

Posttetanic Potentiation at the Crayfish Neuromuscular Junction Is Dependent on Both Intracellular Calcium and Sodium Ion Accumulation

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The fluorescent indicator fura-2 was used to measure cytoplasmic calcium in presynaptic terminals in the crayfish *Procambarus clarkii* under conditions that raise intracellular sodium to examine whether sodium can elevate intracellular calcium concentration ($[Ca^{2+}]_i$) or prolong its efflux and thus influence the magnitude and duration of posttetanic potentiation (PTP).

Sodium was elevated in presynaptic terminals at rest by either (1) injection of sodium into the excitatory axon, (2) application of veratridine to open sodium channels, or (3) application of ouabain to block Na/K exchange, with $[Ca^{2+}]_i$ increasing by either 430, 400, or 180 nM, respectively. Intracellular calcium concentration increased only when external calcium was present, indicating that calcium influx occurred through Na/Ca exchange. Additionally, ouabain enhanced excitatory junctional potentials (EJPs) eightfold. Elevation of sodium using a high-frequency stimulation in zero-calcium Ringer's did not elevate $[Ca^{2+}]_i$ during the train or immediately afterward when calcium-containing Ringer's was re-introduced. This indicates that a physiological sodium load does not release calcium from internal stores or reverse Na/Ca exchange to levels where $[Ca^{2+}]_i$ accumulation is detectable.

We examined the ability of sodium to interfere with calcium efflux from presynaptic terminals by loading boutons with both sodium and calcium or calcium alone using high-potassium depolarization. Elevation of internal sodium slowed calcium efflux from the terminal (12.3 min) compared to calcium removal without a sodium load (4.0 min). When sodium loading was increased during a tetanus by application of ouabain, the time constants for decay of EJP potentiation, 17.3 min, and for $[Ca^{2+}]_i$, 35 min, were longer than control values, 4.4 min and 5.8 min, respectively. In addition, using lithium to inhibit the efflux of calcium by Na/Ca exchange following a PTP-inducing train also lengthened the decay of $[Ca^{2+}]_i$ to 15.7 min. Intracellular sodium accumulation in presynaptic terminals slows the efflux of calcium through Na/Ca exchange, and may therefore augment and prolong PTP.

Transmitter release is dependent on the influx of extracellular calcium into presynaptic terminals (Katz, 1969). At the neuromuscular junction (NMJ) of the crayfish opener muscle, transmitter release shows many forms of frequency-dependent enhancement. Stimulation of the excitatory axon for a few seconds at a frequency greater than 1 Hz will produce enhancement of the excitatory junctional potential (EJP) amplitude that decays in less than a second. This enhancement is termed facilitation and is presumably due to a residual increase in intracellular calcium (Atwood and Wojtowicz, 1986; Zucker, 1989). A longer, more robust stimulation, that is, 20–33 Hz for 5–10 min, will produce two different kinds of long-term enhancement: posttetanic potentiation (PTP), which decays in minutes, and long-term facilitation (LTF), which lasts for tens of minutes to hours. PTP has been linked to the entry and accumulation of calcium and/or sodium ions into the presynaptic terminals during neural activity (Wojtowicz and Atwood, 1988). A direct measurement of $[Ca^{2+}]_i$ following a PTP-inducing train at the crayfish NMJ, using the fluorescent indicator fura-2, has shown that $[Ca^{2+}]_i$ decays at the same rate as PTP (Delaney et al., 1989), suggesting that $[Ca^{2+}]_i$ is important for this process. In addition, there has been strong evidence to suggest that intraterminal sodium ($[Na^+]_i$) accumulation also plays an important role. Atwood et al. (1975) showed at the crayfish NMJ that a lowered external sodium concentration reduced PTP, whereas conditions that led to increased sodium loading through inhibition of Na/K exchange prolonged PTP. Other investigators confirmed that elevating $[Na^+]_i$ by similar methods augmented PTP, both at the frog (Rahamimoff et al., 1978; Lev-Tov and Rahamimoff, 1980; Misler and Hurlbut, 1983; Misler et al., 1987) and rat NMJ (Nussinovitch and Rahamimoff, 1988). Sodium injected directly into presynaptic terminals of the crayfish axon (Wojtowicz and Atwood, 1985) and the squid giant synapse (Charlton and Atwood, 1977) also enhanced transmitter release. Pharmacological agents used to raise $[Na^+]_i$, such as veratridine and monensin, consistently increased transmitter release at both invertebrate (Atwood et al., 1983; Finger and Martin, 1987) and vertebrate NMJ (Meiri et al., 1981).

Various mechanisms by which elevation of $[Na^+]_i$ could enhance transmitter release have been proposed. The first possibility is that internal sodium ions could have a direct effect on transmitter release. Alternatively, sodium ions could increase $[Ca^{2+}]_i$ by release of calcium from intracellular stores, by entry from the external medium through Na/Ca exchange, or by slowing removal of accumulated calcium because of a reduction in the sodium gradient that drives Na/Ca exchange.

To test these possibilities directly, we have used the fluores-

Received Oct. 7, 1991; revised May 14, 1992; accepted June 5, 1992.

We thank Dr. Kerry Delaney and Dr. David Tank, AT&T Bell Laboratories, Murray Hill, NJ, for sharing and allowing us to use their unpublished SBFI data, in addition to Dr. Delaney's valuable discussion. We also thank Russell English for expert technical assistance. This work was supported by NIH Research Grant NS 15114.

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cent indicator fura-2 to measure cytoplasmic [Ca²⁺]_i in presynaptic terminals of crayfish opener muscle under conditions where intracellular sodium was elevated by pharmacological and physiological methods. Simultaneous recordings of EJPs and [Ca²⁺]_i measurements were acquired during sodium elevation in some experiments. The effect of sodium elevation on resting [Ca²⁺]_i was measured along with its effect on the decline of [Ca²⁺]_i during decay of PTP. We have attempted to determine whether intracellular sodium elevation during tetanic stimulation influences the posttetanic decay of [Ca²⁺]_i that lasts for minutes and, if so, by which mechanism it operates.

A preliminary account of these experiments has appeared in Mulkey and Zucker (1991b) and in Zucker et al. (1991).

Materials and Methods

Dye injection. All experiments were performed on the opener muscle of the first walking leg of crayfish, *Procambarus clarkii*, using animals measuring 2–2.5 inches. Viewing the preparation through a Nikon CF-E 10× long-working-distance air objective (5.2 mm), the excitatory axon was penetrated near the Y-branch with standard-wall glass microelectrodes (40 MΩ with 3 M KCl) that were filled with fura-2 (22 mM) pentapotassium salt (Molecular Probes, Eugene, OR) in 200 mM KCl. Fura-2 was iontophoresed into the excitatory axon by injecting 10–12 nA of hyperpolarizing current for approximately 30 min. The excitatory action potential was continuously monitored intracellularly during the filling in response to extracellular stimulation of the nerve at 0.5 Hz. The intensity of the fluorescence was monitored in the presynaptic terminals during the injection to prevent overfilling, as fura-2 is a calcium buffer that can alter the time course and magnitude of calcium transients in neurons when present at high concentrations (Kao and Tsien, 1988). The final concentration of fura-2 in the boutons was estimated by comparing the intensity of fluorescence of a 50 μM fura-2 solution in a 20-μm-pathlength cuvette to the intensity in presynaptic terminals having a pathlength of approximately 5–10 μm. The fluorescent intensity in terminals was about double that in the cuvette, suggesting that the concentration in the boutons was about 200 μM. After filling with fura-2, the injection electrode was gently removed in preparation for imaging.

Electrophysiology. The excitatory axon was dissected from the meopodite segment and placed in a plastic suction electrode for stimulation. While viewing the preparation through a Zeiss 40× water immersion objective (1.6 mm working distance), intracellular electrodes containing 3 M KCl (10 MΩ) were placed in muscle fibers onto which imaged terminals synapsed (if possible) or in adjacent fibers for recording EJPs. In the PTP experiments, following the high-frequency train, the excitatory axon was stimulated at 1 Hz and EJPs were acquired and averaged using a Nicolet 4094 digital oscilloscope during successive 1 min intervals until EJPs returned to control values. In some experiments, LTF was observed: EJPs did not return to their pretetanic level for the time measured (up to 1 hr). Averaged EJPs were stored on floppy disks for later analysis. Individual EJPs obtained during shorter trains were recorded with a Gould chart recorder. An extracellular electrode (25–50 μm inside diameter) was positioned on a distal axonal branch to record the nerve field potential during high-frequency stimulation in zero-calcium solutions to ensure action potential invasion.

