Na^+ REGULATES RELEASE OF Ca^{++} SEQUESTERED IN SYNAPTOSOMAL MITOCHONDRIA

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SUMMARY

The interactions of Na+ and Ca++ were examined in synaptosomes and mitochondria. Na+ decreased both $^{45}Ca^{++}$ binding to synaptosomes and loss of $^{45}Ca^{++}$ from mitochondria. The ability of low concentrations of a local anesthetic, N,N-diethylamino-octyl 3,4,5-trimethoxybenzoate, to inhibit movement of Ca from intracellular bound stores was found to be associated with increased uptake of Ca by synaptosomes. A Na+-dependent regulatory effect was postulated between mitochondrial binding of Ca and influx of Ca into the nerve terminal. In addition, the results suggest difficulties in distinguishing between Na+-dependent and Ca++-dependent processes in synaptosomal function.

Increases in the concentration of intracellular Ca in the nerve terminal are involved in the release of neurotransmitters. There are at least two mechanisms by which cytoplasmic [Ca++] rises in association with depolarization of the nerve membrane: (a) change in Ca++ permeability of the membrane, possibly the opening of a specific Ca channel (1), and (b) release of bound Ca++ from intracellular stores (2). The relative importance of these two processes for Ca-dependent transmitter release is not completely understood. Spontaneous transmitter release can be augmented, when extracellular [Ca]=0, by interfering with mitochondrial sequestration of Ca++ (3). However, the existence of a Ca++ flux from the external milieu into the terminal appears to be essential for depolarization-induced increases in the release of several transmitters (4,5).

Several components of isolated nerve terminals (synaptosomes) are able to bind Ca++ (6-8). Energy-dependent Ca++ binding by synaptosomes may be accounted for almost entirely by mitochondrial sequestration (3,9), although

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a recent report suggests that intrasynaptosomal vesicles also play an important role in this process (10). Sequestration may serve to reduce intracellular [Ca++] after depolarization-coupled influx of extracellular Ca++. The subsequent fate of the Ca++ sequestered from the cytosol by mitochondria is not known. It is not likely that the mitochondria act solely as a Ca++ "sink", or their Ca++ content would continuously increase. Some releasing of Ca++ must occur possibly in association with a phase of nerve terminal ion fluxes. In muscle, increased intracellular [Na+] releases Ca++ from mitochondria (11). In addition, veratridine and ouabain, which increase intracellular [Na+], increase Ca++-dependent release of insulin (12) and acetylcholine (13) from secretory cells and nerve terminals. This action is presumed to result from Na+-induced release of Ca++ from intracellular stores. However, Na+-regulation of Ca++-dependent secretion could result from either Na+ mediation of Ca++ flux at the secretory cell membrane, or at intracellular sites of Ca++ compartmentation. Na+ regulation of Ca++ binding has been shown in synaptosomes and is proposed to result from a gradient-driven ATPase-dependent exchange between Na+ and Ca++ at the membrane (14,15).

The studies decribed here were undertaken to investigate the effects of Na+ on binding and retention of Ca++ by isolated synaptosomes and mitochondria.

METHODS

Synaptosomes and mitochondria were prepared from rat caudate tissue by sucrose gradient methods (16). The mitochondrial and synaptosomal pellets were resuspended in Krebs-Henseleit phosphate (KHP) buffer, pH 7.4, (NaCl, 115 mM; KCl, 4.7 mM; $CaCl_2$, 1.8 mM; $MgSO_4$, 1.2 mM; 0.1 M Na_2HPO_4-HCl buffer, 20 mM Na+), and kept at 2-4° until use. A buffer including physiological salts was used, instead of the Tris buffers sometimes used in studies of ^{45}Ca binding (6), in order to vary salt concentrations in a salt media. In incubations, 100 μl of the mitochondrial suspension (containing 60-80 μg protein) or the synaptosomal suspension (40-60 μg protein) were added to 900 μl of KHP buffer and preincubated at 37° under air for 5 min. The tissue was loaded with ^{45}Ca by adding 4.5-6.0 x 10^{-10} M ^{45}Ca (approximately 2.5 x 10^6 cpm) to the suspensions and incubating under air for 5 min on a Dubnoff metabolic shaker. Binding of ^{45}Ca was terminated by rapid vacuum filtration over 0.450 μm Whatman filter paper. The filters were washed twice with 2-3 ml of 0.9% NaCl

