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UPTAKE OF CALCIUM IONS BY SYNAPTOSOMES FROM RAT BRAIN

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SUMMARY

Rat brain synaptosomes incubated in modified Krebs–Ringer media accumulated ^{45}Ca in amounts dependent upon the medium calcium concentration. At 37 °C, ^{45}Ca uptake was increased by 19 % upon the application of electrical pulses. Uptake was effectively inhibited by ruthenium red, uncouplers of oxidative phosphorylation, antimycin A and rotenone, and fluorocitrate while oligomycin and ouabain increased ^{45}Ca uptake. Transfer to a medium low in Na^+ led to marked increase in ^{45}Ca uptake and this effect was also found in the presence of rotenone and arsenate.

The results indicate the requirement of metabolic energy for a component of calcium uptake by synaptosomes. Effects of low Na^+ incubation and electrical stimulation may be due to events at the outer synaptosomal membrane though the possibility of direct effects at the mitochondrion have not been excluded.

INTRODUCTION

The importance of Ca^{2+} for the process by which a nerve action potential brings about release of neurotransmitter substances has been established for peripheral motor nerves [1]. Evidence has also accumulated suggesting that, upon depolarization of an axon, Ca^{2+} moves into the nerve [2]. The mechanism by which such calcium shifts occur is not well understood.

The present studies focus on Ca^{2+} accumulation by synaptosomes. Several previous studies have established that synaptosomes do accumulate Ca^{2+} in significant amounts [3–6]. Some problems in interpreting the data are due to the varying incubation conditions that have been used. Thus, some investigations have utilized hypotonic media with or without ATP, others more physiological media. These choices may reflect different views about the state of synaptosomal particles: do they resemble cell-containing tissues with semi-permeable membranes that maintain cation gradients and manufacture high-energy phosphates for use in active transport and other processes that require energy, or are they to be regarded as membranes

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Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*-tetraacetic acid.

that bind or take up cations or other substances, but require an external energy source in the form of ATP? How much of the Ca^{2+} uptake simply reflects the ability of mitochondria contained within the synaptosomal particle to take up Ca^{2+} that has leaked through the outer synaptosomal membrane? The present studies suggest that much of the synaptosomal uptake is dependent upon the presence of functioning mitochondria.

MATERIALS AND METHODS

Preparation of synaptosomes

Synaptosomes were prepared from 3–4 rat brains by differential and density gradient centrifugation by the Bradford [7] adaptation of the technique of Gray and Whittaker [8]. The synaptosomal fraction which layered above 1.2 M sucrose after density gradient centrifugation was removed with a Pasteur pipette and was carefully diluted with 1.2 vol. of cold water. This suspension was recentrifuged at $80\,000 \times g$ in a Spinco 30 rotor for 30 min. The resulting pellet was resuspended in cold incubation medium and kept on ice prior to the incubation, which was always carried out the same day, usually within 1 h.

Incubation procedures and ^{45}Ca uptake

Uptake of calcium was carried out at 30 °C in oxygenated media of the following compositions: Medium 1. 124 mM NaCl, 5 mM KCl, 20 mM NaH_2PO_4, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$ and 10 mM glucose, pH 7.4, and $CaCl_2$ as indicated. Medium 2. Similar to Medium 1, with 30 mM Tris–HCl substituting for NaH_2PO_4 as buffer.

After 15 min preincubation, 5–10 μl $CaCl_2$ (2 μCi ^{45}Ca; to the final indicated concentration) was added to synaptosomes (1.5–2 mg protein) suspended in 1.0 ml (in triplicate) medium at 30 °C. Portions (usually 150 μl) were removed at intervals and placed into 500 μl of cold ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetra-acetic acid (EGTA) stopping solution: 120 mM NaCl, 5 mM KCl, 30 mM EGTA, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 20 mM Tris, pH 7.4 [6]. Samples (in stopping solution) were kept on ice for 3 min, unless stated otherwise, and 200 μl were then applied to Schleicher and Schuell membrane filters (0.45 μm) which had been pre-washed with 5 ml of cold 0.25 M KCl. This was done on a manifold (Millipore Sampling Manifold 3025) with 30 filtering positions in the cold room (5 °C) and the manifold was connected to a Duo-Seal vacuum pump. Within 5–10 s all of the fluid passed through the filter and the residue was washed three times with 3 ml of cold 0.25 M sucrose. Then the filters were collected, placed in glass counting vials, and briefly dried at 80–100 °C before addition of 10 ml of toluene-base liquid scintillation counting fluid.

