Calcium Buffering in Presynaptic Nerve Terminals

II. Kinetic Properties of the Nonmitochondrial Ca Sequestration Mechanism

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ABSTRACT The kinetic properties of the nonmitochondrial ATP-dependent Ca sequestering mechanism in disrupted nerve terminal (synaptosome) preparations have been investigated with radioactive tracer techniques; all solutions contained DNP, NaN_3, and oligomycin, to block mitochondrial Ca uptake. The apparent half-saturation constant, K_{Ca}, for the nonmitochondrial Ca uptake is ~0.4 μM Ca; the Hill coefficient is ~1.6. Mg is also required for the Ca uptake, and the apparent K_{Mg} is ~80 μM. ATP and deoxy-ATP, but not CTP, GTP, ITP, UTP, ADP, or cyclic AMP, promote Ca uptake; the K_{ATP}, is ~10 μM. ATP analogs with blocked γ-phosphate groups are unable to replace ATP. Particulate fractions from the disrupted synaptosomes possess Ca-dependent ATPase activity in the presence of Mg; the apparent K_{Ca} for this activity is 0.4–0.8 μM Ca, and the Hill coefficient is ~1.6. The Ca uptake and ATPase kinetic data suggest that the hydrolysis of 1 ATP may energize the transport of two Ca^{2+} ions into the storage vesicles. The second part of the article concerns the intraterminal distribution of Ca in "intact" terminals. When the terminals are disrupted after ^{45}Ca loading, about one-half of the ^{45}Ca is retained in the particulate material; some of this Ca, presumably stored in mitochondria, is released by the uncoupler, FCCP. Some of the ^{45}Ca is released by A-23187, but not by FCCP; this fraction may be Ca stored in the nonmitochondrial sites described above. The proportion of ^{45}Ca stored in the nonmitochondrial sites is increased when the Ca load is reduced or when the mitochondria are blocked with ruthenium red. These data indicate that the nonmitochondrial Ca storage sites are involved in intraterminal Ca buffering; they may play an important role in synaptic facilitation and post-tetanic potentiation, which result from Ca retention after neural activity.

INTRODUCTION

The sequence of events involved in the secretion of neurotransmitter substances (36) is normally initiated by depolarization of the presynaptic nerve terminals;

this induces an increase in the plasmalemmal Ca permeability (38). The terminals then gain Ca because of the large, inwardly directed Ca electrochemical gradient (cf. 16). The consequent rise in the intracellular ionized Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers a burst of neurotransmitter release (cf. 43, 51). The rate of transmitter release then falls off very sharply, within ~1 ms, as the $[Ca^{2+}]_i$ returns toward the resting level (37). The Ca that enters during the period of phasic activity must eventually be extruded, in order that the terminals return to the resting steady state. However, it is unlikely that this Ca is extruded within 1 ms; in fact, there is evidence that some of the Ca may remain in the terminals for a considerable time (seconds or minutes) after a period of activity (37, 59, 69, 71). Inasmuch as the $[Ca^{2+}]_i$ level falls very rapidly, it is clear that the temporarily retained Ca must be buffered in the cytosol.

Two candidates for a role in intraterminal Ca buffering are the mitochondrion and the nonmitochondrial Ca-sequestering organelle, perhaps smooth endoplasmic reticulum, as described in the preceding article (16). Mitochondria appear to have a rather low affinity for Ca ($K_{Ca} > 10^{-5}$ M) when incubated in solutions whose composition approximates the intracellular ionic environment (32, 66).

The ability of mitochondria to accumulate Ca is impaired by a variety of agents including 2,4-dinitrophenol, Na azide, oligomycin, FCCP, and ruthenium red. The second type of Ca-storing organelle can be distinguished by its insensitivity to all of the aforementioned agents (16), and by its high affinity for Ca ($K_{Ca} < 10^{-6}$ M). In this article we describe some of the kinetic properties of the latter (nonmitochondrial) Ca-storing system. The second part of the article is concerned with a discussion of the possible contributions of the mitochondrial and nonmitochondrial Ca-sequestering systems to intraterminal $[Ca^{2+}]$ regulation.

MATERIALS AND METHODS

The preparation of nerve terminals and the methods for measuring ^{45}Ca uptake have all been described in the preceding article (16). Additional details will be given in the figure legends.

Reagents and Solutions

The composition of the solutions used for these experiments were given in Table I of Ref. 16. In all experiments, some of the solutions contained special reagents; most of the reagents were described previously (16). In addition, in the present experiments, we used a variety of nucleoside phosphates; these were all obtained from the Sigma Chemical Co., St. Louis, Mo., with the exception of the nonhydrolyzable ATP analogs (cf. 62, 73), adenylyl (β-γ-methylene) diphosphonate (AMP-PCP) and adenylyl-imidophosphate (AMP-PNP), which were obtained from Boerhinger Mannheim Biochemicals, Indianapolis, Ind. The adenylate kinase inhibitor, diadenosylpentaphosphate (41) was also obtained from Boerhinger Mannheim. In some experiments the ATP level was buffered with phosphoenol pyruvate (PEP) and pyruvate kinase (both from Sigma). In a few instances potato apyrase (from Sigma) was used in an effort to reduce basal (endogenous) ATP levels in the "ATP-free" suspensions.

Intraterminal Ca Distribution

Equilibrated "intact" synaptosomes were incubated in NaCl medium (16, Table I)

containing 1.2 mM or 0.05 mM $CaCl_2$ and 10 mM glucose. After 12 min at 30°C, sufficient KCl medium (16, Table I) was added to raise the KCl concentration of the suspension to 50 mM; the KCl medium contained glucose and 1.2 mM or 0.05 mM $CaCl_2$ labeled with ^{45}Ca. After an additional incubation period (usually 30–60 s) at 30°C, to load the synaptosomes with ^{45}Ca (12), Ca uptake was terminated with an EGTA solution (cf. 12 for details) and the suspensions were diluted with 7.5 ml of ice-cold Ca-free NaCl medium (total volume = 9 ml). The suspensions were centrifuged at 15,000 *g* (max) for 6 min at 4°C; the supernatant solutions were discarded, and the pellets were rinsed with ice-cold NaCl medium containing 1.2 mM $CaCl_2$. Some pellets were resuspended in NaCl medium with 1.2 mM $CaCl_2$, and immediately filtered to determine the amount of ^{45}Ca taken up by the "intact" terminals. Other pellets were resuspended in lysis solution (16, Table I) which often contained other reagents such as FCCP and (or) A-23187; these suspensions were incubated at 30°C, usually for 5 min, and then filtered. The filters were washed with NaCl medium, in the case of the "intact" terminals or with KCl medium, in the case of disrupted terminals, before being prepared for counting; all wash solutions were ice-cold and contained 1.2 mM $CaCl_2$. Further details regarding these experiments will be given in Results.

Ca-Dependent ATPase Activity

The disrupted synaptosomes and some of the partially purified particulate fractions (cf. 16) were assayed for Ca-dependent ATPase activity. Disrupted synaptosomes (~80 mg synaptosome protein in 100 ml lysis solution) were centrifuged at 14,000 *g* for 7 min at 5°C. The resulting pellets were resuspended in a total of 20 ml of lysis solution; 5 ml of this suspension was layered over a discontinuous sucrose density gradient consisting of (top to bottom) 5 ml of 0.4 M, 7.5 ml of 0.6 M, 7.5 ml of 1.0 M, and 5 ml of 1.2 M sucrose. The gradients were centrifuged at 120,000 *g* for 2 h at 5°C. The material from the 0.6–1.0 M sucrose interface was diluted with lysis solution (material from four gradients was diluted to 50 ml) and centrifuged at 36,000 *g* for 30 min at 5°C. The pellets (~2.2 mg total protein) were resuspended in KCl medium (16) and assayed for ATPase activity. Although other fractions from the sucrose gradient were assayed in some experiments, the material from the 0.6–1.0 M sucrose interface showed the highest Ca-dependent ATPase specific activity; the latter fraction was therefore used for most experiments, including the ones described below (see Results for further details).

To determine the amount of inorganic phosphate (P_i) released, 0.4-ml aliquots of the incubation suspensions (see legend to Fig. 6) were added to 0.133 ml of an ice-cold 8% ascorbate-40% trichloracetic acid (TCA) solution. These samples were centrifuged at 8,730 *g* for 3 min, and the supernatant solutions were assayed for P_i by the method of Baginski et al. (3).

