+}\) transients (unpublished observations).

**DISCUSSION**

The present study demonstrates the primary role of mito-
chondrial Ca\(^{2+}\) uptake and the secondary role of plasma-
lemmal Ca\(^{2+}\) extrusion in the [Ca\(^{2+}\)]\(_i\)-dependent Ca\(^{2+}\)
clear-
ance of a small Ca\(^{2+}\) load caused by a relatively short
repetitive activity in frog motor nerve terminals and the boost-
ing role of thapsigargin-sensitive Ca\(^{2+}\) uptake in the clearance
of a heavier Ca\(^{2+}\) load. How Ca\(^{2+}\) extrusion and mitochondrial
Ca\(^{2+}\) uptake depend on [Ca\(^{2+}\)]\(_i\) and how much each of them
 contributes to the total Ca\(^{2+}\) clearance are discussed more in detail within the limitation of understanding of the time course of Ca\(^{2+}\) transients averaged over the whole terminal as in the following text.

### Nonlinear dynamics of Ca\(^{2+}\) and its reactions with Ca\(^{2+}\) indicators in the nerve terminals

Ca\(^{2+}\) entry caused by a nerve impulse first produces a large increase in \([\text{Ca}^{2+}]_i\), to \(>10-100 \ \mu\text{M}\) in a localized region (Ca\(^{2+}\) domain) around Ca\(^{2+}\) channels (Bollmann et al. 2000; Heidelberger et al. 1994; Llinás et al. 1992; Schneggenburger and Neher 2000; Schweizer et al. 1995), which dissipates within a few milliseconds, by binding, diffusion, and uptake (DiGregorio et al. 1999; Sala and Hernández-Cruz 1990; Sinha et al. 1997; Suzuki et al. 2000). On the other hand, high-affinity Ca\(^{2+}\) indicators in the Ca\(^{2+}\) domain quickly bind with Ca\(^{2+}\) and diffuse out immediately after each nerve impulse, while free Ca\(^{2+}\) indicators in neighboring regions would diffuse into the domain and bind Ca\(^{2+}\) (Naraghi and Neher 1997; Suzuki et al. 2000; Tank et al. 1995). Thus during the plateau of Ca\(^{2+}\) transient, Ca\(^{2+}\) entry at a constant rate would be in pseudo-equilibrium with Ca\(^{2+}\) extrusion at the cell membrane and mitochondrial Ca\(^{2+}\) uptake (David 1999), producing standing gradients in \([\text{Ca}^{2+}]_i\), free and bound forms of Ca\(^{2+}\) indicators from the Ca\(^{2+}\) microdomain to the global space. The high \([\text{Ca}^{2+}]_i\) in the Ca\(^{2+}\) domain in a small volume and a short life time would have escaped detection by high-affinity Ca\(^{2+}\) indicators. The measured changes in \([\text{Ca}^{2+}]_i\) during the plateau are therefore largely underestimated, while the changes in \([\text{Ca}^{2+}]_i\) after the dissipation of the Ca\(^{2+}\) domains would faithfully reflect the time course of the averaged change in \([\text{Ca}^{2+}]_i\) in the terminals. Simulation indeed revealed that the fluorescence changes averaged over the whole terminal represent the true impulse-induced changes in \([\text{Ca}^{2+}]_i\) only 20 ms after the end of stimuli (Suzuki et al. 2000).