ATP determination

A sample of the suspension of synaptosomes (usually 0.4 ml) was centrifuged at 3000 rev./min in the Sorvall SS-34 rotor for 5 min in glass 5-ml centrifuge tubes with adaptors. The pellet was resuspended in the same volume of cold 6% trichloroacetic acid and was centrifuged again. The supernatant from this extraction was extracted twice with 0.5 ml of cold diethylether to remove the trichloroacetic acid. A

0.2-ml aliquot of aqueous trichloroacetic acid-free extract was used for ATP assay. ATP was assayed as previously described [9].

Respiration and electrical stimulation

In certain experiments, respiration rates with or without electrical stimulation of synaptosomes were measured. This was done in Warburg flasks fitted with concentric gold electrodes [10] (electrical stimulation studies) at 37 °C in Medium 1 with a final volume of 5 ml. Condenser pulses were used at 100/s with an amplitude of 10 V and a time constant of 0.2 ms [11].

RESULTS

Respiration by synaptosomes

Synaptosomes respired in a linear fashion for at least 90 min in physiological media buffered either with Tris or phosphate ions (data not shown). In either medium, application of electrical pulses was accompanied by a rise of about 40 % in respiration rate in confirmation of the results of Bradford and co-workers [12, 13]. The respiration rate fell to levels equal to or somewhat lower than the pre-stimulation rates after electrical stimulation was stopped. These effects of stimulation are remarkably similar to those with brain slices [14].

Calcium uptake by synaptosomes

In preliminary experiments it was observed that a portion of the ^{45}Ca bound by the synaptosomes during the incubation period was subsequently lost in the cold EGTA stopping solution. The loss was most rapid during the first 2 min and then fell at a slower rate over the subsequent 8 min. For subsequent studies, a standard period of 3 min in EGTA stopping solution was adopted.

When synaptosomes were incubated in oxygenated Tris-buffered medium, uptake of ^{45}Ca was shown to increase with time (data not shown). The rate of uptake and amount of ^{45}Ca accumulated by the particles was dependent upon the medium calcium concentration and the synaptosomes were generally not saturated after 10 min. With media concentrations of 0.1, 0.5, 1.2 and 5 mM $^{45}CaCl_2$, synaptosomes accumulated 0.22, 0.63, 1.19 and 1.70 μg atoms ^{45}Ca/100 mg protein at 10 min, respectively.

The calcium accumulation by synaptosomes in these experiments was also sensitive to electrical stimulation (data not shown). After 10 min at 37 °C electrically stimulated synaptosomes had accumulated 18.6 % more ^{45}Ca than the control unstimulated preparations. The increase appeared to be due to an increase in the early rate of uptake in the stimulated slices.

Effects of La^{3+} and ruthenium red

La^{3+} has been used to displace Ca^{2+} from accessible binding sites, presumably on the plasma membrane [15]. Addition of lanthanum in either the EGTA stopping solution or in the preincubation and incubation media led to apparent stimulation of ^{45}Ca uptake by synaptosomes (Fig. 1). In marked contrast, calcium uptake was markedly inhibited by ruthenium red, an agent that has been shown to inhibit calcium uptake in mitochondria [16] and whole cell systems.

Effects of medium tonicity and ATP

Incubation of synaptosomes in a hypotonic medium had profound effects on the influence of ATP on ^{45}Ca uptake (Fig. 2). In the absence of added ATP, synaptosomes in hypotonic medium contained 54 % more ^{45}Ca than those incubated in isotonic medium for 10 min. Addition of $MgCl_2$+ATP brought about a striking stimulation in ^{45}Ca uptake by the preparation in hypotonic medium, so that at 10 min the synaptosomes had accumulated 182 % more ^{45}Ca than in the isotonic medium and 45 % more than synaptosomes in the hypotonic medium without ATP or $MgCl_2$. This differed from the control isotonic preparation where ATP and $MgCl_2$ clearly inhibited ^{45}Ca uptake. Similar differences are observed if initial rates of ^{45}Ca uptake are calculated rather than net uptakes.