Injection of sodium and potassium ions. Sodium ions were injected into the excitatory axon with standard-wall glass microelectrodes (40 MΩ) filled with 3 M NaCl and 0.3 M KCl, using 5–10 nA depolarizing current. KCl was included in the NaCl pipette, as it improved the recording characteristics of the electrodes so the action potential could be monitored throughout the injection. Potassium ions were injected in the same manner, but with microelectrodes filled with 3 M KCl.

Solutions. Normal Van Harreveld's solution contained (in mM) 195 NaCl, 13.5 CaCl₂, 5.4 KCl, 2.6 MgCl₂, and 10 Na-HEPES at pH 7.4. The EGTA Ringer's contained (in mM) 185 NaCl, 5.4 KCl, 30 MgCl₂, 2 Na₂EGTA, and 10 Na-HEPES at pH 7.4. Cobalt Ringer's contained CoCl₂ substituted for CaCl₂ (13.5 mM), along with (in mM) 175 NaCl, 5.4 KCl, 30 MgCl₂, and 10 Na-HEPES at pH 7.4. In lithium Ringer's lithium replaced sodium and Na-HEPES was reduced from 10 to 5 mM. Zero-sodium saline contained choline chloride instead of NaCl, plus (in mM) 13.5 CaCl₂, 2.6 MgCl₂, and 6 K-HEPES. Zero-sodium, zero-calcium saline consisted of choline chloride substituted for NaCl, plus (in

mM) 16.1 MgCl₂, 6 K-HEPES, and 2 K₂EGTA at pH 7.4. The high-potassium saline solutions contained 40 mM KCl, with choline chloride substituted for NaCl in the zero-sodium, high-potassium saline. Veratridine was obtained from Sigma, and stock solutions were made by dissolving the drug in 0.1 N HCl and storing it in the freezer. Final concentrations were obtained by dissolving veratridine in the appropriate saline. Ouabain (Aldrich) was made up fresh daily by dissolving it in the appropriate saline solution at its final concentration.

The temperature of the preparation was kept at 18 ± 2°C with a stage-mounted Peltier device. A gravity perfusion system controlled by a solenoid four-way valve (General Valve, series 18, Fairfield, NJ) was used to switch between normal and modified salines. An inflow was positioned above the surface of the opener muscle, and solutions flowed under the 40× water immersion lens. However, to ensure complete removal of calcium from the bath, the objective was lifted out of the bath and the surface washed vigorously with a syringe having a 1-mm-diameter plastic tube attached to the tip, while also continuing to perfuse with the gravity system. A perfusion time of about 10 min in cobalt Ringer's was needed to eliminate calcium influx during a high-frequency train. With zero-calcium EGTA, perfusion for 45–60 min with repeated vigorous washing was usually required.

Imaging of presynaptic terminals. Individual presynaptic terminals were imaged using a 40× Zeiss water immersion lens and a Nikon Optiphot upright compound microscope equipped with a silicon intensified target (SIT) camera (Dage MTI, model 66). Bandpass filters of 350 ± 10 nm and 385 ± 5 nm (Omega Optical, Brattleboro, VT) were used to pass the selective excitation wavelengths for fura-2. The filters were mounted on a wheel driven by a computer-controlled stepper motor with an electronic shutter (also computer driven) placed proximally in the light path. A dichroic mirror (450 nm) separated the excitation and emission wavelengths, and a barrier filter (510 ± 10 nm) passed only the peak emission wavelength, reducing interference from autofluorescence. Before each experiment, images for both the camera background (which includes tube dark current, optical autofluorescence, etc.) and tissue background were taken, along with a shading image, which corrects for the variations in intensity of the two excitation wavelengths over the field of view. A tissue background consisted of an image of a muscle fiber whose fluorescence was similar to that of the muscle fiber onto which the imaged terminals made synapses. An image processor (Gould FD 5000, Fremont, CA) automatically subtracted each background and shading correction before displaying an image. Sequential 350 and 385 nm images were stored on an optical disk recorder (Panasonic TQ-2028F, Secaucus, NJ). The image processor, optical disk recorder, filter wheel, and shutter were under the control of a Scientific Microsystems SMS 1000 computer (Mountain View, CA), using software written by Dr. Roger Tsien (Pharmacology Department, University of California at San Diego).

Ratios were acquired using averages of 32 or 64 frames at each excitation wavelength with the SIT camera. *In vivo* calibrations to estimate the 350:385 nm fluorescence ratios at saturated calcium (R_{max}) and at zero-calcium (R_{min}) were done using 50 μM digitonin (obtained from Sigma and dissolved in distilled water) added to normal saline or zero-calcium saline containing 10 mM Na₂EGTA. An R_{min} was hard to achieve, as the intracellular calcium concentration of the presynaptic terminal increased in the application of digitonin. It is possible the calcium stores from muscle were leaking into the external medium, raising the extracellular calcium concentration and leading to an increase in intracellular calcium in the terminals. From experiments in which the preparation was soaked in an EGTA Ringer's for approximately 50 min, we found R_{min} to be about 0.8. It was possible to determine R_{max} using 50 μM digitonin in normal saline. This is a rather high concentration of digitonin (Negulescu and Machen, 1990), but lower concentrations were not effective in raising intracellular calcium concentration. R_{max} was measured as 19. A dissociation constant for fura-2 could not be calculated from these *in vivo* methods, as the possibility that calcium leaks from muscle fibers would raise the external calcium to unknown values and give an inaccurate value. Therefore, the K_D of fura-2 in crayfish axoplasm was estimated at 865 nm by measuring the ratio of fluorescence to excitation at 350 and 385 nm of 50 μM fura-2 mixed in a solution resembling crayfish cytoplasm (250 mM K-D-gluconate, 15 mM NaCl, 15 mM K-HEPES at pH 7.02). The calcium was buffered to 500 nM using 10 mM Na₂EGTA and 5 mM CaCl₂ at pH 7.02, calculated from the measured affinity of EGTA (Gryniewicz et al., 1985). Pairs of ratioed images obtained with 350 and 385 nm excitation were used to calculate intracellular calcium concentration from Equation 5 of Gryniewicz et al. (1985) using the above values for R_{min} , R_{max} , and K_D .

Movement of the preparation due to muscle contraction was often a problem during or following PTP-inducing stimulation, especially in the ouabain-containing solutions. Movement during acquisition of image pairs will give incorrect values for $[Ca^{2+}]_i$. Therefore, in experiments in which high-frequency stimulation was used, the preparation was stabilized by gluing the distal two segments of the shell to the Sylgard surface of the recording dish using a cyanoacrylate glue (Histoacryl Blau, Tri Hawk Industries, Montreal, Canada).

Data analysis. To determine $[Ca^{2+}]_i$ from stored images, circular masks, encompassing 100–300 pixels, were positioned over terminals and the $[Ca^{2+}]_i$ was spatially averaged.

Time constants were calculated for the decline in $[Ca^{2+}]_i$ and EJPs following PTP-inducing trains in both normal and ouabain solutions and tested for statistical significance using a paired *t* test. Similar statistical tests were done on calculated time constants for $[Ca^{2+}]_i$ decay in control versus lithium Ringer's following PTP-inducing trains.

Results

Elevation of $[Na^+]_i$ in the axon and presynaptic terminals elevates $[Ca^{2+}]_i$

It is thought that elevation of sodium ions intracellularly can lead to an increase in $[Ca^{2+}]_i$ (Rahamimoff et al., 1980). There are two ways the sodium ion accumulation could elevate $[Ca^{2+}]_i$: (1) by releasing calcium from internal stores, or (2) by calcium entering from the external medium through reversal or reduced extrusion of Na/Ca exchange. To test these two possibilities, we chose to elevate intracellular sodium ions and see what effect this had on the $[Ca^{2+}]_i$. To elevate the $[Na^+]_i$, three different methods were used. The $[Na^+]_i$ was raised either (1) by injection of sodium ions directly from a microelectrode that had been inserted into the excitatory axon, or (2) by using the drug veratridine, which shifts the activation curve for sodium channels such that they are partially open at resting membrane potentials (Catterall, 1980), or (3) by eliminating sodium extrusion by blocking Na/K exchange with ouabain.