RESULTS

Kinetic Aspects of the Nonmitochondrial ATP-dependent Ca Uptake

DIVALENT CATION DEPENDENCE The nonmitochondrial ATP-dependent Ca sequestration mechanism described in the preceding article appears to have a high affinity for Ca (16; Fig. 2 and Table IV). This aspect was further investigated by determining the rate of Ca uptake as a function of the $[Ca^{2+}]_i$ in the incubation medium; the $[Ca^{2+}]$ was controlled with Ca-EGTA buffers, and uptake was measured in the absence and presence of ATP. Data from a representative experiment are shown in Fig. 1. Note that the ATP-dependent Ca uptake (▲) has an apparent mean half-saturation constant for Ca (K_{Ca}) of

~0.4 μM, which is consistent with the data in Table IV of Ref. 16. A Hill plot of the data from Fig. 1 yields a least square regression line with a slope (Hill coefficient) of 1.6 for [Ca^{2+}] between 0.1 and 3.7 μM; consistent with this is the observation that the square law relationship provides a good fit to the data. This suggests that two or more Ca^{2+} ions act cooperatively, and may be transported

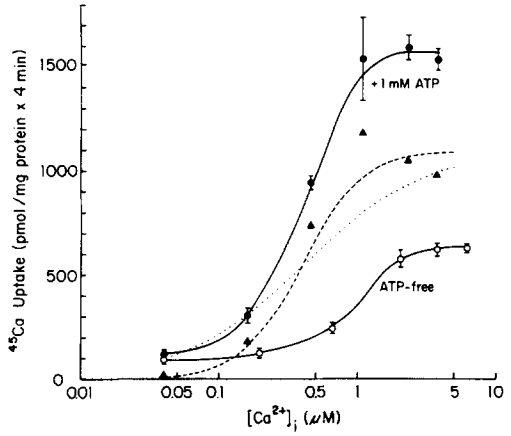

FIGURE 1. The effect of ionized Ca^{2+} concentration on ^{45}Ca uptake by disrupted synaptosomes. Equilibrated-pelleted synaptosomes were suspended in hypotonic lysis solution (cf. 16, Table I); in the case of the ATP-free samples, the lysis solutions contained 1 mg/ml apyrase to hydrolyze the endogenous ATP. Solutions containing EGTA and ^{45}Ca-labeled $CaCl_2$ ± 1 mM ATP were added to start the Ca uptake. These solutions also contained sufficient pH buffer, KCl, NaCl, and $MgCl_2$ to give a final composition identical to "KCl medium" (16, Table I); 0.1 mM NaN_3, 0.1 mM DNP, and 0.7 μg/ml oligomycin were also present. The synaptosome protein concentration was 0.42 mg/ml. The Ca in the incubation medium was buffered to the indicated ionized Ca^{2+} concentration by keeping $[Ca]_{total}$ constant at 25 μM, and varying $[EGTA]_{total}$; the nominal free [Ca^{2+}] was calculated using a CaEGTA stability constant of 7.6×10^6 M^{-1} (56). The samples were incubated for 4 min at 30°C. The data are graphed on semi-logarithmic coordinates; each circle is the mean of 3 determinations ± SEM. The solid curves, fitted to the circles, were drawn by eye. The dashed and dotted (derived) curves fitted to the ATP-dependent Ca uptake data (▲) are of the form:

$$J^*_{Ca} = J_{Ca}/[1 + (K_{Ca}/Ca^{2+})^n],$$

where J_{Ca} is the rate of Ca uptake at any [Ca^{2+}], and J^*_{Ca} is the maximal rate of uptake (1,100 pmol/mg protein per 4 min in this experiment). The apparent half-saturation for Ca, K_{Ca}, has a value 0.4 μM. The exponent n has a value of 1 (dotted curve) or 2 (dashed curve); the square law relationship ($n = 2$) is based on the Hill coefficient value for these data, of 1.6 (see text).

simultaneously by the uptake mechanism (see below).

Magnesium ions are also required for Ca uptake, as is illustrated by the data in Fig. 2. In the absence of Mg, the ATP-dependent Ca uptake (▲) is virtually abolished. The apparent half-saturation constant for Mg (K_{Mg}) is ~80 μM. At the higher Mg (total) concentrations, a considerable fraction of the Mg must be

free i.e., complexed with ATP; for example, cf. 39. Inasmuch as the ATP-dependent Ca uptake is not inhibited at the high Mg concentrations, this must mean that the Ca transport mechanism has a very high Ca/Mg selectivity ratio.

NUCLEOTIDE REQUIREMENTS A variety of nucleotides were tested for their ability to promote Ca uptake. The bar graph in Fig. 3 shows that the only really effective nucleotides were ATP, ADP, and deoxy-ATP; 1 mM CTP and ITP exhibited only a slight stimulatory effect, whereas GTP, UTP, and cyclic AMP reduced the Ca uptake to below the nucleotide-free control level.

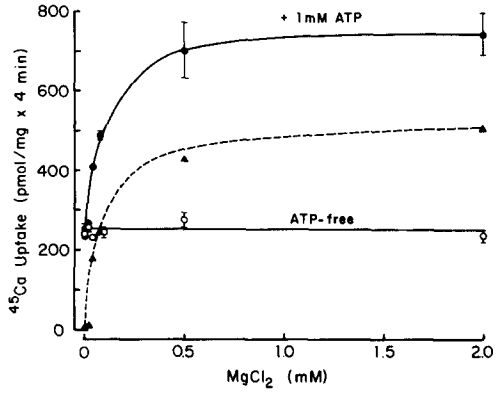

FIGURE 2. Effect of $MgCl_2$ concentration on ^{45}Ca uptake by disrupted synaptosomes. Synaptosomes were disrupted in lysis solution; after 1 min, ^{45}Ca-labeled solutions containing 15 μM $CaCl_2$ were added to start the Ca uptake. These solutions also contained buffer, KCl, NaCl, NaN_3, DNP, oligomycin, and in some cases (see graph), $MgCl_2$ and ATP. The final K and Na concentrations were 145 and 5 mM, respectively (KCl medium in Table 1 of Ref. 16). The synaptosome protein concentration in the suspensions was 0.33 mg/ml. The suspensions were incubated for 4 min at 30°C. Each circle indicates the mean of three determinations; SE bars are shown where they extend beyond the symbols. The solid curves, fitted to the circles, were drawn by eye. The broken (derived) curve relating the ATP-dependent Ca uptake data (▲) to the Mg concentration was calculated from the equation:

$$J_{Ca} = J^*_{Ca}/(1 + K_{Mg}/[Mg]),$$

where J^*_{Ca} is the maximal rate of Ca uptake, with a value of 525 pmol/mg protein per 4 min; K_{Mg} is the apparent half-saturation constant for Mg, with a value of 80 μM.

To determine whether ADP stimulates Ca uptake directly, or indirectly as a consequence of conversion to ATP via adenylate kinase, the experiment of Fig. 4 was made. These data show that the adenylate kinase inhibitor, diadenosylpentaphosphate (41), greatly reduced the ADP-stimulated Ca uptake, but had no effect on the ATP-stimulated Ca uptake. We conclude that ADP does not directly promote Ca uptake; clearly, the adenine ring and the three phosphates are required for activity. The structure of the sugar is apparently not so crucial, since deoxyribose can substitute for the ribose.

The effect of the ATP concentration on Ca uptake is shown in Fig. 5. The

apparent half-saturation concentration for ATP (K_{ATP}) is ~10 μM. From a comparative point of view, it is interesting to note that both the sarcoplasmic reticulum Ca transport system (68) and the plasma membrane Na-K exchange pump (17) share comparably high affinities for ATP. Moreover, deoxy-ATP can also substitute for ATP in powering Na extrusion in squid axons (17).

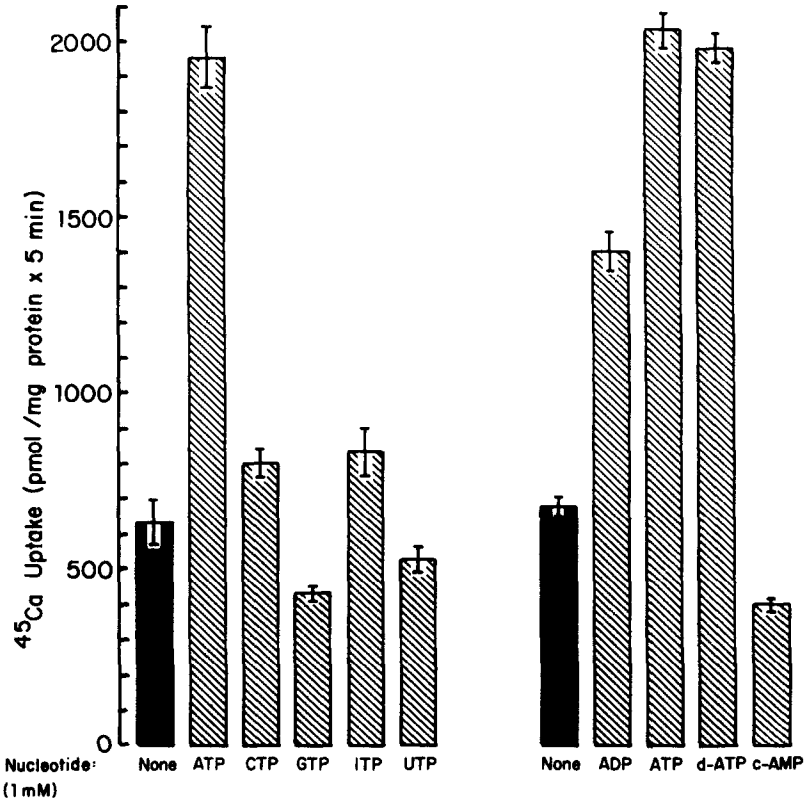

FIGURE 3. The effects of various nucleotides on ^{45}Ca uptake by disrupted synaptosomes. Equilibrated synaptosomes were suspended in lysis solution; 1 min later solutions containing KCl, NaCl, $CaCl_2$ (labeled with ^{45}Ca), and in some instances, nucleotides (see graph) were added. All solutions contained 0.1 mM NaN_3, 0.1 mM DNP, and 0.7 μg/ml oligomycin. The final composition of the incubation solutions was similar to that of KCl medium (16; Table I), $[Ca]_{total}$ was 10 μM, and the nucleotide concentrations were all 1 mM. Incubations were carried out for 5 min at 30°C; the protein concentration in the suspensions was 0.5 mg/ml. Each bar represents the mean of three determinations ± SEM.