Effects of metabolic inhibitors

The actions of a number of potential inhibitors on ^{45}Ca uptake and on ATP content of synaptosomes were investigated (Tables I–III) in order to assess the possible contribution of mitochondria to calcium uptake processes. These studies were done in the presence of glucose as a primary substrate or in a medium containing glucose and supplemented with pyruvate and malate. In the latter medium, after 10 min incubation, ^{45}Ca uptake was stimulated 138 % over that in medium containing glucose, while ATP levels rose by 49 % (Table II). As in brain slices [17], ouabain stimulated ^{45}Ca uptake by synaptosomes, in this case a stimulation of 33 % (Table III). The presence of $MgCl_2$ lowered ^{45}Ca uptake by only 21 %. Classical uncoupling

TABLE I

EFFECTS OF INHIBITORS ON CALCIUM UPTAKE BY SYNAPTOSOMES

The studies were carried out in Medium 2, as described in the text. Unless indicated otherwise, means from three experiments, each at least in triplicate, are given ±S.E. at 10 min after addition of 1.2 mM ^{45}Ca. The inhibitors were also present during the preincubation period.

Additions	Calcium uptake (μg atoms ^{45}Ca taken up/100 mg protein)	
	Glucose in medium	Glucose+10 mM pyruvate+5 mM malate in medium
None	0.976±0.047(23)	2.326±0.140(6)
$MgCl_2$, 10 mM	0.770±0.045	—
2,4-Dinitrophenol, 0.1 mM	0.545±0.047	0.435±0.054
Rotenone, 10 μM	0.496±0.042	0.415±0.041
Fluorocitrate, 2 mM	0.116±0.018	0.486±0.209
NaF, 10 mM	0.645±0.065	1.920±0.188
NaF, 10 mM+2,4-dinitrophenol, 0.1 mM	0.568±0.063	0.476±0.082
NaCN, 2 mM	0.758±0.081	1.993±0.045
NaF, 10 mM+NaCN, 2 mM	0.958(2)	1.659(2)
Dicoumarol, 50 μM	0.392±0.039	0.390±0.032
Antimycin A, 15 μg/ml	0.694(2)	—
Oligomycin, 5 μg/ml	1.347±0.090	2.800±0.257
Ouabain, 0.1 mM	1.300(2)	—

TABLE II

EFFECTS OF INHIBITORS ON ATP CONTENT OF SYNAPTOSOMES

A portion of the samples reported in Table I were analyzed at 0 and 10 min for ATP as described in Materials and Methods. Mean values (number of experiments in parentheses) are reported ±S.E. where appropriate. The zero time values were determined at the beginning of the incubation period.

Additions	ATP (pmoles/mg protein)			
	Glucose in medium		Glucose +10 mM pyruvate +5 mM malate in medium	
	0	10 min	0	10 min
None	1.05±0.11(11)	1.16±0.10(11)	1.34(2)	1.73(2)
2,4-Dinitrophenol	0.36(2)	0.34(2)	—	—
Fluorocitrate	1.15(2)	0.55(2)	—	—
NaF	0.16(2)	0(2)	0.93(2)	0.99(2)
NaCN+NaF	0(2)	0(2)	0.54±0.06(3)	0.55±0.05(3)
Dicoumarol	0.39(2)	0.41(2)	—	—
Antimycin A	0.09(2)	0.05(2)	—	—
Oligomycin	0.26(2)	0.28(2)	—	—

TABLE III

ATP CONTENT OF SYNAPTOSOMES

The samples reported in Fig. 3 were analyzed at the 0- and 10-min points for ATP as described under Materials and Methods. Mean values are reported ±S.E. with the number of individual experiments in parentheses; each experiment represents 3–6 individual samples. The zero time values were determined at the beginning of the incubation period.