Figure 1 shows an experiment in which sodium ion injection into the excitatory axon led to an increase of $[Ca^{2+}]_i$ in the axon by 210 nM. Intracellular calcium concentration was measured in the excitatory axon 100–150 μ m from the injection electrode. The electrode was placed in the proximal axon, where it is the largest in diameter, to ensure success of multiple penetrations with minimal damage. Since most presynaptic terminals synapse onto muscle fibers more distally from the proximal axon, imaging of these terminals becomes more difficult with a microelectrode inserted into the axon, and injected sodium might not reach distant terminals. Therefore, we imaged $[Ca^{2+}]_i$ near the injection electrode. The result in Figure 1 is typical of seven preparations in normal Ringer's showing a $[Ca^{2+}]_i$ rise in the axon having a mean \pm SE equal to 430 ± 80 nM. Upon perfusion of the preparation with an EGTA Ringer's, containing no added calcium, 2 mM Na_2EGTA , and 30 mM $MgCl_2$, a similar injection of sodium ions (using the same electrode and current) did not increase the $[Ca^{2+}]_i$. Two of the four sodium injection experiments performed in zero-calcium saline were done in a cobalt Ringer's (cobalt substituted for calcium). In some preparations, the $[Ca^{2+}]_i$ increased slightly (11.0 ± 7.0 nM; $N = 4$) in either a cobalt or an EGTA Ringer's, which is most likely due to an inadequate wash in zero-calcium Ringer's. Perfusion with zero-calcium Ringer's was difficult to do satisfactorily, as the electrode inserted in the axon was not always stable throughout a vigorous perfusion. Following the sodium ion injection, the NaCl electrode was gently removed and a microelectrode containing 3 M KCl was inserted into the axon to ensure that the injection of depolarizing current was not admitting calcium ions by opening voltage-dependent calcium channels. A potassium ion in-

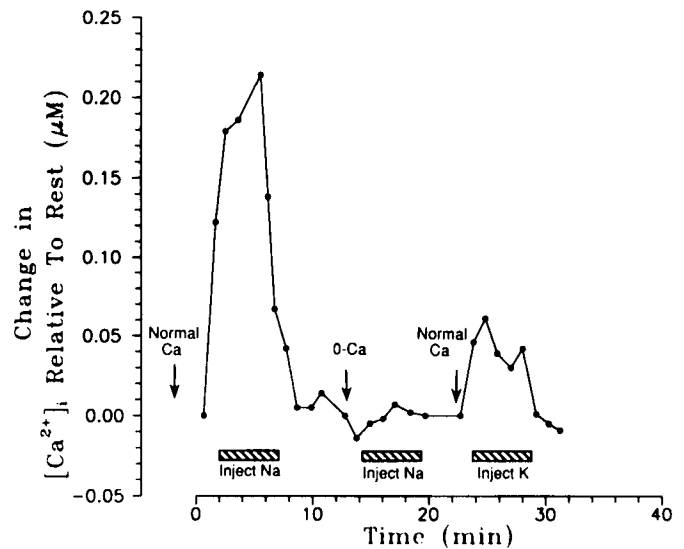


Figure 1. Effects of sodium and potassium injection on $[Ca^{2+}]_i$ in a normal and calcium-free Ringer's. In normal Ringer's, sodium ions were injected into the excitatory axon using +10 nA current with the injection time depicted by the hatched bar. The $[Ca^{2+}]_i$ rose by 210 nM in the axon in response to the increase in $[Na^+]_i$. The $[Ca^{2+}]_i$ returned to preinjection values when the injection current was halted. The preparation was then bathed in a zero-calcium EGTA solution, and sodium ions were again injected using +10 nA current. There was no observed increase in $[Ca^{2+}]_i$. Calcium-containing Ringer's was added, and potassium ions were subsequently injected into the same area of the excitatory axon as the sodium ions, using +10 nA of current. The $[Ca^{2+}]_i$ rose by 40 nM and returned to its resting value when the current was stopped.

jection control was done in all seven experiments. In Figure 1, the injection of potassium ions using +10 nA current elevated $[Ca^{2+}]_i$ by 40 nM (mean \pm SE, 130 ± 30 nM; $N = 7$). In some preparations, the potassium injection did elevate $[Ca^{2+}]_i$, but in all seven preparations the sodium injection elevated $[Ca^{2+}]_i$ to substantially greater values (between 1.5 and 13 times greater effect). These results suggest that sodium ion accumulation in the excitatory axon can elevate $[Ca^{2+}]_i$ in the axon, but only when external calcium is present. Therefore, a localized high concentration of intracellular sodium ions probably increases $[Ca^{2+}]_i$ by reversal of Na/Ca exchange with no indication of any release of calcium from internal stores.

Veratridine, at doses (10–100 μ M) used by previous investigators (Finger and Martin, 1987; Martin and Finger, 1988), is known to depolarize the axonal membrane to levels above that needed to activate voltage-gated calcium channels (Ohta et al., 1973; Wojtowicz and Atwood, 1984). When $[Ca^{2+}]_i$ was measured in the presence of veratridine (10–100 μ M) in normal saline, the $[Ca^{2+}]_i$ increased to 4–10 μ M. In order to test the possibility intracellular sodium ion accumulation via open sodium channels leads to $[Ca^{2+}]_i$ elevation either by release of calcium from internal stores or Na/Ca exchange, lower doses of veratridine had to be used. Veratridine at doses of 0.5–1 μ M has been shown to depolarize crayfish axon approximately 2–6 mV (Ohta et al., 1973). When axonal membrane voltage was measured during application of veratridine at 0.5 μ M, the axon depolarized 4–5 mV and the $[Ca^{2+}]_i$ increased to about 3 μ M. Using high-potassium saline solutions to depolarize the presynaptic membrane uniformly, Delaney et al. (1991) have shown in crayfish that membrane depolarizations of 13–14 mV in the axon result in $[Ca^{2+}]_i$ accumulation between 20 and 40 nM in the presynaptic terminals, with depolarizations around 17–19 mV

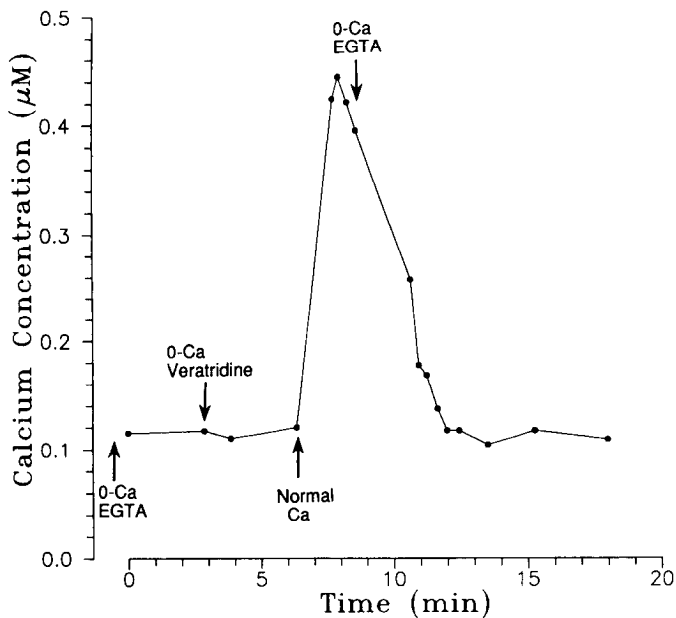


Figure 2. Effects of veratridine on $[Ca^{2+}]_i$ in a calcium-free and normal Ringer's. The preparation was bathed in a zero-calcium EGTA Ringer's prior to veratridine application. Zero-calcium Ringer's containing 0.5 μM veratridine was added at the arrow and produced no measurable change in $[Ca^{2+}]_i$. A calcium-containing Ringer's was added approximately 3 min later, and $[Ca^{2+}]_i$ rose by 320 nM. Washing in zero-calcium EGTA Ringer's brought the $[Ca^{2+}]_i$ back to the resting value.

increasing $[Ca^{2+}]_i$ sharply. Therefore, at low doses of veratridine, membrane depolarizations of 4–5 mV are probably not sufficient to activate enough voltage-gated calcium channels to lead to measurable $[Ca^{2+}]_i$ accumulation.