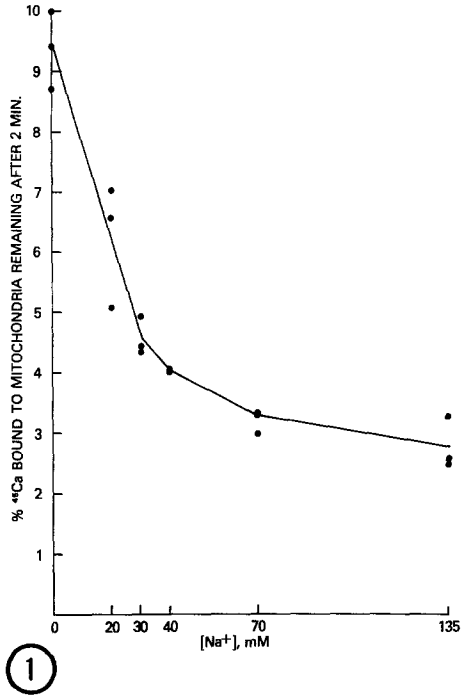
(37° C to avoid "cold shock" release). The filters were then placed in Erlenmeyer flasks with 2 ml experimental buffer and incubated for 2 min at 37° C. The releasing incubation was terminated by filtration and two washes over another filter to trap any dislodged tissue. The dried filters were transferred to vials and, after addition of 1.5 ml water and 15 ml Aquasol, counted on a liquid scintillation counter. Synaptosomal binding of ^{45}Ca was studied by the methods used for loading mitochondria. In the 6 experimental buffers, NaCl was replaced by LiCl and the 0.1 M Na_2HPO_4-HCl buffer by a 0.1 M phosphoric acid-tris base buffer.

RESULTS AND DISCUSSION

In the presence of 0.45-0.6 nmol ^{45}Ca, mitochondria bind 33.68 $\pm$ 10.45 pmol ^{45}Ca/mg protein/5 min. The loss of ^{45}Ca from preloaded mitochondria is time-dependent, with 80-90% released by 2 min. This is comparable to the rate of loss reported for synaptosomes (15). The loss of ^{45}Ca may result from both exchange with the $^{40}CaCl_2$ in the media as well as net loss of Ca++ from the mitochondria. ^{45}Ca efflux from mitochondria in Ca++-free buffer with EDTA was less than that in normal Ca++ (1.8 mM) buffer, which suggests that ^{45}Ca loss includes homoexchange between the two isotopic species or that extramitochondrial Ca++ may stimulate mitochondrial Ca++ efflux, possibly through activation of a Ca++-dependent ATPase similar to that described in rat brain microsomes (17).

The effects of altering [Na+] are shown in Fig. 1. As [Na+] decreases from 135 mM, loss of ^{45}Ca from mitochondria is significantly decreased. No effect of reduced Na+ was seen on mitochondrial ^{45}Ca binding. The effects of altering [Na+] on synaptosomal ^{45}Ca binding are shown in Fig. 2. When [Na+]=135 mM, synaptosomes bind 11.5 pmol ^{45}Ca/mg protein/4 min. The amount of ^{45}Ca bound decreases significantly as [Na+] increases to 40 mM. Between 40 and 70 mM [Na+], the rate of change in ^{45}Ca binding is less, and above 70 mM further increase in [Na+] does not affect ^{45}Ca binding.

Considering both Figs. 1 and 2, it appears that as ^{45}Ca loss from mitochondria is increased by Na+, binding of ^{45}Ca into synaptosomes is inhibited. Conversely, as mitochondrial Ca++ loss is reduced in the absence of Na+, binding of ^{45}Ca to synaptosomes increases. It is therefore suggested that binding (and entry) of Ca++ into the nerve terminals, in the absence of