Media contents		pmoles ATP/mg protein	
Preincubation (15 min)	Incubation (10 min)	0	10 min
130 mM Na^+	130 mM Na^+	1.05 ±0.11(11)	1.16 ±0.10(11)
130 mM Na^+	0 Na^+, 130 mM choline chloride	1.18 ±0.04(3)	1.25 ±0.04(3)
130 mM Na^+ +10 μM rotenone	130 mM Na^+ +10 μM rotenone	0.121±0.03(3)	0.112±0.06(3)
130 mM Na^+ +10 μM rotenone	0 Na^+, 130 mM choline chloride +10 μM rotenone	0.176±0.04(6)	0.208±0.05(6)
130 mM Na^+ +10 μM rotenone + 5 mM arsenate	130 mM Na^+ +10 μM rotenone + 5 mM arsenate	0.12(2)	0.07(2)
130 mM Na^+ +10 μM rotenone + 5 mM arsenate	0 Na^+ +130 mM choline chloride +10 mM rotenone + 5 mM arsenate	0.19(2)	0.10(2)

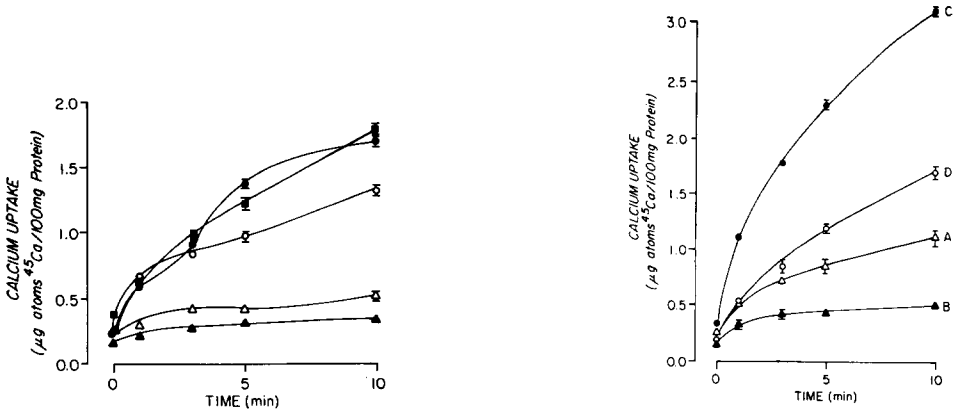

Fig. 1. Effects of La^{3+} and ruthenium red on calcium uptake. Synaptosomes were preincubated and incubated in Medium 2. Ruthenium red, when indicated was added at the beginning of preincubation at 21.5 μg/ml (△) or 215 μg/ml (▲) (single experiment); control (○). La^{3+} was present at 2 mM either from the outset of the preincubation (●) or was added to the stopping solution (■). The Ca^{2+} was 1.2 mM at zero time. Mean values for three experiments ±S.E. are shown.

Fig. 2. Effects of medium tonicity and ATP on calcium uptake. Synaptosomes were preincubated and incubated as follows: (A) Medium 2 (control), (B) Medium 2+3 mM ATP+3 mM $MgCl_2$, (C) 50 mM Tris, pH 7.4+3 mM ATP+3 mM $MgCl_2$, (D) 50 mM Tris, pH 7.4. In all cases ^{45}Ca (1.2 mM) was added at zero time. Means for 3–8 experiments ±S.E. are given. When S.E. bars are not shown they are within the dimensions of the symbol.

agents such as 2,4-dinitrophenol and dicoumarol which do not inhibit respiration, but which prevent the associated phosphorylation in mitochondria, inhibited ^{45}Ca uptake in synaptosomes by 44–59 % with glucose as substrate and by 81–83 % in the supplemented medium. The inhibitors antimycin A and rotenone, which are specific for the terminal electron transport chain in mitochondria, inhibited ^{45}Ca uptake 29–49 % with glucose and 82 % in the medium supplemented with pyruvate and malate. NaCN, which also belongs to this class of inhibitors, was less effective in lowering ^{45}Ca uptake, but its concentration in the medium is difficult to control. Oligomycin, which acts primarily on energy coupling mechanisms, stimulated ^{45}Ca uptake, unlike the other substances studied. Fluorocitrate, a potent inhibitor of the aconitase reaction, was the most effective inhibitor studied with only glucose present, lowering uptake by 88 %. In the presence of pyruvate plus malate its action was about equivalent to other inhibitors or uncoupling agents. Its action cannot be explained on the basis of possible chelation of Ca^{2+} since citrate did not exhibit similar inhibition (data not shown). All of these inhibitors were effective in lowering ATP levels in synaptosomes (Table II). Dicoumarol and 2,4-dinitrophenol decreased ATP by 65–71 %, fluorocitrate by 53 %, oligomycin by 76 %, and antimycin A by 96 % after 10 min incubation. Since these inhibitors have little or no effect on substrate level phosphorylation, attempts were made to deplete ATP levels further by inhibiting the glycolytic pathway. NaF which inhibits enolase lowered ^{45}Ca uptake by 34 % (Table I), and ATP by 100 % in this group of experiments. The combination of NaF and NaCN lowered ^{45}Ca uptake by only about 2 % (glucose medium), while completely depleting ATP. In a subsequent group of experiments some difficulty was found in reproducing these observations with NaF. Apparently the high concentration of