EVIDENCE THAT A CA-DEPENDENT ATPASE IS INVOLVED IN THE CA UPTAKE The requirements for Mg and ATP, or deoxy-ATP, raise the possibility that the Ca uptake is mediated by a Mg-dependent ATPase. Indirect support for the idea that an ATPase is involved comes from the observation (Table I) that two analogs of ATP with blocked γ-phosphate groups, AMP-PCP and AMP-PNP (62, 73), are relatively ineffective as substitutes for ATP. The reason for the

small stimulation of Ca uptake by these analogs is uncertain; thin layer chromatography of ATP-PNP on DEAE-cellulose gave two spots, but the small Ca uptake promoted by ATP-PNP was not inhibited by diadenosylpentaphosphate, which indicates that the extra uptake is not due to contaminating ADP (see above). The data imply (cf. 74) that cleavage of the terminal phosphate bond, and not simply ATP binding, may be required for the Ca sequestration process. Of related interest is the fact that AMP-PCP cannot substitute for ATP in promoting Ca uptake by sarcoplasmic reticulum (50). As shown in Table I,

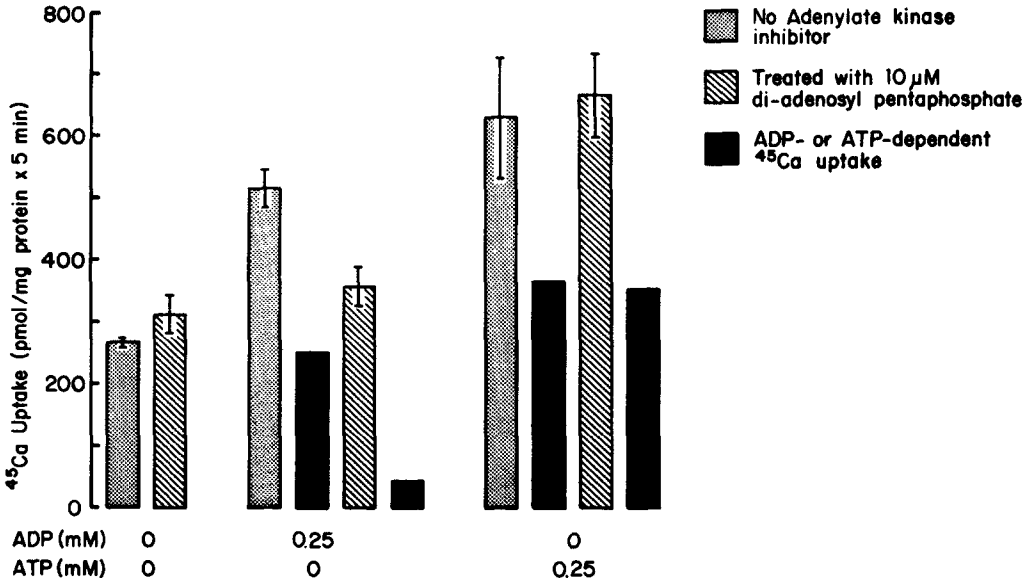

FIGURE 4. The effects of the adenylate kinase inhibitor, di-adenosyl pentaphosphate on ADP- and ATP-dependent ^{45}Ca uptake by disrupted synaptosomes. Equilibrated synaptosomes were suspended in 1.0 ml of lysis solution; 1 min later, 1.0 ml of ^{45}Ca-containing salt solution was added to give a final composition similar to "KCl medium" with 10 μM Ca. All solutions contained 0.1 mM NaN_3, 0.1 mM DNP, and 0.7 μg/ml oligomycin. Some of the solutions also contained ADP or ATP (250 μM final concentration) ± di-adenosyl pentaphosphate to give a 10 μM solution. The suspensions were incubated for 5 min at 30°C; the protein concentration in the suspensions was 0.54 mg/ml. Each bar represents the mean of three Ca uptake determinations ± SEM.

AMP-PNP, but not AMP-PCP, inhibits the mitochondrial poison-insensitive ATP-dependent Ca uptake; this is consistent with the fact that AMP-PNP is structurally and chemically more closely related to ATP than AMP-PCP (73, 74).

More direct information was obtained by testing for Ca-dependent ATPase activity in our preparations. We found that the particles obtained from the intermediate-density layers of the discontinuous sucrose gradient at the 0.6–1.0 and 1.0–1.2 M sucrose interfaces, upon subfractionation of disrupted synaptosomes (16; Methods), exhibited a Ca-dependent ATPase activity in the presence of Mg, oligomycin (to inhibit the mitochondrial ATPase), and ouabain (to

inhibit Na + K-dependent ATPase). The activity was particularly enriched in the material from the 0.6–1.0 M sucrose interface, the fraction that also has the highest nonmitochondrial ATP-dependent Ca uptake specific activity (16). Unfortunately, the Ca-dependent ATPase activity is difficult to measure accurately in the original disrupted synaptosome suspensions or in most of the other sucrose gradient fractions, because the Ca-independent ("background") ATPase activity is far greater than the Ca-dependent activity (cf. Fig. 6 legend and Ref. 19).

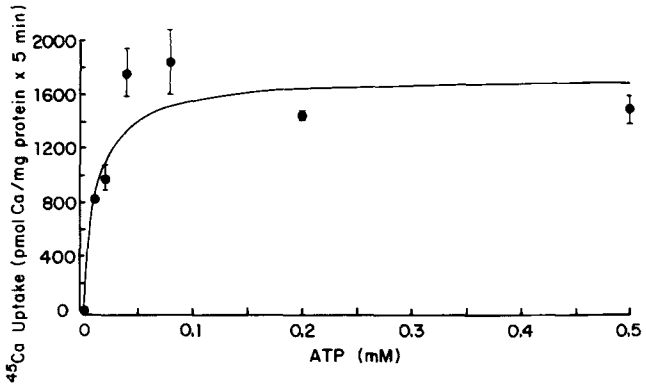

FIGURE 5. The effect of ATP concentration on ^{45}Ca uptake by disrupted synaptosomes. Equilibrated, lysed synaptosome suspensions were diluted with K-rich solutions containing ^{45}Ca-labeled $CaCl_2$ to give a final concentration of 8 μM Ca in a solution with the composition of "KCl medium" (16, Table I). All solutions contained NaN_3, DNP, and oligomycin. ATP was added to most of the solutions, as indicated on the abscissa; the ATP concentrations were buffered with an enzymatic ATP-regenerating system consisting of 5 mM phosphoenol pyruvate (PEP) and 10 U/ml of pyruvate kinase. The ATP-dependent component of the Ca uptake is plotted on the ordinate: the Ca uptake from ATP-free incubation solution (103 pmol/mg protein per 5 min) was substracted from each of the gross Ca uptake values. The suspensions were incubated for 5 min at 30°C; the synaptosome protein concentration in the suspensions was 0.42 mg/ml. Each symbol indicates the mean of three determinations ± SEM (where the error bars extend beyond the symbols). The ATP-dependent Ca uptake (J_{Ca}) data have been fitted by a curve of the form:

$$J_{Ca} = J^{*}_{Ca}/(1 + K_{ATP}/[ATP]),$$

where J^{*}_{Ca} is the maximal Ca uptake, with a value of 1,700 pmoles/mg protein per 5 min in this experiment. The apparent half-saturation constant for ATP, K_{ATP}, is 10 μM.

The data in Fig. 6 indicate that the apparent K_{Ca} for the Ca-dependent ATPase activity is ~0.8 μM; in one other similar experiment, K_{Ca} was ~0.4 μM. Indeed, Duncan (22) recently reported that "synaptic plasma membranes" from rat brains have a Ca-dependent ATPase activity with an apparent K_{Ca} of 0.4 μM; Otsuka et al. (55) and de Meis et al. (19) have also observed Ca-dependent ATPase activity in "brain microsomes."

The data from Fig. 6, when graphed as a Hill plot, fit a least squares

regression line with a slope (Hill coefficient) of 1.6; Duncan (22) reported that the Hill coefficient for his data was 1.4. Note that the square law relationship gives a better fit to the data in Fig. 6 than the first order curve. These findings may indicate that at least two Ca^{2+} ions are required to activate the hydrolysis of

TABLE I

EFFECTS OF NUCLEOTIDES WITH BLOCKED γ-PHOSPHATE ON ^{45}Ca UPTAKE BY DISRUPTED SYNAPTOSOMES

Nucleotide	Concentration	^{45}Ca uptake	
		Total*	Nucleotide-dependent
	mM	*pmol/mg protein × 3 min*	
Experiment A: effect of AMP-PNP			
None	–	226±14	–
AMP-PNP	0.5	366±9	140±17
ATP	0.1	624±10	398±17
ATP	0.1		
+AMP-PNP	0.5	536±14	310±14
ATP	0.5	939±39	713±41
ATP	0.5		
+AMP-PNP	0.5	757±17	531±22
Experiment B: effect of AMP-PCP			
None	–	634±76	–
AMP-PCP	0.5	780±46	154±89
ATP	0.1	1,307±20	677±79
ATP	0.1		
+AMP-PCP	0.5	1,258±12	624±77
ATP	0.5	1,255±43	621±87
ATP	0.5		
+AMP-PCP	0.5	1,372±33	738±83

* Lysed synaptosome suspensions were diluted with salt solution to give a final composition identical to "KCl medium" (16; Table I). All solutions contained 0.1 mM NaN_3, 0.1 mM DNP, and 0.7 μg/ml oligomycin. The diluted solutions also contained ~15 μM $CaCl_2$ (checked by atomic absorption), labeled with ^{45}Ca, and nucleotides (as indicated in columns 1 and 2). The suspensions were incubated for 3 min at 30°C; the protein concentrations were (A) 0.29 and (B) 0.21 mg/ml in the suspensions. Each value in column 3 is the mean of three determinations ± SEM.

one ATP molecule. Furthermore, the Hill coefficients for ATP-dependent Ca uptake (1.6; cf. Fig. 1) and for Ca-dependent ATP hydrolysis (1.6; cf. Fig. 6) are compatible with the hypothesis that the stoichiometry of the nonmitochondrial Ca uptake system may be two Ca^{2+} ions transported per ATP hydrolyzed. In this context, it may be worth noting that the Ca transport system in skeletal muscle sarcoplasmic reticulum has a stoichiometry of 2 Ca^{2+} accumulated per

ATP hydrolyzed (27, 68).