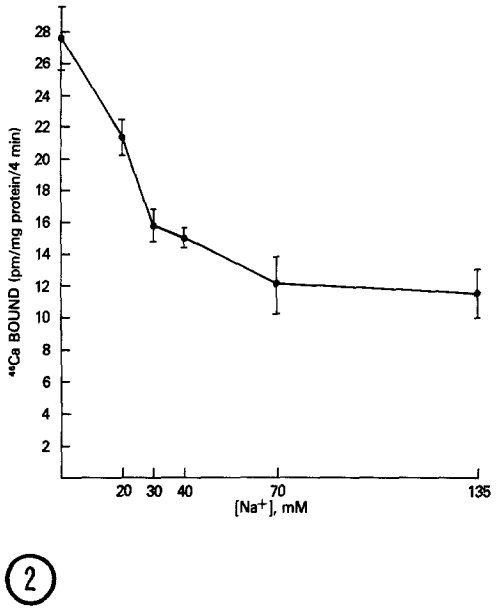


Fig. 1. Effects of Na+ on efflux of ^{45}Ca from mitochondria, preloaded as described in the test and incubated for 2 min in media deficient in Na+. Results are the amount of ^{45}Ca remaining in tissue after 2 min expressed as percent of the ^{45}Ca bound to mitochondria before incubating. Points are the means of triplicate determinations.

Fig. 2. Effect of Na+ on binding of ^{45}Ca to synaptosomes. Points are means of three experiments, each done in triplicate, lines are S.E.M.

depolarization, is regulated by Na+ through its extracellular effects on Na+-Ca++ exchange mechanisms at the membrane level and through the intracellular effects on binding of Ca++ to intracellular compartments, specifically mitochondria.

It has been hypothesized that local anesthetics block intracellular Ca++ movement from bound sites, such as mitochondria, to free cytosolic pools (18). As a consequence, the influx of extracellular Ca++ might be increased, since the gradient between high external [Ca++] and low internal [Ca++] (9) would not be lessened by any contribution of intracellularly bound Ca++ to the internal unbound Ca++ pool. The local anesthetic,

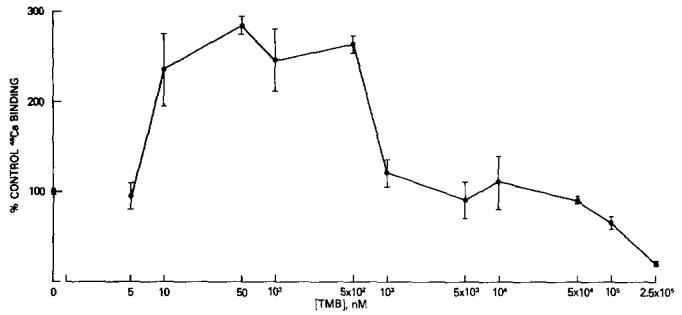


Fig. 3. Effect of TMB-8 (5×10^{-9} - 2.5×10^{-4} M) on ^{45}Ca binding by synaptosomes. Results are expressed as the percentage of binding in the absence of TMB-8. Points are means of four experiments, each done in triplicate; lines are S.E.M.

N,N-diethylamino-octyl 3,4,5-trimethoxybenzoate (TMB-8) has been shown to block intracellular Ca++ metabolism in platelets (19) and muscle (20). As shown in Fig. 3, at low concentrations, TMB-8 increased synaptosomal ^{45}Ca binding (Fig. 3) while at concentrations greater than 1×10^{-5} M, TMB-8 reduced ^{45}Ca binding. The latter result is in accord with that reported for a high concentration (3 mM) of another local anesthetic, procaine (15). This effect may result from interactions of the drug with the synaptosomal membrane. The increase in Ca binding at low TMB-8 concentrations provides evidence for the hypothesis (18) that internal [Ca++] can be influenced by internal Ca++ movements.

These results indicate that Na+ plays an important role in the regulation of Ca++-dependent processes in the nerve terminal. Changes in Na+ flux which accompany the action potential at the terminal may thus be involved in depolarization-associated intracellular Ca++ movements, in addition to changes in conductance of the specific Ca++ channel (1). As a consequence of this hypothesis, it becomes more complex to define Ca++-dependent and Na+-dependent processes. This potential problem was addressed in studies of norepinephrine and γ-aminobutyric acid release (21). For example, reduction of Na+ reduces high affinity synaptosomal binding of norepinephrine (22) and

choline (23); this has been interpreted to demonstrate a Na+ effect on the carrier affinity for its substrate. However, these observations could be explained at least in part, by an increase in Ca++ influx associated with reduced Na+. Increased Ca++ influx might cause an increase in transmitter release, indistinguishable from decreased transmitter binding (uptake) by the methods generally used to study these processes.

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