### \([\text{Ca}^{2+}]_i\)-dependent mitochondrial Ca\(^{2+}\) uptake

The \([\text{Ca}^{2+}]_i\) dependence of mitochondrial Ca\(^{2+}\) uptake, seen as CCCP-sensitive component of Ca\(^{2+}\) efflux (\(J_{\text{cat}}\)) induced by 100 impulses or that of the Ca\(^{2+}\) efflux remaining in the presence of Li\(^+\) at high pH, is nicely fitted by the Hill’s equation (Eq. 1) assuming the cooperative binding of two Ca\(^{2+}\) ions to a Ca\(^{2+}\) transporter. This is consistent with the recent findings in bullfrog sympathetic ganglion cells (Colegrove et al. 2000) and also with the known property of mitochondrial Ca\(^{2+}\) uniporters binding two Ca\(^{2+}\) for translocation (Bygrave et al. 1971; Scarpa and Grassi 1973; see Gunter and Pfeiffer 1990). The threshold level of \([\text{Ca}^{2+}]_i\) for mitochondrial Ca\(^{2+}\) uptake to occur, however, was found to be 0.2 \(\mu\text{M}\) in the present study (see also Colegrove et al. 2000), which is smaller than the known value of 0.5 \(\mu\text{M}\) (see Carafoli 1987; Gunter and Pfeiffer 1990). The discrepancy was explained by the ignorance of Ca\(^{2+}\) release at \([\text{Ca}^{2+}]_i\) <0.5 \(\mu\text{M}\) in the previous measurement of mitochondrial Ca\(^{2+}\) uptake (see Colegrove et al. 2000).

The increase in the third, slowest component by blocking mitochondrial Ca\(^{2+}\) uptake is unexpected because the blockade of Ca\(^{2+}\) uptake would have also reduced mitochondrial Ca\(^{2+}\) release that occurs on restoration of \([\text{Ca}^{2+}]_i\), close to the resting level (Colegrove et al. 2000; David et al. 1998; Duchén et al. 1990; Friel and Tsien 1994). The increase in the slowest component could be explained by a large \([\text{Ca}^{2+}]_i\) load exceeding the capacity of Ca\(^{2+}\) extrusion as a result of the blockade of mitochondrial Ca\(^{2+}\) uptake. Presumably, mitochondrial Ca\(^{2+}\) release may have occurred after the end of 100 stimuli at 100 Hz but must have been too small to apparently affect the slowest decay component.

\([\text{Ca}^{2+}]_i\)-dependent Ca\(^{2+}\) extrusion at the cell membrane

The component of Ca\(^{2+}\) efflux via Ca\(^{2+}\) extrusion (\(J_{\text{cat}}\)) after 100 impulse-induced Ca\(^{2+}\) load was dependent on \([\text{Ca}^{2+}]_i\) and amounted to be \(\sim 30\%\) of the total Ca\(^{2+}\) efflux caused by 100 impulses. The \(J_{\text{cat}}\), seen as the Li\(^+\) and high-alkaline-sensitive component or the Ca\(^{2+}\) efflux remaining in the presence of CCCP, was well fitted by Eq. 1 assuming the Hill number of two (Fig. 2D). This is consistent with the known property of the Ca\(^{2+}\)-ATPase having two Ca\(^{2+}\)-binding sites at the cell membrane (Ferreira and Lew 1976; Lew et al. 1982) but not with the binding of a single Ca\(^{2+}\) to a Na/Ca exchanger molecule (see Carafoli 1987). Therefore there must be several other possible mechanisms for the apparent cooperative \([\text{Ca}^{2+}]_i\) dependence of \(J_{\text{cat}}\).

First, the Ca\(^{2+}\) affinity of Ca\(^{2+}\) pumps at the cell membrane is markedly enhanced by the binding of calmodulin (see Carafoli 1991). Thus rises in \([\text{Ca}^{2+}]_i\) promote the action of calmodulin and amplify the \([\text{Ca}^{2+}]_i\) dependence of Ca\(^{2+}\) pump. Second, a rise in \([\text{Ca}^{2+}]_i\), should increase the driving force for Na/Ca exchangers. For instances, 10-fold increase in \([\text{Ca}^{2+}]_i\) would shift the equilibrium potential for Na/Ca exchange (\(E_{\text{Na/Ca}}\)) to a more positive value by 56 mV [using the equation, \(E_{\text{Na/Ca}} = 3E_{\text{Na}} - 2E_{\text{Ca}}\) (Mullins 1977)]. These two effects would have changed the \([\text{Ca}^{2+}]_i\)-dependent property of \(J_{\text{cat}}\) to that more closely expected from Eq. 1. The deviation of the relationship of Ca\(^{2+}\) efflux to \([\text{Ca}^{2+}]_i\), in the presence of CCCP from the equation in the range of \([\text{Ca}^{2+}]_i\) > 1.5 \(\mu\text{M}\) (see Fig. 2C) might presumably be explained by the recruitment of another Ca\(^{2+}\) clearance mechanism unidentified yet.