The one disturbing feature is that the maximum rate of Ca-dependent ATP hydrolysis, ~50 nmol P_i per mg protein per min (Fig. 6), is nearly 70 times larger than the ATP-dependent Ca uptake activity of the 0.6–1.0 M sucrose

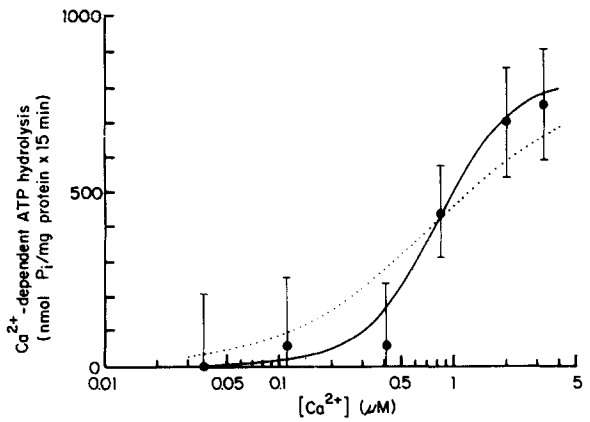

FIGURE 6. Effect of ionized Ca^{2+} concentration on ATP hydrolysis by fractionated synaptic membranes. Lysed synaptosomes were fractionated on a discontinuous sucrose density gradient (see Methods). Material from 0.6–1.0 M sucrose interface was diluted with a salt solution to give the following concentrations: 145 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM ATP, 25 μM $CaCl_2$, 20 mM HEPES (buffered to pH 7.4 with Tris base), 1 mM ouabain, and 0.7 μg/ml oligomycin. A variable concentration of EGTA was also included, to buffer the ionized Ca^{2+} concentration; the nominal $[Ca^{2+}]$ values shown on the abscissa were calculated on the basis of a CaEGTA stability constant of 7.6×10^6 M^{-1} (56). The ouabain and oligomycin were included to inhibit contaminating Na + K-dependent, and mitochondrial ATPases, respectively. The suspensions, which contained 76 μg protein per ml, were incubated at 30°C. At 1 and 16 min, aliquots were diluted with an ascorbate-TCA solution (3) and assayed for inorganic phosphate (P_i). The 1-min P_i values were used as internal controls, to correct for P_i contamination (<2 μmol/mg protein in all cases); these values were subtracted from the 16-min values, to give the total ATP hydrolysis in 15 min. The Ca-dependent ATP hydrolysis was then obtained by subtracting the 15-min P_i value in the 0.037 μM Ca^{2+} solution (2,764 nmol P_i/mg protein per 15 min) from each of the P_i values at the higher Ca^{2+} concentrations. The data have been fitted by a curve of the form:

$$v = V^*/[1 + (K_{Ca}/Ca^{2+})^n],$$

where v is the rate of P_i production at any $[Ca^{2+}]$, and V^* is the maximal rate (817 nmol/mg protein per 15 min). The apparent half-saturation constant for Ca, K_{Ca}, has a value of 0.8 μM. The exponent, n, has a value of 1 (dotted curve) or 2 (solid curve); the square law relationship ($n = 2$) is based on the Hill coefficient value for these data, of 1.6 (see text).

gradient fraction of ~800 pmol per mg protein per min (Table V in Ref. 16). Two factors may contribute to this discrepancy between the Ca uptake and ATP hydrolysis rates. One is that the initial rate of Ca uptake is somewhat larger than the uptake rate measured at 5 min, as in Table V of Ref. 16, perhaps by a factor

of 2 or 3, according to our preliminary data. Furthermore, some of the Ca-storing organelles may become irreversibly leaky as a consequence of the osmotic lysis; but from the data described below (and see Discussion), we estimate that, at most, about half or three-quarters of these organelles are damaged. Therefore, these factors can account for only about a 10-fold discrepancy between the rate of Ca uptake and of ATP hydrolysis, unless the rate of ATP hydrolysis is enhanced in the damaged vesicles where there is no Ca gradient to work against. An additional possibility is that other Ca-dependent ATPases, i.e., not associated with the Ca uptake mechanism of Fig. 1, may contaminate our preparations. The contractile proteins, known to be present in presynaptic nerve terminals (7), may be prime candidates for this role. Thus, some of the Ca-dependent ATPase activity may not be involved in the ATP-dependent Ca sequestration system; in this case, the apparent similarity of the kinetic dependence on Ca concentration, for the respective processes, may simply be fortuitous.

TEMPERATURE EFFECTS: ACTIVATION ENERGY FOR CA UPTAKE To obtain some additional information about the nature of the Ca uptake mechanism, the temperature dependence of the ATP-dependent uptake was determined in several experiments. An Arrhenius plot of the data from a representative experiment is shown in Fig. 7. From the slope of the line, we calculate that, below 20°C, the activation energy for the nonmitochondrial ATP-dependent Ca uptake is about 10 Kcal per mol of Ca taken up. This value, although somewhat smaller than the activation energy for Ca uptake by sarcoplasmic reticulum

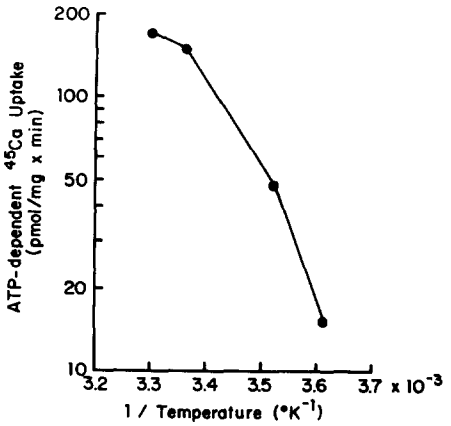

FIGURE 7. Arrhenius plot of the ATP-dependent ^{45}Ca uptake by disrupted synaptosomes. After lysis, the tissue suspensions were diluted with KCl-rich solutions to give a final solution composition similar to that of "KCl medium" (16, Table I); the solutions also contained NaN_3, DNP, oligomycin, and 12.5 μM Ca labeled with ^{45}Ca. The protein concentration in the suspensions was 0.5 mg/ml. The samples were incubated for 3 min at 4, 11, 24, or 30°C, as indicated on the abscissa. Each point represents the mean of the difference between three determinations of Ca uptake in the presence of 1 mM ATP, and three determinations in the absence of ATP.

(~25–30 Kcal/mol; 25), is, nevertheless, sufficiently large to be consistent with a process involving active transport.

Distribution of Ca in Intraterminal Organelles

While the aforementioned observations are in accord with the hypothesis (16) that synaptosomes contain (nonmitochondrial) organelles with a high-affinity Ca uptake mechanism, the critical question is: what role, if any, do these organelles play in the regulation of $[Ca^{2+}]_i$ in intact terminals? One approach to the solution of this problem is to determine what happens to Ca after it enters the terminals. Of course, as mentioned in the Introduction, in the long run the (net) Ca that enters during activity must be extruded. However, previous studies (14) have indicated that this extrusion may take several minutes, especially if the Ca load is large. Furthermore, if ^{45}Ca is used, even after all of the Ca load is extruded from the terminals, some of the radioactive tracer will have exchanged for ^{40}Ca in the intraterminal compartments. Thus, the distribution of tracer within the terminals should provide information about which organelles are involved in intracellular Ca buffering. To distinguish between mitochondrial and nonmitochondrial Ca storage sites, we have taken advantage of the fact that FCCP rapidly and completely releases Ca from mitochondria, but not from the NaN_3 + DNP + oligomycin-insensitive sites; this is exemplified by the data in Fig. 8. On the other hand, Ca stored in the latter (nonmitochondrial) sites can be released by A-23187 (16).

To obtain information about the disposition of the Ca that enters during a period of activity, "intact" terminals were loaded with Ca by incubating them for 30 or 60 s in normotonic ^{45}Ca-containing, K-rich (depolarizing) solutions (12). The Ca uptake was stopped with EGTA (12) and, after centrifugation and rinsing (see Methods), the pellets were lysed; in some instances the lysis solutions contained FCCP or A-23187 + FCCP, to separate the mitochondrial and nonmitochondrial vesicular stores of Ca, respectively. Data from two representative experiments are illustrated in Figs. 9 and 10.

As shown in Fig. 9 A and B, only about half of the previously accumulated ^{45}Ca load is retained in the particulate material, i.e., on the Millipore filters, after osmotic shock. The loss of about half of the ^{45}Ca load may be due to: (*a*) efflux of Ca from the intraterminal organelles during the 4- or 5-min incubation in the lysis solutions (cf. 16, Fig. 7); (*b*) the destruction of some of the Ca-storing organelles during lysis; (*c*) loss of free Ca^{2+}, and of Ca bound to soluble proteins or to the surfaces of the nonfilterable particles; and (*d*) loss of Ca sequestered in small, filterable organelles or vesicles. This loss makes it difficult to quantitate precisely the intraterminal Ca distribution. It is clear from Figs. 9 A and 10 A, however, that some of the accumulated ^{45}Ca is sequestered in mitochondria, because about half of the ^{45}Ca retained in the particulate material is released by FCCP. Moreover, Fig. 9 B shows that the fraction of ^{45}Ca stored in the mitochondria (i.e., releasable by FCCP) can be reduced markedly if the "intact" terminals are incubated with the mitochondrial Ca uptake inhibitor ruthenium red (cf. 16), before and during ^{45}Ca loading.