NaF used here causes aggregation of the synaptosomes which seems to entrap some ^{45}Ca and may account for the lack of inhibition of calcium uptake shown in Table I. We have discontinued using fluoride for this reason and instead, we have used 5 mM arsenate which interferes with glycolysis at the level of the glyceraldehyde-3-phosphate dehydrogenase reaction through an arsenolytic reaction at the substrate level. In these experiments (Table III) ATP was lowered by 91–94 % in the presence of rotenone and arsenate. In medium containing 130 mM Na^+, calcium uptake was lowered 54 % in the presence of these latter inhibitors (Figs 3C and 3E).

Effects of Na^+ concentration

Earlier studies with cerebral slices showed an increase in calcium uptake by slices incubated in media which contained LiCl or choline chloride instead of NaCl [11, 17]. ^{45}Ca uptake by synaptosomes was also altered by substitution of other cations for Na^+. Synaptosomes which were transferred to sodium-free medium accumulated 103 % more ^{45}Ca than synaptosomes incubated in sodium-containing medium (Fig. 3).

In order to assess the possible contribution of mitochondria to this process, rotenone was added. As shown in Fig. 3C, in standard incubation medium rotenone led to a 57 % inhibition of ^{45}Ca uptake and may eliminate or greatly reduce the contribution of mitochondria to the overall uptake process since synaptosomal ATP was reduced by 90 % compared to controls after the 10-min incubation (Table III). Also, rotenone or antimycin at the concentration used in Tables I and III inhibited respiration of glucose by 75–85 % (data not shown). In the presence of rotenone, transfer of synaptosomes to Na^+-free medium increased ^{45}Ca uptake by about 390 % (Figs 3C and 3D). In the presence of rotenone and arsenate, transfer to Na^+-free medium again increased ^{45}Ca uptake by synaptosomes by about 380–390 % (Figs 3E and 3F) while ATP was reduced by 91–94 % of control values. Synaptosomes

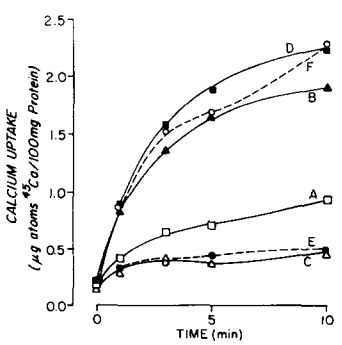

Fig. 3. Effects of Medium Na^+. Synaptosomes were preincubated in Medium 2 (Tris) for 15 min. The suspension was centrifuged for 3 min at 12 000 rev./min (Sorvall SS-34 rotor) and the pellet was resuspended to the same initial volume (1.2 ml) as follows: (A) Medium 2 (130 mM Na^+), Control, (B) Medium 2 with equimolar choline chloride to replace Na^+, (C) Medium 2+10 μM rotenone, (D) Medium 2 with equimolar choline chloride to replace Na^+ and with 10 μM rotenone, (E) Medium 2 with 10 μM rotenone and 2 mM arsenate, (F) Medium 2 with equimolar choline chloride to replace Na^+, 10 μM rotenone and 2 mM arsenate. $^{45}CaCl_2$ was 1.2 mM. Mean values for 2–11 experiments are reported. S.E. was within the dimensions of the symbols used.

were also incubated in medium containing pyruvate and malate+rotenone with similar results (data not shown).