Blocking either Na/Ca exchangers or Ca\(^{2+}\) pumps alone at the cell membrane affected only slightly or not obviously the decay rate of the clearance of a small Ca\(^{2+}\) load produced by a short tetanus (100 stimuli), while blocking both indeed slowed the decay rate. Presumably, a greater increase in \([\text{Ca}^{2+}]_i\) in the submembrane region as a result of the blockade of one mechanism would have enhanced another in compensation for the \([\text{Ca}^{2+}]_i\) dependence of their rate. Such a greater increase in \([\text{Ca}^{2+}]_i\) would have escaped detection by high-affinity Ca\(^{2+}\) indicators (see preceding text). Although the operations of Na/Ca exchanger and Ca\(^{2+}\) pumps are complementary to each other, the contribution of the former is stronger than the latter, as seen in the effect of Li\(^+\) on Ca\(^{2+}\) transients induced by 200 stimuli. This is consistent with the higher rate of Na/Ca exchanger than that of Ca\(^{2+}\) pumps at the cell membrane (see Carafoli 1987). A question remains, however, how such complementary operations of Na/Ca exchanger and Ca\(^{2+}\) pumps can be explained by their different Ca\(^{2+}\) affinity and transport rate (see Carafoli 1987) and the possible location of the latter close to Ca\(^{2+}\) channels as suggested for ciliary ganglion synapses (Juhászova et al. 2000).

The apparent absence of changes in the plateau phase of
Ca\(^{2+}\) transients induced by either 100 or 200 stimuli under the blockade of Ca\(^{2+}\) extrusion may be explained in part by the secondary role of Ca\(^{2+}\) extrusion in Ca\(^{2+}\) clearance and in part by the failure of detection of the high Ca\(^{2+}\) in the submembrane regions with high-affinity Ca\(^{2+}\) indicators. This contrasts with the successful recording of an increase in the [Ca\(^{2+}\)], in the bulk phase due to the blockade of mitochondrial Ca\(^{2+}\) uptake. The little effect of blocking Ca\(^{2+}\) extrusion as well as mitochondrial Ca\(^{2+}\) uptake on the resting level of [Ca\(^{2+}\)] could be accounted for presumably by small Ca\(^{2+}\) entry under the resting condition.

Comparison with Ca\(^{2+}\) clearance in other terminals and physiological significance

The predominant role of mitochondrial Ca\(^{2+}\) uptake in the clearance of the increased [Ca\(^{2+}\)], found in the present study is consistent with the previous studies on the rat neurohypophysial nerve endings (Stuenkel 1994) and the motor nerve terminals of the crayfish (Ohnuma et al. 1999; Tang and Zucker 1997) and the lizard (David et al. 1998). On the other hand, the involvement of Na/Ca exchanger in Ca\(^{2+}\) clearance in frog motor nerve terminals conforms to its similar role in rat brain synaptosomes (Nachshen et al. 1986) and hippocampal presynaptic terminals (Reuter and Poerzig 1995). Furthermore, Na/Ca exchangers were shown to exist in the membrane of the rat motor nerve terminal (Lather et al. 1992).

The significance of the present study is twofold. First, the modes of clearance of Ca\(^{2+}\) load via both mitochondrial Ca\(^{2+}\) uptake and Ca\(^{2+}\) extrusion are Ca\(^{2+}\) dependent in frog motor nerve terminals. Second, the operations of Na/Ca exchangers and Ca\(^{2+}\) pumps at the cell membrane to a small Ca\(^{-}\) load are complementary to each other with the slight predominance of the former. The effective operation of Ca\(^{2+}\) extrusion machinery, Na/Ca exchangers and Ca\(^{2+}\) pumps, is really physiologic for the presynaptic terminals of small size whose surface/volume ratio is quite large and therefore effective for Ca\(^{2+}\) clearance. This contrasts to the negligible role of Ca\(^{2+}\) extrusion for the clearance of Ca\(^{2+}\) load caused by Ca\(^{2+}\) entry in the large-sized cell soma of bullfrog sympathetic neurons (Colegrove et al. 2000) and rat adrenal chromaffin cells (Park et al. 1996).

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