Some of the ^{45}Ca also appears to be stored in another type of membrane-

bound organelle, because A-23187 releases most of the ^{45}Ca that is not released by FCCP (Figs. 9 and 10). Although we cannot yet positively identify the organelles in question, it seems reasonable to assume that they may comprise those organelles described above, and in Ref. 16, that can accumulate Ca at the expense of ATP hydrolysis in the presence of mitochondrial poisons. When the mitochondrial Ca uptake mechanism is blocked, e.g., with ruthenium red, as shown in Fig. 9B, the amount of Ca stored in these organelles is increased, although the total amount of Ca retained within the terminals is diminished.

The Ca load imposed upon terminals exposed to K-rich solutions containing

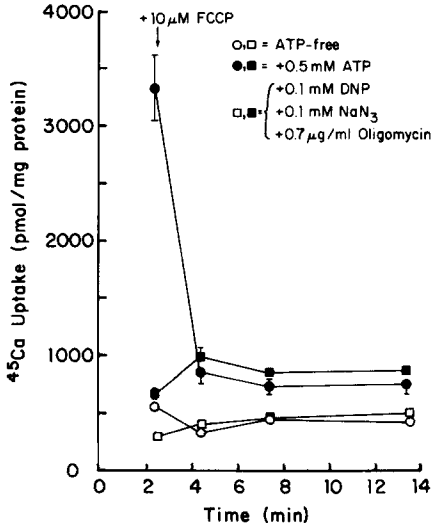

FIGURE 8. Effect of FCCP on the amount of ^{45}Ca accumulated by disrupted synaptosomes. Equilibrated-lysed synaptosomes were incubated in KCl medium containing 7 μM $CaCl_2$ (labeled with ^{45}Ca) ± 0.5 mM ATP; the incubation media of the samples indicated by the square symbols also contained NaN_3, DNP, and oligomycin. Aliquots of the suspensions were sampled for ^{45}Ca uptake after 2.4 min of incubation at 30°C. Immediately thereafter, 4 μl of 5 mM solution of FCCP in ethanol was added to each sample, and the incubation was continued for an additional 2, 5, or 11 min as indicated on the graph. Each symbol indicates the mean of three Ca uptake determinations; error bars are shown where the SEM extends beyond the symbol.

1.2 mM Ca, for 30–60 s, is rather massive; the total Ca content of the terminals may even be doubled under these circumstances (12). When the Ca concentration in the K-rich solutions is much lower (e.g. 50 μM), so that the Ca load imposed on the terminals is reduced, a larger fraction of the ^{45}Ca appears in the nonmitochondrial sites; concomitantly, the fraction associated with the mitochondria is reduced (Fig. 10B, as compared to Fig. 10A). The implication is that with a smaller, more physiological Ca load, the nonmitochondrial sites may sequester a larger proportion of the entering Ca. This may be comparable to the uptake data from disrupted synaptosomes (16; Table IV) which show that, at low free Ca^{2+} levels ($<10^{-6}$ M), the nonmitochondrial stores accumulate Ca

more effectively than the mitochondria. If the Ca-sequestering organelles in the disrupted preparations are, indeed, intraterminal structures, as we suggest (cf. 16), these observations may indicate that $[Ca^{2+}]_i$ is normally buffered at $<10^{-6}$ M in the terminals.

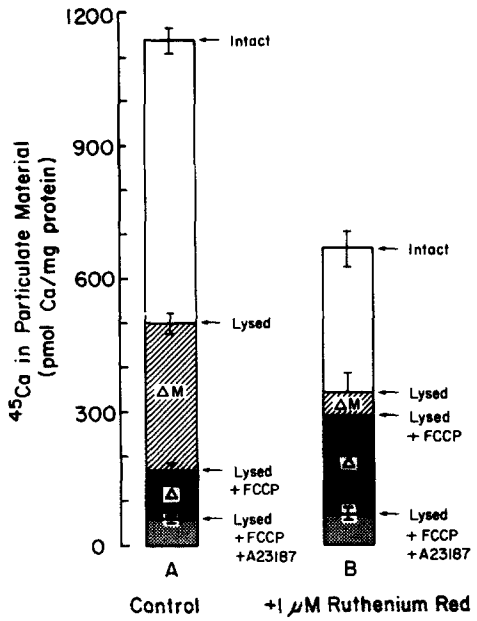

FIGURE 9. Effect of ruthenium red on the intracellular distribution of ^{45}Ca in K-stimulated synaptosomes. "Intact" synaptosomes were incubated in normotonic solution containing 50 mM K, 100 mM Na, and 1.2 mM Ca (labeled with ^{45}Ca) for 60 s at 30°C; in some instances (B) the solutions also contained 1 μM ruthenium red. The synaptosomes were pelleted, washed, and resuspended in normotonic solution to measure the total ^{45}Ca load, or in lysis solution; in some instances the lysis solution contained 10 μM FCCP or 10 μM A-23187 + 10 μM FCCP. The tops of the bars indicate the total amount of ^{45}Ca accumulated by the intact terminals; the tops of the hatched bars show the amount of ^{45}Ca remaining in the particulate fractions after osmotic shock. The heights of the hatched segments (ΔM) show the amount of ^{45}Ca in the mitochondria, i.e., released by FCCP. The heights of the black segments (Δ) indicate the amount of ^{45}Ca released by A-23187, but not FCCP, perhaps the Ca stored in the nonmitochondrial compartment described the text. The residual, nonfilterable ^{45}Ca, after lysis and incubation with FCCP + A-23187, is indicated by the stippled segments. Each error bar indicates the mean of three determinations ± SEM.

DISCUSSION

Kinetics of Nonmitochondrial Ca Uptake; Comparison with Sarcoplasmic Reticulum

The Ca sequestration system described above and in the preceding article (16) bears a striking resemblance to the Ca transport system of skeletal muscle

sarcoplasmic reticulum. In particular, the kinetic properties such as the half-saturation values for Ca and ATP, K_{Ca} and K_{ATP}, respectively, are very similar. The parallelism extends to the behavior of these uptake systems in response to a variety of poisons and other agents. In view of these numerous similarities, summarized in Table II, it seems possible that identical mechanisms may be involved in Ca sequestration in these two organelles.

In order extend the comparison even further, Dr. D. H. MacLennan

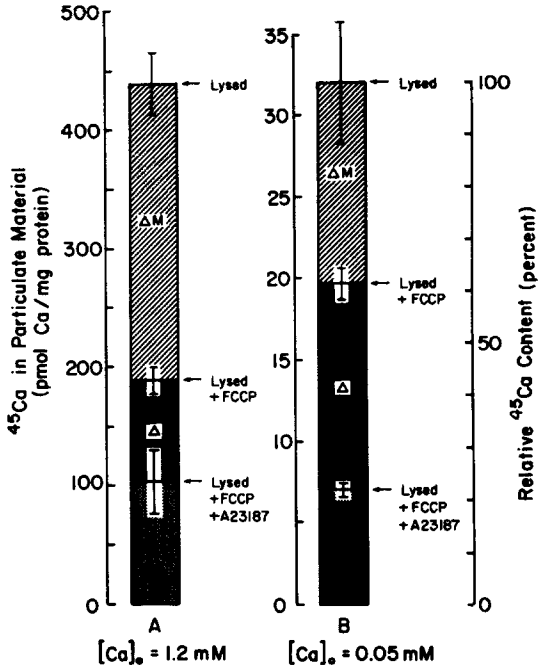

FIGURE 10. Effect of the concentration of Ca in the Ca-loading solution on the distribution of ^{45}Ca in K-stimulated "intact" synaptosomes. The protocol was similar to that of the experiment of Fig. 9, except that some K-rich loading solutions (B) contained only 0.05 mM $CaCl_2$, and no ruthenium red was used. The loading period was 36 s in this experiment. In this figure the total bar heights (100% values) indicate the amount of ^{45}Ca remaining in the particulate material after a 4-min incubation in lysis solution without FCCP or A-23187. 100% is equivalent to (A) 54% of the initial ^{45}Ca load of 813 pmol Ca/mg protein and (B) 44% of the load of 73 pmol Ca/mg protein. The explanation of the hatched (ΔM), black (Δ), and stippled segments is given in the caption to Fig. 9. Each error bar indicates the mean of three determinations ± SEM.