Figure 2 shows the effect of 0.5 μM veratridine in an EGTA Ringer's followed by a rinse in normal saline that contained calcium. Veratridine in a zero-calcium external medium did not increase $[Ca^{2+}]_i$ in four preparations, and in three preparations the $[Ca^{2+}]_i$ increased slightly, accounting for the variation recorded in the means (30 ± 10 nM; 14 terminals in seven preparations, designated $N = 14, 7$). As mentioned previously, effective perfusion in zero-calcium saline was not achieved in all experiments, even though the preparation was soaked in EGTA Ringer's for 40–60 min. Veratridine does, however, increase $[Ca^{2+}]_i$ when calcium was present in the external medium. As shown in Figure 2, $[Ca^{2+}]_i$ increased to 440 nM from a resting value of 120 nM, showing a 320 nM increase in $[Ca^{2+}]_i$, with a mean increase of 400 ± 30 nM, $N = 14, 7$.

To test whether calcium ions were going through sodium channels activated by veratridine, we measured $[Ca^{2+}]_i$ in a saline solution in which choline was substituted for sodium. The $[Ca^{2+}]_i$ did not increase significantly in the veratridine mixture containing no sodium ions (15.0 ± 6.0 nM; $N = 8, 5$), but when sodium ions were added, the $[Ca^{2+}]_i$ increased to 4.8 ± 1.2 μM ($N = 8, 5$). In these experiments, veratridine concentrations of 1.0–100 μM were used. These results suggest that elevation of the $[Na^+]_i$ by means of veratridine application at 0.5–1.0 μM , increases $[Ca^{2+}]_i$ most likely through an effect on Na/Ca exchange.

Ouabain, at a dose of 0.5 mM, increased the resting $[Ca^{2+}]_i$ in presynaptic terminals by 180 ± 30 nM ($N = 12, 8$), with a 30–40 min delay between ouabain application and the increase in $[Ca^{2+}]_i$. This delay in ouabain's action has previously been

reported in reference to transmitter release at the frog NMJ (Baker and Crawford, 1975), and it was suggested this reflected the slow binding time of ouabain to the Na/K exchanger, as Baker and Crawford reported no delay in the increased frequency of miniature endplate potentials (MEPPs) when a potassium-free medium was used to block Na/K exchange. However, if low-frequency stimulation was given in the presence of ouabain, this 30–40 min delay could be shortened slightly, presumably by increasing the sodium entry, as also observed by Baker and Crawford (1975). K. R. Delaney and D. W. Tank (unpublished observations) measured $[Na^+]_i$ in crayfish presynaptic terminals using the ratiometric fluorescent dye SBFI in the presence of 5 mM ouabain. The absolute value for $[Na^+]_i$ in crayfish presynaptic terminals is not known from the SBFI (sodium-binding benzofuran isophthalate) data, because reliable calibration data were not obtained. They showed $[Na^+]_i$ accumulation occurred within minutes of ouabain application, but $[Ca^{2+}]_i$ accumulation took 30–40 min to occur, suggesting that this 30–40 min delay in calcium rise was not due to binding of ouabain to the pump but the time it took for the sodium accumulation to lead to $[Ca^{2+}]_i$ accumulation.

Ouabain (0.5 mM) enhanced EJPs by 8.2 ± 2.7 times (eight preparations) when stimulating at frequencies of 1–10 Hz. Since ouabain increased EJP amplitude more than expected for a 180 nM increase in $[Ca^{2+}]_i$ (Delaney et al., 1989), we looked for an effect of ouabain on calcium influx during action potentials. Figure 3 shows the resting $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ during 5 Hz stimulation, and the accompanying EJPs (consisting of the mean of 20 EJP amplitudes at 5 Hz) in a preparation bathed in normal saline, followed by the addition of ouabain and finally the wash-out of the drug. Ouabain increased the resting level of calcium in this preparation by 300 nM (Fig. 3) along with a 15-fold enhancement in EJP amplitude. There was a very slight increase in calcium accumulation that occurred during a 5 Hz stimulation in ouabain (20–50 nM), but this was probably not large enough to account for a 15-fold enhancement in EJP amplitude.

Ouabain (0.5–1 mM) has been reported to depolarize crayfish axons from 5 to 10 mV and to reduce the action potential amplitude by 16 mV (Wojtowicz and Atwood, 1985). In one experiment, we left the fura-injecting electrode in the presynaptic axon to measure the effect of 0.5 mM ouabain on presynaptic potential. The axon depolarized 7 mV and increased the resting $[Ca^{2+}]_i$ by 120 ± 10 nM ($N = 2, 1$). The action potential peak decreased by 4.2 mV, probably due to sodium ion accumulation, and the duration, measured at the base of the action potential, increased by 1.3 msec. As mentioned previously, depolarization of 13–14 mV in this preparation leads to $[Ca^{2+}]_i$ accumulation between 20 and 40 nM in presynaptic terminals (Delaney et al., 1991). Therefore, the large increase in $[Ca^{2+}]_i$ that occurs in presynaptic terminals in the presence of ouabain is most likely due to an effect on Na/Ca exchange and not calcium influx through voltage-gated calcium channels, as a 7 mV depolarization does not lead to detectable calcium accumulation (Delaney et al., 1991). It might be due to internal sodium accumulation releasing calcium from intracellular stores, but we measured the effect of ouabain (0.5 mM) in an EGTA Ringer's in five preparations and found that the resting $[Ca^{2+}]_i$ did not change for the time it was measured (up to 100 min; 3 ± 6 nM; $N = 8, 5$). To be confident that the ouabain was blocking the Na/K pump in these zero-calcium experiments, we stopped the perfusion and watched the $[Ca^{2+}]_i$ slowly increase by 90 ± 20 nM ($N = 7, 4$) in 3–5 min and by 240 nM when perfusion was stopped for 30 min (one preparation). When perfusion with

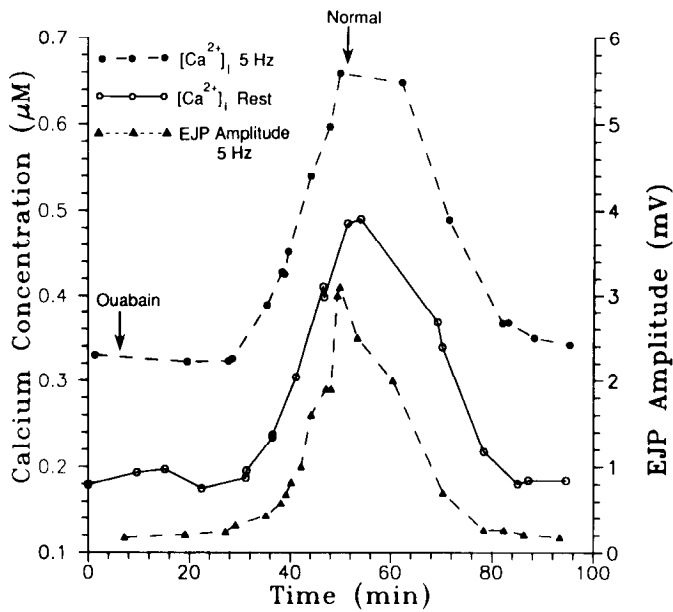


Figure 3. The effects of ouabain on $[Ca^{2+}]_i$ and transmitter release. Ouabain (0.5 mM), introduced at the arrow, increased the resting $[Ca^{2+}]_i$ (open circles) by about 300 nM, and the EJPs evoked by 5 Hz stimulation were increased 15-fold (triangles). The increase in $[Ca^{2+}]_i$ during 5 Hz stimulation in ouabain (solid circles) was 20–50 nM greater than the increase in $[Ca^{2+}]_i$ during stimulation without ouabain. This indicates that the calcium influx was increased slightly by ouabain application. Removal of ouabain by washing in normal Ringer's at the second arrow brought $[Ca^{2+}]_i$ and EJP amplitude back to preouabain values.

EGTA Ringer's was resumed, the $[Ca^{2+}]_i$ returned to the control resting values. A ouabain application in a zero-sodium choline Ringer's (one preparation) was ineffective in elevating $[Ca^{2+}]_i$, indicating that ouabain's effect on intracellular calcium was sodium dependent.