(Department of Biochemistry, University of Toronto) very kindly tested the antigenic response of some of our preparations (the material from the "light" and "heavy" bands of the discontinuous sucrose gradients; see 16); he used antibodies to purified rat skeletal muscle sarcoplasmic reticulum Ca + Mg ATPase (31) and to rabbit calsequestrin (75). The results were negative; no precipitin reactions were observed. However, the anti-ATPase antibodies blocks neither ATP hydrolysis nor Ca uptake in the sarcoplasmic reticulum. Thus, the

TABLE II

COMPARISON OF THE Ca UPTAKE MECHANISMS OF SKELETAL MUSCLE SARCOPLASMIC RETICULUM AND THE PRESYNAPTIC NERVE TERMINAL "NONMITOCHONDRIAL" Ca SEQUESTERING SITES

Property	Presynaptic "nonmitochondrial" system*	Skeletal muscle sarcoplasmic reticulum	Reference
Nucleotide requirements	ATP, deoxy ATP are effective	ATP>ITP >GTP>CTP >UTP (≃0.25/ATP)	26, 46
	CTP, ITP, UTP, ADP, 3′5′-cyclic-AMP are inactive		
	AMP-PCP and AMP-PNP cannot replace ATP. AMP-PNP inhibits ATP-dependent uptake	AMP-PCP is inactive	53
K_{ATP}	~19 μM	<10 μM	67, 68
K_{Ca} (Ca uptake)	~0.4 μM	~0.5 μM	68
		~0.3–0.5 μM (Ca binding)	26
K_{Ca} (Ca-ATPase)	~0.4–0.8 μM	~0.3–0.5 μM	26, 68
Activation energy	~10 Kcal/mol	~25–30 Kcal/mol	26
Ca:ATP Stoichiometry	2:1?	2:1	26, 68
Mg^{2+}	Required	Required	25, 26
K_{Mg}	~80 μM	?	
Effects of anions	Oxalate increases net Ca uptake	Oxalate and phosphate increase net Ca uptake	26, 45
Effect of A-23187	Prevents Ca uptake	Prevents Ca uptake	61
	Releases stored Ca	Releases stored Ca	61
Action of:			
Mitochondrial uncouplers	Insensitive	Insensitive	61
NaN_3	Insensitive	Insensitive	63
Oligomycin	Insensitive	Insensitive	67, 68
Ruthenium red	Insensitive	Insensitive	65
Saponins	Insensitive to low concentrations	Insensitive to low concentrations	48
Mersalyl	Inhibited	Inhibited	50
Tetracaine	Inhibited	Inhibited	33
Trypsin	Destroys uptake mechanism	Destroys uptake mechanism	49
Presciptin reaction with antibody to:			
Rat muscle sarcoplasmic reticulum ATPase	Negative	Positive	31
Rabbit calsequestrin	Negative	Positive	75

* Data from this and the preceding article (16).

antigenic determinants are not functional parts of the transport system. Consequently, the data do not yet eliminate the possibility that the Ca transport mechanism in our system is identical to a component of sarcoplasmic reticulum.

In the preceding article (16) we suggested that the organelles responsible for the mitochondrial poison-insensitive Ca uptake in the synaptosome preparations are probably located within the presynaptic terminals and may be the smooth endoplasmic reticulum (SER). Similar Ca uptake mechanisms have been associated with SER from a variety of other types of cells (see Introduction to 16). We should, therefore, seriously consider the possibility that some SER in many types of cells may function to sequester Ca and may play a role in intracellular Ca regulation. Perhaps the sarcoplasmic reticulum is simply an expanded and specialized form of this class of SER.

The apparent similarity between the responses of intracellular Ca storage sites in muscle and in presynaptic nerve terminals to caffeine and to dantrolene may provide further support for the aforementioned hypothesis. Caffeine, which promotes the release of Ca from sarcoplasmic reticulum (e.g., 23), is known to increase the spontaneous and evoked release of neural transmitter quanta at the neuromuscular junction; these effects have been attributed to caffeine-induced release of Ca from intraterminal Ca stores (e.g., 54, 70). Moreover, dantrolene, which counteracts the effect of Ca and apparently inhibits the release of Ca from sarcoplasmic reticulum (e.g., 20, 71), also depressed the frequency of spontaneous miniature endplate potentials (MEPPs) at the neuromuscular junction, presumably by inhibiting Ca release from intraterminal, nonmitochondrial Ca stores (64). It even seems possible that some Ca release from intraterminal stores may be triggered by depolarization, by analogy with excitation-contraction coupling in muscles (e.g., 18), if there are endoplasmic reticulum-plasma membrane junctions in nerve terminals similar to those observed in neuronal cell bodies and dendrites (29). Such evoked Ca release could possibly account for the increased frequency of spontaneous MEPPs observed at the neuromuscular junction, bathed in Ca-free medium, when the motor nerve is stimulated tetanically (52, 60).

Calcium Buffering in Presynaptic Nerve Terminals

The data presented above, and in the preceeding article (16), strongly support the view that there are at least two types of organelles within presynaptic nerve terminals capable of sequestering Ca. Moreover, our observations on Ca uptake kinetics and intracellular Ca distribution provide a basis for speculation on how the terminals handle the Ca that enters during a period of neuronal activity. The proposed sequence of events is pictured diagrammatically in Fig. 11.

In resting terminals, $[Ca^{2+}]_i$ is buffered at about 10^{-7} M (4, 11, 21, and see above; the precise value is unimportant for present purposes). Upon depolarization, the membrane Ca conductance increases, Ca enters the terminals, and $[Ca^{2+}]_i$ rises. If the Ca entry sites are confined to the "active zone" of the plasma membrane, i.e., the region of synaptic contact and vesicle release, as suggested by Llinas (42; and see 38), $[Ca^{2+}]_i$ in the cytoplasm adjacent to the active zone may increase to 10^{-6} or perhaps even 10^{-5} M (13). After repolarization, the Ca

conductance returns to the resting level within about 1–2 ms, causing Ca entry to decline. Because a Ca^{2+} concentration gradient is set up within the cytosol with the highest level near the active zone membrane, Ca will diffuse away from the membrane and toward the center of the terminal; along the way, the Ca may bind to high-affinity binding sites, as indicated by reaction 1 in Fig. 11. The binding sites

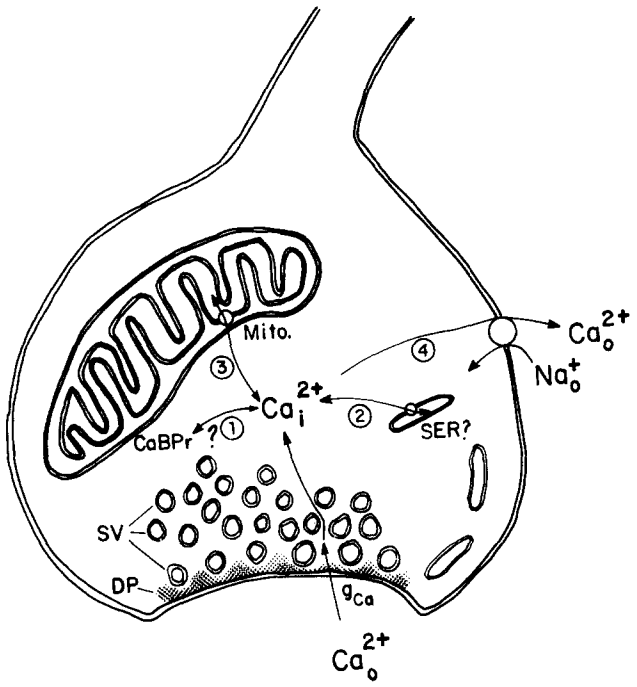

FIGURE 11. Diagram of the proposed pathways of Ca movement and sites of Ca buffering in the presynaptic nerve terminals. Depolarization triggers an increase in Ca conductance (g_{Ca}) in the "active zone" of the plasmalemma, i.e., in the region of synaptic contact, where synaptic vesicles fuse with the plasmalemma. The Ca which then enters, raising $[Ca^{2+}]_i$, rapidly diffuses to high-affinity Ca binding sites (Ca-BPr, reaction 1). Intraterminal Ca sequestration sites, both nonmitochondrial (perhaps smooth endoplasmic reticulum, or "SER"; reaction 2) and mitochondrial ("Mito", reaction 3), take up Ca, further lowering $[Ca^{2+}]_i$. Ultimately, the Ca which entered during activity must be extruded from the terminal; a Na-Ca exchange mechanism in the plasmalemma (reaction 4) appears to mediate this net Ca extrusion (14, 15). SV, synaptic vesicles; DP, dense projections in the region of the "active zone".

may be specific Ca-binding proteins such as those identified in squid axoplasm by Baker and Schlaepfer (5, 6); perhaps proteins such as the Ca-dependent phosphodiesterase regulator from brain (35) may be candidates for this role in vertebrate neurons, provided that they have appropriately high affinity and capacity for Ca. Alternatively, or in addition, the binding may be the initial step in the process of Ca sequestration by the mitochondrial and nonmitochondrial Ca transport systems. In any event, the diffusion and binding will cause $[Ca^{2+}]_i$

in the neighborhood of the active zone plasmalemma to fall dramatically. This would account for the rapid termination of the period of phasic transmitter release, with a time constant for decay of ~1 ms (37).

The second stage of Ca buffering is the sequestration of Ca by intraterminal organelles, once the Ca has diffused to, and been bound by the transport sites. Our kinetic and Ca-distribution data (e.g., Fig. 10 B), as well as morphological considerations, indicate that the nonmitochondrial sites may play a particularly important role here. The fact that the nonmitochondrial sites have a higher affinity for Ca than do the mitochondrial sites (cf. 16, Table IV), and the fact that intraterminal mitochondria are generally not found within 0.3–1 μm of the active zone (cf. 10, 24, 30, 34), whereas smooth endoplasmic reticulum may be more diffusely distributed in neuronal (including nerve terminal)[1] cytoplasm (cf. 28, 29), provide circumstantial evidence that the nonmitochondrial sites may be the first to sequester Ca (reaction 2 in Fig. 11). This view is supported by the observation (Fig. 10 B) that a large fraction of the retained Ca is sequestered in the nonmitochondrial sites when the Ca load is small. Eventually, of course, the entering Ca will diffuse to the neighborhood of the mitochondria, and these organelles will also take up some of the excess Ca (reaction 3 in Fig. 11). Moreover, the mitochondria appear to have a much larger capacity, perhaps 10- to 30-fold larger, for Ca storage than do the nonmitochondrial sites (cf. 16, Fig. 5); thus, with large Ca loads, e.g., after a long tetanus, the mitochondria may play a more important role in Ca buffering (cf. Figs. 9 and 10 A).

It is well known that mitochondrial poisons increase the spontaneous release of transmitter quanta (e.g. 1, 40). This effect has been interpreted as the consequence of a rise in $[Ca^{2+}]_i$ due to blockage of mitochondrial Ca buffering (1, 57). Our observations suggest that the situation may actually be much more complex, and that other factors also may contribute to the rise in $[Ca^{2+}]_i$ and the consequent stimulation of transmitter release. For example, inhibition of mitochondrial respiration will reduce the ATP level in the terminals, and may therefore interfere with Ca sequestration at the nonmitochondrial sites. By slowing Na extrusion through the Na-K exchange pump, the reduced ATP level should lower the Na gradient across the plasmalemma; this may also lead to a net gain of Ca by the terminals due to reduced Ca efflux and increased Ca influx via Na-Ca exchange (14, 15, and see reaction 4 in Fig. 11).

There is considerable evidence that the facilitated release of neurotransmitter, after a conditioning stimulus, is due to transient retention of Ca by the terminals (37, 72), and that facilitation decays with several time constants of about 1, 25–50, and 200–300 ms, respectively (cf. 37, 44, 47, 72). These findings may be compatible with our suggestion that at least three mechanisms with different properties contribute to intraterminal Ca buffering; perhaps the diffusion and binding, sequestration at non-mitochondrial sites, and sequestration at mitochondrial sites are associated with the short, intermediate, and long time constant facilitation decay processes, respectively.

[1] McGraw, C. F., A. V. Somlyo, and M. P. Blaustein. 1978. Ultrastructural localization of calcium by electron probe analysis in presynaptic nerve terminals. *Soc. Neurosci. Symp.* In press.

These buffering mechanisms will return $[Ca^{2+}]_i$ toward, but not quite to, the resting level. Ultimately, the plasma membrane extrusion mechanism(s) must be responsible for restoring $[Ca^{2+}]_i$ to the normal resting level and for maintaining normal Ca balance; previous studies from our laboratory (14, 15) indicate that net Ca extrusion from presynaptic terminals may involve an obligatory exchange of Na^+ ions for Ca^{2+} (reaction 4 in Fig. 11). Irrespective of the mechanism involved, Ca efflux may be expected to increase when $[Ca^{2+}]_i$ rises, e.g., as a result of depolarization and Ca entry. This extrusion will also lower $[Ca^{2+}]_i$. As $[Ca^{2+}]_i$ falls toward the resting level, the intracellular bound and sequestered Ca will tend to leave the buffering sites, and will become available for extrusion; this process will continue until the original Ca balance is restored. Consequently, the net extrusion must be the slowest of the $[Ca_{2+}]_i$ regulatory processes; it may, therefore, be associated with the decay of post-tetanic potentiation (PTP), which occurs with a time constant of tens of seconds to minutes (e.g., 44, 69), inasmuch as PTP also results from accumulation of Ca by the terminals (e.g., 59, 69). Indeed, there is evidence that experimental manipulations which may be expected to alter the transmembrane Na gradient and thereby interfere with Na-coupled Ca extrusion (14, 15) do, in fact, alter long-term facilitation (e.g., 2, 8, 9). Of considerable interest is the possibility that these processes may underlie so-called "short-term memory", which appears to be selectively blocked by inhibitors of Na transport (58).

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REFERENCES

1. ALNAES, E., and R. RAHAMIMOFF. 1975. On the role of mitochondria in transmitter release from motor nerve terminals. *J. Physiol. (Lond.)*. **248**:285–306.
2. ATWOOD, H. L., L. E. SWENARCHUK, and C. R. GRUENWALD. 1975. Long-term synaptic facilitation during sodium accumulation in nerve terminals. *Brain Res.* **100**:198–204.
3. BAGINSKI, E. S., P. P. FOA, and B. ZAK. 1967. Microdetermination of inorganic phosphate, phospholipids and total phosphate in biological materials. *Clin. Chem.* **13**:326–332.
4. BAKER, P. F. 1972. Transport and metabolism of calcium ions in nerve. *Prog. Biophys. Mol. Biol.* **24**:177–223.
5. BAKER, P. F., and W. SCHLAEPFER. 1975. Calcium uptake by axoplasm extruded from giant axons of *Loligo*. *J. Physiol. (Lond.)*. **249**:37–38P.
6. BAKER, P. F., and W. SCHLAEPFER. 1978. Uptake and binding of calcium by axoplasm isolated from giant axons of *Loligo* and *Myxicola*. *J. Physiol. (Lond.)*. **276**:103–125.
7. BERL, S., S. PUSZKIN, and W. J. NICKLAS. 1973. Actomyosin-like protein in brain. *Science (Wash. D. C.)*. **179**:441–446.

8. BIRKS, R. I., P. G. R. BURSTYN, and D. R. FIRTH. 1968. The form of sodium-calcium competition at the frog myoneural junction. *J. Gen. Physiol.* **52**:887–907.
9. BIRKS, R. I., and M. W. COHEN. 1968. The influence of internal sodium on the behaviour of motor nerve endings. *Proc. R. Soc. Lond. B Biol. Sci.* **170**:401–421.
10. BIRKS, R. I., H. E. HUXLEY, and B. KATZ. 1960. The fine structure of the neuromuscular junction of the frog. *J. Physiol. (Lond.)*. **150**:134–144.
11. BLAUSTEIN, M. P. 1974. The interrelationship between sodium and calcium fluxes across cell membranes. *Rev. Physiol. Biochem. Pharmacol.* **70**:33–82.
12. BLAUSTEIN, M. P. 1975. Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals, *in vitro*. *J. Physiol. (Lond.)*. **247**:617–655.
13. BLAUSTEIN, M. P. 1978. The role of calcium in catecholamine release from adrenergic nerve terminals. *In* The Release of Catecholamines from Adrenergic Neurons. D. M. Paton, editor. Pergamon Press, London. In press.
14. BLAUSTEIN, M. P., and A. C. ECTOR. 1976. Carrier-mediated sodium-dependent and calcium-dependent calcium efflux from pinched-off presynaptic nerve terminals (synaptosomes) *in vitro*. *Biochim. Biophys. Acta.* **419**:295–308.
15. BLAUSTEIN, M. P., and C. J. OBORN. 1975. The influence of sodium on calcium fluxes in pinched-off nerve terminals *in vitro*. *J. Physiol. (Lond.)*. **247**:657–686.
16. BLAUSTEIN, M. P., R. W. RATZLAFF, N. C. KENDRICK, and E. S. SCHWEITZER. 1977. Calcium buffering in presynaptic nerve terminals. I. Evidence for involvement of a nonmitochondrial ATP-dependent sequestration mechanism. *J. Gen. Physiol.* **72**:15–41.
17. BRINLEY, F. J., JR., and L. J. MULLINS. 1968. Sodium fluxes in internally dialyzed squid axons. *J. Gen. Physiol.* **52**:181–211.
18. COSTANTIN, L. L. 1975. Contractile activation in skeletal muscle. *Prog. Biophys. Mol. Biol.* **29**:197–224.
19. DE MEIS, L., B. M. RUBIN-ALTSCHUL, and R. D. MACHADO. 1970. Comparative data of Ca^{2+} transport in brain and skeletal muscle microsomes. *J. Biol. Chem.* **245**:1183–1189.
20. DESMEDT, J. E., and K. HAINAUT. 1977. Inhibition of the intracellular release of calcium by dantrolene in barnacle giant muscle fibres. *J. Physiol. (Lond.)*. **265**:565–585.
21. DIPOLO, R., J. REQUENA, F. J. BRINLEY, JR., L. J. MULLINS, A. SCARPA, and T. TIFFERT. 1976. Ionized calcium concentrations in squid axons. *J. Gen. Physiol.* **67**:433–467.
22. DUNCAN, C. J. 1976. Properties of Ca^{2+}-ATPase activity of mammalian synaptic membrane preparations. *J. Neurochem.* **27**:1277–1279.
23. ENDO, M. 1975. Mechanism of action of caffeine on the sarcoplasmic reticulum of skeletal muscle. *Proc. Jpn. Acad.* **51**:479–484.
24. GRAY, E. G. 1963. Electron microscopy of presynaptic organelles of the spinal cord. *J. Anat.* **97**:101–106.
25. HASSELBACH, W. 1974. Sarcoplasmic membrane ATPases. *In* The Enzymes. P. D. Boyer, editor. Academic Press, Inc., New York. **10**:431–467.
26. HASSELBACH, W., and M. MAKINOSE. 1961. Die Calciumpumpe der "Erschlaffungsgrana" des Muskels und ihre Abhängigkeit von der ATP-Spaltung." *Biochem. Z.* **333**:518–528.
27. HASSELBACH, W., and M. MAKINOSE. 1963. Über den Mechanismus des Calcium-

transportes durch die Membranen des sarkoplasmatischen Reticulums. *Biochem. Z.* **339**:94–111.