Elevation of $[Na^+]_i$ by tetanic stimulation does not elevate $[Ca^{2+}]_i$

To test what effect a physiological sodium load had on $[Ca^{2+}]_i$, we stimulated the excitatory axon at high frequencies (ranging from 20 to 50 Hz) for 5–20 min in an EGTA Ringer's while monitoring the nerve field potential with an extracellular electrode placed on a distal axonal branch to ensure action potential invasion. As shown in Figure 4, a 33 Hz, 10 min train had no effect on $[Ca^{2+}]_i$, as in three other preparations (-8 ± 7 nM; $N = 8, 4$), whereas one preparation showed a slight elevation, probably due to an insufficient wash in EGTA Ringer's (60 ± 4 nM; $N = 4, 1$). Following the tetanus, the bathing solution was quickly changed to one containing normal calcium to see if the physiological sodium load could elevate $[Ca^{2+}]_i$ through reversal of the Na/Ca exchange once external calcium was available. There was no $[Ca^{2+}]_i$ accumulation measured in all five preparations (-2 ± 5 nM; $N = 12, 5$).

To ensure that the external calcium concentration near the imaged boutons was equal to normal within 1 min following the tetanic train, two things were done. First, before starting the tetanic stimulation, surface terminals were selected for imaging, so solution changes would reach them quickly. Second, a stimulation, for example, 10 Hz for 20 sec, was initially delivered in normal Ringer's, and then EGTA Ringer's was washed in and the nerve was stimulated again; immediately afterward normal Ringer's was washed in and the nerve was stimulated a

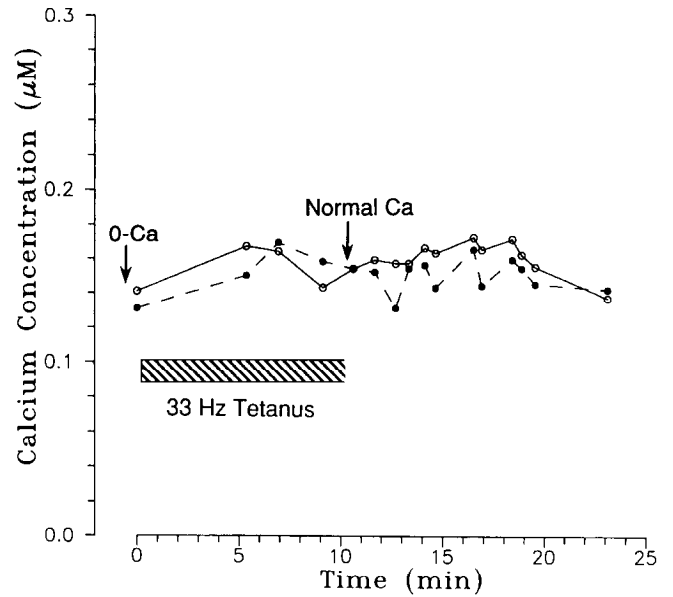


Figure 4. Sodium accumulation during high-frequency stimulation in zero-calcium Ringer's does not elevate $[Ca^{2+}]_i$. In this preparation, $[Ca^{2+}]_i$ was measured in two terminals, depicted by the solid and broken lines. The preparation was perfused in zero-calcium EGTA Ringer's prior to stimulation. A 10 min, 33 Hz tetanus was delivered, depicted by the bar, and the $[Ca^{2+}]_i$ was measured during the train. An extracellular electrode was positioned on a distal axonal branch to ensure invasion of the action potential. There was no change in the $[Ca^{2+}]_i$ during the stimulation. Immediately following the tetanus, normal-calcium Ringer's was added, shown by the arrow, and there was no measurable change in the $[Ca^{2+}]_i$ in either terminal.

third time 1–2 min later. In all cases the $[Ca^{2+}]_i$ accumulation, and therefore the external calcium concentration, had reached the initial control value of the first train shortly after replacement of the EGTA Ringer's and stimulation by the third train. Apparently, sodium accumulation that occurs during a tetanus neither releases calcium from internal stores nor leads to $[Ca^{2+}]_i$ accumulation due to reversal of the Na/Ca exchange.

Two points must be considered before concluding that the Na/Ca exchange did not reverse, admitting calcium from the outside. During the high-frequency stimulation in zero external calcium, little if any calcium influx occurred through calcium channels; therefore, calcium buffers and removal systems remained unsaturated. Under these conditions, perhaps some calcium influx did occur via reversal of Na/Ca exchange once external calcium was replaced, but removal was rapid and did not lead to detectable calcium accumulation. Second, Delaney and Tank (unpublished observations), using the ratiometric fluorescent dye SBFI, measured relative $[Na^+]_i$ during high-frequency stimulation (30 Hz, 7 min) in zero-calcium, 30 mM Mg^{2+} solution and showed that sodium did reach the same concentration as during stimulation in normal external solution. About 1 min after stimulation had ceased, $[Na^+]_i$ fell to approximately two-thirds of its peak value reached during stimulation. $[Na^+]_i$ values may have dropped below that necessary to reverse Na/Ca exchange during the 1 min interval needed to replace external calcium. Therefore, under conditions of high-frequency stimulation in normal external solution, it remains possible that sodium accumulation reverses Na/Ca exchange and admits some calcium from the outside.

Another way to approach the effect of intracellular sodium would be to load terminals tetanically with sodium while phar-

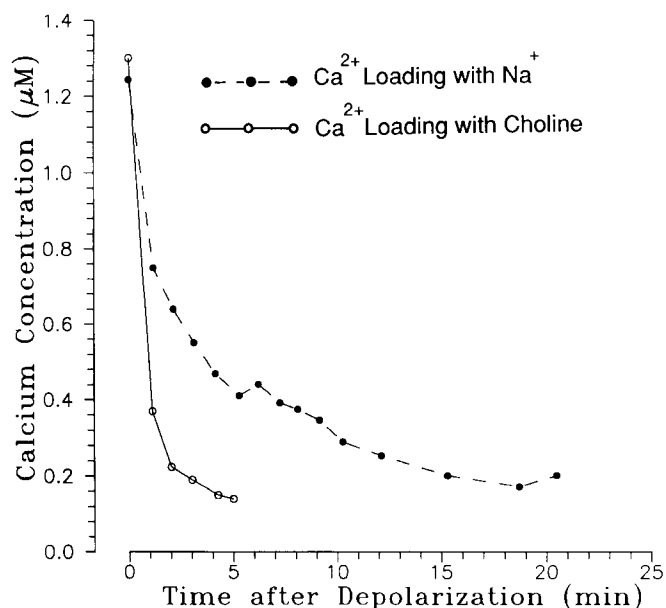


Figure 5. Loading presynaptic terminals with sodium slows the extrusion of elevated $[Ca^{2+}]_i$. Presynaptic terminals were depolarized with high potassium (40 mM) to admit both sodium and calcium ions. Once $[Ca^{2+}]_i$ had reached a criterion value (1.3 μM), normal-potassium Ringer's was added and the decay of $[Ca^{2+}]_i$ was monitored (broken line). Elevated $[Ca^{2+}]_i$, under conditions of sodium loading, took approximately 18.5 min to return to the resting value. Depolarization of the terminals was repeated with a high-potassium (40 mM) choline Ringer's, where choline was substituted for sodium. Normal Ringer's was added when the $[Ca^{2+}]_i$ had reached the same criterion level as in the Na/Ca loading trial. The decline of $[Ca^{2+}]_i$ (solid line) was faster under conditions of no sodium load, taking about 4 min to return to the resting value.

macologically blocking calcium influx through calcium channels, and look for an effect on $[Ca^{2+}]_i$. However, ionic blockers of calcium channels also interfere with Na/Ca exchange (Baker and Dipolo, 1984), and we found that other calcium channel blockers (ω -conotoxin, dihydropyridines) were ineffective against calcium channels in these terminals.

Elimination of sodium loading speeds the extrusion of elevated $[Ca^{2+}]_i$

During a normal PTP-inducing train, presynaptic terminals would be loaded with both sodium and calcium ions. It is possible a sodium load could slow the calcium removal process by reducing the sodium gradient that drives the calcium extrusion via Na/Ca exchange. Since a physiological load of intracellular sodium ions does not lead to $[Ca^{2+}]_i$ accumulation (Fig. 4), we chose to test whether loading with both sodium and calcium would affect the rate of calcium extrusion. Preparations were depolarized with high potassium (40 mM) to admit both sodium and calcium ions through voltage-dependent channels. The $[Ca^{2+}]_i$ was monitored during the depolarization and the high potassium was washed out when an arbitrary criterion value for $[Ca^{2+}]_i$ was reached. The decay of $[Ca^{2+}]_i$ was measured while washing in normal saline. This decay time provides a measurement for calcium extrusion from the presynaptic terminal in the presence of elevated $[Na^+]_i$. Preparations were then bathed in high potassium with choline substituted for sodium, while $[Ca^{2+}]_i$ was monitored. Saline containing normal potassium and sodium was added when the $[Ca^{2+}]_i$ had reached the same criterion level as in the sodium/calcium loading trial, and the decline in

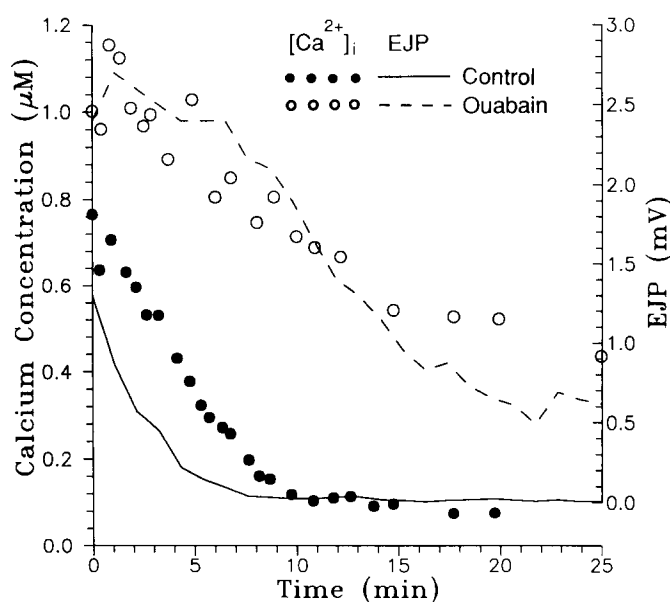


Figure 6. Increased sodium loading during a tetanus prolongs the posttetanic decay of $[Ca^{2+}]_i$ and evoked release. A 33 Hz, 5 min train was delivered in normal Ringer's and the posttetanic decay of EJP amplitude (solid line) and $[Ca^{2+}]_i$ (solid circles) were measured. The time constant for posttetanic decay was calculated as 4 min for EJPs and about 6 min for $[Ca^{2+}]_i$. Ouabain (0.5 mM) was added, and an identical tetanic stimulation was delivered in the same preparation. Tetanic stimulation in ouabain enhanced and prolonged EJP amplitude and $[Ca^{2+}]_i$ during following the tetanus, with the time constant for EJP posttetanic decay (broken line) calculated as 15.5 min and the decay time constant for $[Ca^{2+}]_i$ calculated as 22 min (open circles).

$[Ca^{2+}]_i$ was followed. We found (Fig. 5) that calcium extrusion was slower in the presence of elevated intracellular sodium (removal time constant of 12.3 ± 1.8 min; $N = 8, 7$) compared to the removal of $[Ca^{2+}]_i$ without an intracellular sodium load (time constant of 4.0 ± 0.4 min; $N = 8, 7$). We conclude that a sodium load interferes with the extrusion of calcium, probably by reducing the sodium gradient, which in turn slows the Na/Ca exchange rate. Because action potentials are blocked in choline Ringer's, we could not record transmitter release in these experiments.

Increased sodium loading during a tetanus prolongs the posttetanic decay of $[Ca^{2+}]_i$ and evoked release

Ouabain was used to increase the sodium loading during tetanic stimulation to test the possibility that PTP would be prolonged under these conditions. In the same preparation, posttetanic decay of EJPs and $[Ca^{2+}]_i$ was measured following a 33 Hz, 5 min stimulation in normal Ringer's and one that contained 0.5 mM ouabain. Ouabain was added for 40 min prior to the high-frequency stimulation trial, and EJPs and resting $[Ca^{2+}]_i$ were measured to ensure both had increased (a known effect of ouabain, as mentioned previously; e.g., Fig. 3). A saline solution, with no added calcium, was washed over the preparation to reduce the ouabain-enhanced EJP to an amplitude close to that of EJPs in normal Ringer's. The resting $[Ca^{2+}]_i$ also decreased with the addition of the low-calcium saline. Figure 6 shows the decay of $[Ca^{2+}]_i$ and EJP amplitude following a 33 Hz, 5 min train in normal and ouabain Ringer's. In this experiment the time constant for decay of the EJP amplitude was 4 min in normal (4.4 ± 0.9 min; $N = 5$) versus 15.5 min in ouabain (17.3 ± 3.8 min; $N = 5$). In this preparation, the $[Ca^{2+}]_i$ decayed with

a time constant of 5.7 min (5.8 ± 3.6 min; $N = 6, 5$) versus 22 min (35 ± 7.3 min; $N = 6, 5$) in normal and ouabain solutions, respectively. In the five preparations in which paired data were collected, the posttetanic decay time constant for $[Ca^{2+}]_i$ in ouabain was significantly slower than normal ($p < 0.01$), along with a significantly slower EJP decay time constant ($p < 0.02$). From these results, it appears sodium loading augments and prolongs PTP by slowing the extrusion of calcium by Na/Ca exchange.

During tetanic stimulation in ouabain, the $[Ca^{2+}]_i$ reached higher values than during the control stimulation, but accurate measurements were hard to obtain as muscle contraction caused movement artifacts during fura-2 image acquisition. The EJPs became very large (i.e., 15–20 mV) initially and in some cases became smaller later in the stimulation, perhaps due to depression. Upon completion of the train, the averaged EJP at 1 Hz (in three preparations) grew for approximately 2 or 3 min and then decayed.

The PTP decay time constant for EJPs was faster than that of $[Ca^{2+}]_i$ for ouabain solutions (17.3 vs 35 min), but the time constants were similar in normal solution (Delaney et al., 1989). A disappearance of EJPs occurred in some preparations following the PTP-inducing train in ouabain, probably due to ouabain causing such an increase in presynaptic $[Na^+]_i$ that the action potential was blocked. Balnave and Gage (1974) reported a similar disappearance of endplate potentials (EPPs) at the toad NMJ following 120 min exposure to ouabain. A gradual block of the action potential following a train could explain the faster decay of the EJP compared to the decay of $[Ca^{2+}]_i$ in ouabain.

Blocking the Na/Ca exchange following a tetanus prolongs the posttetanic decay of $[Ca^{2+}]_i$

Lithium substitution for extracellular sodium is a satisfactory way to inhibit calcium efflux through Na/Ca exchange, as lithium ions cannot replace sodium in the exchanger (Hermoni et al., 1987). A high-frequency stimulation, 33 Hz for 5 min, was delivered in normal Ringer's and the $[Ca^{2+}]_i$ decay following the train was measured. An identical PTP-inducing train was delivered, after $[Ca^{2+}]_i$ had returned to rest from the first trial, and lithium Ringer's (lithium substituted for sodium) was washed in immediately after the stimulation ceased. Both PTP-inducing trains were delivered in normal Ringer's, with lithium Ringer's washed in only after stimulation ended, to avoid loading the terminal with lithium, as lithium is known to have effects on intracellular second messenger systems (Berridge and Irvine, 1989).

Figure 7 shows the posttetanic decay of $[Ca^{2+}]_i$ in normal and lithium Ringer's. The time constant of decay in normal Ringer's is equal to 6.3 min, compared to 15 min for lithium Ringer's. Normal and lithium posttetanic decay of $[Ca^{2+}]_i$ were measured in seven preparations and 13 terminals, with a mean of 6.5 ± 1.4 min for a control decay time constant and 15.7 ± 3.9 min for a decay time constant in lithium. The time constant for $[Ca^{2+}]_i$ decay in lithium was significantly slower ($p < 0.05$).

EJPs were measured following stimulation in normal and lithium Ringer's, but lithium is known to have a depressant effect on the postsynaptic potential at the crayfish NMJ (Ortiz and Junge, 1978). This depressant effect was observed in these experiments, and therefore measurement of EJP posttetanic decay was not possible.

Upon addition of lithium Ringer's in four out of seven preparations, the $[Ca^{2+}]_i$ jumped by 450 ± 60 nM ($N = 9, 4$) for the first 30 sec to 1 min and then decayed, as shown in Figure 7.