28. HENKART, M. 1975. The endoplasmic reticulum of neurons as a calcium sequestering and releasing system: morphological evidence. *Biophys. J.* **15**(2, Pt. 2):267a. (Abstr.).
29. HENKART, M., D. M. LANDIS, and T. S. REESE. 1976. Similarity of junctions between plasma membranes and endoplasmic reticulum in muscle and neurons. *J. Cell Biol.* **70**:338–347.
30. HEUSER, J. E., and T. S. REESE. 1977. Structure of the synapse. *Handb. Physiol.* **1**(Sect. 1. The Nervous System):261–294.
31. HOLLAND, P. C., and D. H. MACLENNAN. 1976. Assembly of the sarcoplasmic reticulum. Biosynthesis of the adenosine triphosphatase in rat skeletal muscle cell culture. *J. Biol. Chem.* **251**:2030–2036.
32. HUTSON, S. M., D. R. PFEIFFER, and H. A. LARDY. 1976. Effect of cations and anions on the steady state kinetics of energy-dependent Ca^{2+} transport in rat liver mitochondria. *J. Biol. Chem.* **251**:5251–5258.
33. JOHNSON, P. N., and G. INESI. 1969. The effect of methylxanthines and local anesthetics on fragmented sarcoplasmic reticulum. *J. Pharmacol. Exp. Ther.* **169**:308–314.
34. JONES, D. G. 1975. Synapses and Synaptosomes. Chapman and Hall, London. 258 pp.
35. KAKIUCHI, S., and R. YAMAZAKI. 1970. Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain. *Biochem. Biophys. Res. Commun.* **41**:1104–1110.
36. KATZ, B. 1968. The Release of Neural Transmitter Substances. Charles C. Thomas, Springfield, Ill. 55 pp.
37. KATZ, B., and R. MILEDI. 1968. The role of calcium in neuromuscular facilitation. *J. Physiol. (Lond.).* **195**:481–492.
38. KATZ, B., and R. MILEDI. 1969. Tetrodotoxin-resistant electrical activity in presynaptic terminals. *J. Physiol. (Lond.).* **203**:459–487.
39. KENDRICK, N. C., R. W. RATZLAFF, and M. P. BLAUSTEIN. 1977. Arsenazo III as an indicator for ionized calcium in physiological salt solutions: its use for determination of the CaATP dissociation constant. *Anal. Biochem.* **83**:433–450.
40. KRAATZ, H. G., and W. TRAUTWEIN. 1957. Die Wirkung von 2,4-Dinitrophenol (DNP) auf die neuromuskulare Erregung Subertragung. *Arch. Exp. Pathol. Pharmakol.* **231**:419–439.
41. LIENHARD, G. E., and I. L. SECEMSKI. 1973. P^1,P^5-di(adenosine-5′)pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. *J. Biol. Chem.* **248**:1121–1123.
42. LLINAS, R. R. 1977. Calcium and transmitter release in squid synapse. *Soc. Neurosci. Symp.* **2**:139–160.
43. LLINAS, R., and C. NICHOLSON. 1975. Calcium role in depolarization-secretion coupling: an aequorin study in squid giant synapse. *Proc. Natl. Acad. Sci. U. S. A.* **72**:187–190.
44. MAGLEBY, K. L., and J. E. ZENGEL. 1976. Stimulation-induced factors which affect augmentation and potentiation of transmitter release at the neuromuscular junction. *J. Physiol. (Lond.).* **260**:687–717.
45. MAKINOSE, M., and W. HASSELBACH. 1965. Der Einfluss von Oxalat auf den Calcium-Transport isolierter Vesikel des sarkoplasmatischen Reticulum. *Biochem. Z.* **343**:360–382.

46. MAKINOSE, M., and R. THE. 1965. Calcium-Akkumulation und Nucleosidtriphosphat-Spaltung durch die Vesikel des sarkoplasmatischen Reticulum. *Biochem. Z.* **343**:383–393.

47. MALLART, A., and A. R. MARTIN. 1967. An analysis of facilitation of transmitter release at the neuromuscular junction of the frog. *J. Physiol. (Lond.).* **193**:679–694.

48. MARTONOSI, A. 1968. Sarcoplasmic reticulum. IV. Solubilization of microsomal adenosine triphosphatase. *J. Biol. Chem.* **243**:71–81.

49. MARTONOSI. A. 1968. Sarcoplasmic reticulum. V. The structure of sarcoplasmic reticulum membranes. *Biochim. Biophys. Acta.* **150**:694–704.

50. MARTONOSI, A., and R. FERETOS. 1964. Sarcoplasmic reticulum. I. The uptake of Ca^{2+} by sarcoplasmic reticulum fragments. *J. Biol. Chem.* **239**:648–658.

51. MILEDI, R. 1973. Transmitter release induced by the injection of calcium ions in nerve terminals. *Proc. R. Soc. Lond. B Biol. Sci.* **183**:421–425.

52. MILEDI, R., and R. E. THIES. 1971. Tetanic and post-tetanic rise in frequency of miniature end-plate potentials in low calcium solution. *J. Physiol. (Lond.).* **212**:245–257.

53. OGAWA, Y. 1972. Relationship between rates of Ca uptake and affinities for Ca of the sarcoplasmic reticulum. *J. Biochem.* **71**:571–573.

54. ONDERA, K. 1973. Effect of caffeine on the neuromuscular junction of the frog, and its relation to external calcium concentration. *Jpn. J. Physiol.* **23**:587–597.

55. OTSUKA, M., I. OTSUKI, and S. EBASHI. 1965. ATP-dependent Ca binding of brain microsomes. *J. Biochem.* **58**:188–190.

56. PORTZEHL, H., P. C. CALDWELL, and J. C. RUEGG. 1964. The dependence of contraction and relaxation of muscle fibres from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. Biophys. Acta.* **79**:581–591.

57. RAHAMIMOFF, R., S. D. ERULKAR, E. ALNAES, H. MEIRI, S. ROTSHENKER, and H. RAHAMIMOFF. 1976. Modulation of transmitter release by calcium ions and nerve impulses. *Cold Spring Harbor Symp. Quant. Biol.* **40**:107–116.

58. ROGERS, J. L., R. OETTINGER, J. SZER, and R. F. MARK. 1977. Separate chemical inhibitors of long-term and short-term memory: contrasting effects of cycloheximide, ouabain and ethacrynic acid on various learning tasks in chickens. *Proc. R. Soc. Lond. B Biol. Sci.* **196**:171–195.

59. ROSENTHAL, J. 1969. Post-tetanic potentiation at the neuromuscular junction of the frog. *J. Physiol. (Lond.).* **203**:121–133.

60. ROTSHENKER, S., S. D. ERULKAR, and R. RAHAMIMOFF. 1976. Reduction in the frequency of miniature end-plate potentials by nerve stimulation in low calcium solutions. *Brain Res.* **101**:362–365.

61. SCARPA, A., J. BALDASSARE, and G. INESI. 1972. The effect of calcium ionophores on fragmented sarcoplasmic reticulum. *J. Gen. Physiol.* **60**:735–749.

62. SIMONS, T. J. B. 1975. The interaction of ATP-analogues possessing a blocked-phosphate group with the sodium pump in human red cells. *J. Physiol. (Lond.).* **244**:731–739.

63. SRETER, F. A., and J. GERGELY. 1964. Comparative studies of the Mg activated ATPase activity and Ca uptake of fractions of red and white muscle homogenates. *Biochem. Biophys. Res. Commun.* **16**:438–443.

64. STATHAM, H. E., and C. J. DUNCAN. 1976. Dantrolene and the neuromuscular junction: evidence for intracellular calcium stores. *Eur. J. Pharmacol.* **39**:143–152.

65. VALE, M. G. P., and A. P. CARVALHO. 1973. Effects of ruthenium red on Ca^{2+} uptake

and ATPase of sarcoplasmic reticulum of rabbit skeletal muscle. *Biochim. Biophys. Acta.* **325**:29–37.

66. VINOGRADOV, A., and A. SCARPA. 1973. The initial velocities of calcium uptake by rat liver mitochondria. *J. Biol. Chem.* **248**:5527–5531.
67. WEBER, A. 1966. Energized calcium transport and relaxing factors. *Curr. Top. Bioenerg.* **1**:203–254.
68. WEBER, A., R. HERTZ, and I. REISS. 1966. Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem. Z* **345**:329–369.
69. WEINREICH, D. 1971. Ionic mechanism of post-tetanic potentiation at the neuromuscular junction of the frog. *J. Physiol. (Lond.).* **212**:431–446.
70. WILSON, D. F. 1973. Effects of caffeine on neuromuscular transmission in the rat. *Am. J. Physiol.* **225**:862–865.
71. YAMAMOTO, Y., A. SUZUKI, and K. HOTTA. 1977. Dissociation of excitation and contraction in skeletal muscle induced by deuterium oxide and dantrolene-sodium. *Jpn. J. Physiol.* **27**:95–109.
72. YOUNKIN, S. G. 1974. An analysis of the role of calcium in facilitation at the frog neuromuscular junction. *J. Physiol. (Lond.).* **237**:1–14.
73. YOUNT, R. G., D. BABCOCK, W. BALLANTYNE, and D. OJALA. 1971. Adenylyl imidophosphate, an adenosine triphosphate analog containing a P-N-P linkage. *Biochemistry.* **10**:2484–2489.
74. YOUNT, R. G., D. OJALA, and D. BABCOCK. 1971. Interaction of P-N-P and P-C-P analogs of adenosine triphosphate with heavy meromyosin, myosin and actomyosin. *Biochemistry.* **10**:2490–2496.
75. ZUBRZYCKA, E., and D. H. MACLENNAN. 1976. Assembly of sarcoplasmic reticulum. Biosynthesis of calsequestrin in rat skeletal muscle cell cultures. *J. Biol. Chem.* **251**:7733–7738.