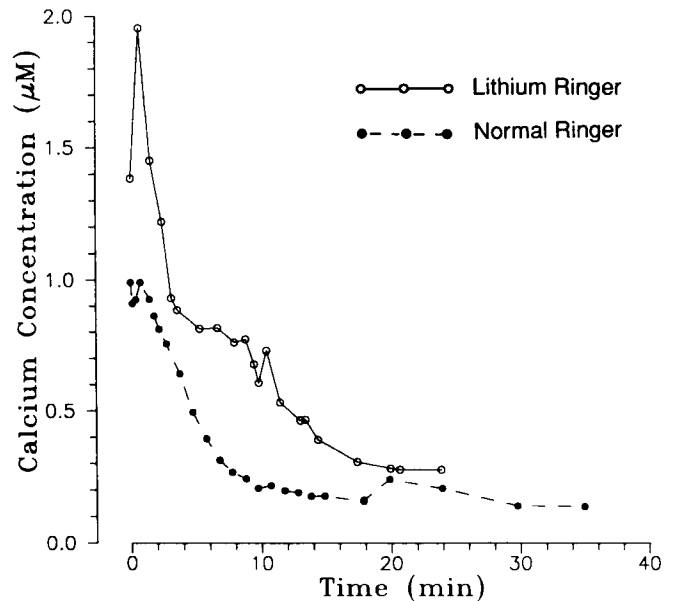


Figure 7. Blocking the Na/Ca exchange following a tetanus with lithium prolongs the posttetanic decay of $[Ca^{2+}]_i$. Tetanic stimulation, 33 Hz for 5 min, was delivered in normal Ringer's and the decay of $[Ca^{2+}]_i$ (broken line) was measured following the train. The time constant for decay of $[Ca^{2+}]_i$ in normal Ringer's was equal to about 6 min. In the same preparation, a second train was delivered in normal Ringer's. Following this tetanus, lithium Ringer's (in which lithium replaced sodium) was added and the $[Ca^{2+}]_i$ decline was monitored (solid line). The time constant for $[Ca^{2+}]_i$ decay was calculated to be 15 min.

This sudden rise in $[Ca^{2+}]_i$ may be due to a reversal in the Na/Ca exchange because of low external sodium and the high internal sodium. This sudden increase in intracellular calcium was also observed in one preparation, five terminals (300 ± 110 nM) upon washing in choline Ringer's following a PTP-inducing train. In contrast, experiments using a zero-sodium Ringer's without sodium loading showed little increase in $[Ca^{2+}]_i$ for up to 30 min (20 ± 5 nM; $N = 23, 12$). We conclude that when calcium efflux through Na/Ca exchange was inhibited by using lithium, the jump in $[Ca^{2+}]_i$ seen once high-frequency stimulation had ceased was due to the reversal of the exchanger. This reversal is only detected when $[Na^+]_i$ has been elevated, followed by removal of external sodium. In addition, the posttetanic decay of $[Ca^{2+}]_i$ was slowed by inhibiting calcium efflux through Na/Ca exchange with lithium.

Complications in using nonspecific Na/Ca exchange blockers

Blocking the Na/Ca exchange during tetanic and posttetanic phases was attempted using the amiloride derivative 2',4'-dimethylbenzamil (DMB), which has been used to block Na/Ca exchange in smooth muscle cells (Smith et al., 1987). DMB was the generous gift of Merck, Sharp & Dohme. Unfortunately, DMB partitioned into the cell membrane and fluoresced intensely, interfering somewhat with the fura-2 signal. However, because we had intracellularly injected fura-2, our images were bright and we were able to overcome this problem. At the high doses (100 μ M) reported to be effective in blocking Na/Ca exchange (Siegl et al., 1984), DMB appeared to cause photodamage, even from a single image. When DMB was used without imaging calcium, and only EJPs were recorded, it appeared to block sodium channels in the axon, as the spike failed. DMB was used at a lower dose (20 μ M) and did not cause photodamage

during fura-2 imaging, but led to a 10-fold increase in EJP amplitude without an increase in resting [Ca²⁺]_i. Due to the complications that arose from using DMB, we tried amiloride, at a dose (2 mM) reported to block the Na/Ca exchange (Kleyman and Cragoe, 1988). At this dose, we found that amiloride completely quenched the fura-2 signal. Therefore, we chose to record only EJPs without calcium imaging and found the amiloride decreased the EJP amplitude. Amiloride has been reported to block T-type calcium channels in mouse neuroblastoma and chick dorsal root ganglion neurons at a lower dose (500 μM) than needed to block the Na/Ca exchange (Tang et al., 1988), and this could account for the decrease we saw in EJP amplitude. Thus, amiloride derivatives did not prove useful as inhibitors of Na/Ca exchange.

Discussion

Reversal of the Na/Ca exchange elevates [Ca²⁺]_i when internal sodium is raised by pharmacological methods

Experimental methods that led to the elevation of internal sodium ions, that is, injection of intracellular sodium and exposure to ouabain and veratridine, increased [Ca²⁺]_i, but only if calcium ions were present in the external medium, suggesting that it was operating via Na/Ca exchange. All three pharmacological methods have previously been used at the crayfish NMJ and other synaptic preparations. First, the injection of sodium ions intracellularly into the crayfish axon (as reported by Wojtowicz and Atwood, 1985) produced an increase in transmitter release that was long lasting in one preparation, compared to a transient increase obtained by intracellular injection of potassium ions. These experiments are in agreement with our findings that show a significantly higher elevation of [Ca²⁺]_i during sodium injection compared to potassium injection in the crayfish axon, assuming the increase in transmitter release observed by Wojtowicz and Atwood (1985) was due to [Ca²⁺]_i elevation in the presynaptic terminals.

Second, previous experimenters have used ouabain in various synaptic preparations and found that ouabain enhances transmitter release. At the crayfish NMJ, ouabain prolonged PTP (Atwood et al., 1975) and greatly enhanced EJP amplitude (Wojtowicz and Atwood, 1985). Prolongation of PTP was also obtained at the frog (Rahamimoff et al., 1978; Lev-Tov and Rahamimoff, 1980; Misler and Hurlbut, 1983; Misler et al., 1987) and rat NMJ (Nussinovitch and Rahamimoff, 1988). Ouabain experiments performed in zero-calcium solutions to determine mechanism of sodium elevation were done at the mammalian and frog NMJ. In agreement with our experiments were those of Elmqvist and Feldman (1965) and Vizi and Vyskocil (1979) at the mammalian NMJ, where the preparation was exposed to ouabain in a zero-calcium solution, and transmitter release, measured as frequency of MEPPs, remained unchanged compared to an increase in release when external calcium was present. It is assumed that the frequency of MEPPs reflects the resting [Ca²⁺]_i. In contrast, Baker and Crawford (1975) at the frog NMJ and Nussinovitch and Rahamimoff (1988) at the mammalian NMJ have shown that the frequency of MEPPs increased in the presence of ouabain in a zero-calcium EGTA solution and posttetanic decay of MEPP frequency was also increased. This discrepancy may be due to a difference between intracellular calcium stores at the crayfish versus the mammalian and frog NMJ. More likely, it can be attributed to an ineffective zero-calcium EGTA perfusion. In our experiments, we

found ouabain to be quite effective in raising [Ca²⁺]_i in a zero-calcium EGTA solution when our continuous perfusion was halted. The fact we could achieve a positive result in both a ouabain or a sodium injection experiment in zero-calcium medium if the EGTA rinse was not done vigorously suggests that previous experiments showed an increased frequency of MEPPs in a zero-calcium ouabain-containing medium only because calcium was still present in the extracellular space surrounding nerve terminals, where it is effectively buffered to intermediate levels (Ginsburg and Rahamimoff, 1983).

Third, experimental studies using veratridine at the crayfish NMJ conflict with our findings. We found, using the drug veratridine, that the elevation of intracellular sodium ions proved to be dependent on external calcium ions in order to raise [Ca²⁺]_i, whereas Finger and Martin (1987) and Martin and Finger (1988), using high doses of veratridine (100 μM), reported an increase in spontaneous transmitter release at the crayfish NMJ in a zero-calcium medium. These investigators used no chelating agent in their zero-calcium saline, and, as it is virtually impossible to eliminate external calcium under these conditions (Mulkey and Zucker, 1991a), it is likely that intracellular calcium increased simply due to influx through voltage-gated calcium channels, known to be open at these high, depolarizing doses of veratridine (Ohta et al., 1973; Delaney et al., 1991).

Posttetanic potentiation is dependent on calcium influx from the external media

Previous experimenters have suggested that PTP is independent of calcium entry from the external medium. Nussinovitch and Rahamimoff (1988) in the mammal; Lev-Tov and Rahamimoff (1980), Misler and Hurlbut (1983), and Misler et al. (1987) in the frog; and Swenarchuk and Atwood (1975) in the crayfish showed that stimulation in a calcium-free medium produced some PTP after normal calcium was restored. Stimulation at high frequencies, like that needed to induce PTP, in a nominally calcium-free Ringer's can lead to calcium entry and even transmitter release, as shown recently at the crayfish NMJ (Mulkey and Zucker, 1991a). Therefore, it is difficult to ensure that calcium influx did not occur during stimulation in a calcium-free solution unless direct measurements of [Ca²⁺]_i can be made. Experiments by Wojtowicz and Atwood (1988) in the crayfish showed that PTP (or the tetanic phase of LTF, as they refer to it) does not occur when high-frequency stimulation is given in solutions effective in blocking calcium influx. Since PTP is not observed under these conditions (Wojtowicz and Atwood, 1988), this suggests that sodium alone has no direct effect on transmitter release. However, since the crayfish NMJ is extremely difficult to wash free of calcium, the return of normal external calcium to all synaptic terminals (beneath muscle fibers) is also slow. This time lapse may therefore prevent the observation of a calcium-independent component to PTP. We do know, however, from fura-2 intracellular calcium measurements that trains in calcium-blocking solutions do not lead to accumulation of [Ca²⁺]_i during or following the train, once external calcium has been restored, ruling out the possibility of calcium release from internal stores (Fig. 4). In support of this result, Connor et al. (1986) used arsenazo III to measure [Ca²⁺]_i during PTP in L10 cells of *Aplysia*, while bathing the preparation in either 4 mM cadmium or zero-calcium, 4 mM EGTA. They also observed no increase in [Ca²⁺]_i during tetanic stimulation, but found that the EGTA solution required many washings before they recorded no change.

Reversal of Na/Ca exchange under physiological conditions occurs only when internal sodium is high and external sodium is low

It has been suggested PTP may be due to a reversed Na/Ca exchange that takes place during and following tetanic stimulation (Misler and Hurlbut, 1983; Misler et al., 1987). The equation for the reversal potential for a 3:1 Na/Ca exchanger is $3E_{Na} - 2E_{Ca} = E_{Na/Ca}$. At resting conditions with a membrane potential of -70 mV, external sodium at 200 mM, internal sodium at 17 mM (Wallin, 1967), and external calcium at 13.5 mM, Na/Ca exchange should transport calcium inward as long as internal calcium is less than 500 nM. The fact that resting calcium is less than 500 nM (Mulkey and Zucker, 1991a) indicates that some other process keeps intracellular calcium low at rest, perhaps the Ca^{2+} pump, known to have a higher affinity for calcium than the Na/Ca exchanger (Blaustein, 1988). Sodium loading by electrical stimulation should also increase the inward flow of calcium via Na/Ca exchange. If $[Ca^{2+}]_i$ rises to 1 μ M and $[Na^+]_i$ goes above 21 mM, the Na/Ca exchange should transport calcium inward. It is known that action potential amplitude decreases during high-frequency stimulation, suggesting a significant accumulation of sodium occurring during repetitive stimulation (Wojtowicz and Atwood, 1985). $[Na^+]_i$ probably reaches values above 21 mM during a PTP-inducing train, since resting values have been estimated to be around 17 mM (Wallin, 1967). However, our evidence suggests that a net calcium influx, presumably due to a Na/Ca exchange, is seen under physiological conditions of sodium loading *only* when sodium outside is low and intracellular sodium is elevated (Fig. 7). This is in agreement with work done in squid axon (Dipolo, 1979; Baker and Dipolo, 1984; Mullins et al., 1985), synaptosomes (Blaustein and Oborn, 1975), and vascular smooth muscle (Bova et al., 1990), where in all cases, calcium influx occurred only when external sodium was low.

We have looked for $[Ca^{2+}]_i$ accumulation due to reversal of Na/Ca exchange under two other conditions: (1) by loading sodium with tetanic stimulation in zero-calcium normal-sodium Ringer's and then restoring external calcium, and (2) in a zero-sodium bathing solution when internal sodium is low. Under neither of these conditions was $[Ca^{2+}]_i$ accumulation detected. As mentioned previously in the results, the first experiment, high-frequency stimulation in zero external calcium, may not detect $[Ca^{2+}]_i$ accumulation because of the time needed to replace external calcium once the stimulation has ceased. In this 1 min interval, $[Na^+]_i$ had dropped to approximately two-thirds of its peak value reached during the high-frequency stimulation (Delaney and Tank, unpublished observations). Second, it is possible we could not record $[Ca^{2+}]_i$ accumulation because of the action of calcium buffers and removal systems. An example of the effect of cell buffers on the detection of $[Ca^{2+}]_i$ accumulation was reported in the squid axon by Swandulla et al. (1991). When the calcium buffer EGTA was injected into the presynaptic terminal, transmitter release was not affected but changes in $[Ca^{2+}]_i$ accumulation, measured using fura-2, were eliminated. In contrast, under normal conditions, $[Ca^{2+}]_i$ reached values of 1 μ M. Therefore, conditions in which intracellular buffers are not saturated may affect the $[Ca]_i$ accumulation that is measured by the fura-2. In the second group of experiments, there was no increase in $[Ca^{2+}]_i$ detected when $[Ca^{2+}]_i$ and $[Na^+]_i$ were at resting values and external sodium was removed. Similar to the high-frequency stimulation experiment, $[Ca^{2+}]_i$ remained at rest

and buffers and removal systems were unsaturated, which may explain why calcium accumulation was not measured under these conditions. Therefore, calcium influx through Na/Ca exchange under physiological conditions is detected using fura-2 measurements only when external sodium is low *and* internal sodium and calcium are elevated.

Pharmacological methods used to elevate sodium (ouabain, veratridine, and sodium injection) appeared quite successful in reversing the Na/Ca exchange under conditions when $[Ca^{2+}]_i$ was at rest. This discrepancy between sodium loading by pharmacological methods and physiological methods may be the result of $[Na^+]_i$ not reaching as high a value in the physiological condition. Another possibility is that perhaps ouabain and veratridine had some nonspecific effect on the calcium pump or buffering systems such that a detectable rise in $[Ca^{2+}]_i$ was really the result of an inhibition of the calcium removal systems.

Intraterminal sodium accumulation enhances and prolongs posttetanic potentiation

We have shown that an internal sodium load slows calcium removal from the presynaptic terminal, in addition to prolonging PTP. This is evidence that sodium ions contribute to the gradual decline in $[Ca^{2+}]_i$ following tetanic stimulation. Blocking calcium efflux via Na/Ca exchange (by reducing the external sodium concentration with lithium substitution) prolongs the decay of $[Ca^{2+}]_i$, and is additional support that Na/Ca exchange may play an important role in PTP. This evidence, though it is indirect, suggests that PTP is the result of an intracellular calcium and sodium load, and that the subsequent decay of $[Ca^{2+}]_i$ is dependent on the Na/Ca exchange rate. Delaney and Tank (unpublished observations) have measured $[Na^+]_i$ in crayfish presynaptic terminals using the fluorescent dye SBFI, and have shown that following a PTP train (33 Hz, 7 min), $[Na^+]_i$ decays with a time constant of around 3 min. This is similar to the time constant of $[Ca^{2+}]_i$ decline following a PTP train (i.e., 5.8 min reported in Results). Since we know that sodium accumulation that occurs as a result of high-frequency stimulation stays elevated for minutes following the train, it may therefore be partly responsible for the time constant of decay for $[Ca^{2+}]_i$ following a PTP-inducing stimulus. Additional support for PTP being sodium dependent is given by experiments in crayfish in which sodium influx is blocked by TTX and trains of depolarizing pulses equivalent to action potentials in releasing transmitter were delivered through an intracellular electrode. These experiments revealed reduced PTP under conditions where sodium accumulation was prevented (Wojtowicz and Atwood, 1988).

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