DISCUSSION

Two current views of calcium transport in cells have been recently reviewed [18]. One suggests that passive influx and an active efflux process at the plasma membrane chiefly determine the cytoplasmic calcium concentration. The other view postulates that mitochondria are the main regulators of Ca^{2+} in cells. A few studies have been done with nervous tissues. Work with squid nerve axons suggests a mechanism linking Ca^{2+} and Na^{+} transport at the plasma membrane [19, 20]. In this process inward movement of Na^{+} provides the energy for outward Ca^{2+} movement and this Na^{+}–Ca^{2+} exchange also works in the reverse direction. Uphill movement of Ca^{2+} would depend on ATP only in the sense that energy is required to maintain a low internal Na^{+} concentration. Blaustein and Wiesmann [5] extended these studies to synaptosomes. Replacement of external Na^{+} by Li^{+} or choline or increase of internal Na^{+} by ouabain treatment all led to increased Ca^{2+} influx. Ca^{2+} efflux from synaptosomes was reduced when external Na^{+} was replaced by Li^{+}. Our previous studies [11, 17] with cerebral cortex slices also supported the idea of a Na^{+}–Ca^{2+} exchange process.

One mechanism for Ca^{2+} extrusion at the outer membrane, shown in red blood cells [21], directly uses ATP for pumping calcium. Ohashi et al. [22] and Ohtsuki [23] suggest that a low intracellular concentration of Ca^{2+} in brain could be maintained by a $(Mg^{2+}+Ca^{2+})$-ATPase system. This enzyme has been demonstrated in synaptosomes [22] and based on its specific activity could be involved in Ca^{2+} extrusion. Ruthenium red inhibits this enzyme in red cells [24]. Since in our studies this agent did not increase Ca^{2+} uptake by synaptosomes, there is no direct evidence from our data that links this system to Ca^{2+} extrusion.

An important role of mitochondria in the control of intracellular Ca^{2+} concentration has been suggested by Cittadini et al. [25] who studied Ca^{2+} uptake in Ehrlich ascites tumor cells and showed inhibition by an uncoupler of oxidative phosphorylation. Borle [18] after considering the rate constants of Ca^{2+} fluxes in mitochondria, concluded that in many cells plasma membranes contribute only 3 % and mitochondria 97 % to the control of cytoplasmic Ca^{2+} concentration.

In the present study we have used synaptosomal preparations for examination of cerebral ^{45}Ca uptake as a reasonable way of isolating the events occurring in neuronal elements from those of non-neuronal structures. The synaptosomes used in the present study, in confirmation of the findings of Bradford [12], responded to electrical stimulation with an increase in respiration rate. This supports the view that these organelles are able to maintain an internal environment that differs sufficiently from that of the surrounding medium to enable them to depolarize in response to electrical excitation, and thereby to increase their metabolic requirements for active cation transport.

The present studies confirmed that synaptosomal ^{45}Ca uptake is markedly increased when tissues are transferred to low Na^{+} media, supporting the presence of a Na^{+}–Ca^{2+} exchange mechanism at the outer membrane. In addition, however, striking changes were found when incubations were carried out in the presence of

various metabolic inhibitors, most of which produced a decrease in ^{45}Ca uptake. The most potent inhibition was produced by fluorocitrate, but uncouplers of oxidative phosphorylation and inhibitors of electron transport also reduced ^{45}Ca uptake. Contrasting effects were seen only with oligomycin which produced a consistent increase in synaptosomal ^{45}Ca uptake.

The requirement of metabolic energy for a component of the observed ^{45}Ca uptake by synaptosomes suggests that this component may reflect the ^{45}Ca accumulation ability of the intrasynaptosomal mitochondria. There has not been a great deal of study of Ca^{2+} accumulation by mitochondria isolated from brain. Mitochondria from liver have been extensively studied and contain a system for Ca^{2+} uptake that includes the following properties [26, 27]: (1) uptake can be stoichiometrically related to electron transport and can also be driven by ATP, (2) respiration-driven uptake is inhibited by uncouplers of oxidative phosphorylation and by blockers of respiration such as cyanide, antimycin A or Amytal, (3) oligomycin has no effect on respiration-dependent uptake but inhibits ATP-dependent uptake.

One of the most difficult problems in interpreting data from Ca^{2+} uptake studies on synaptosomes is to decide to what extent the uptake is influenced by the mitochondria contained within the synaptosomes. Although the decrease in ^{45}Ca uptake brought about by metabolic inhibitors does suggest an important role of mitochondria, the effects of electrical stimulation and low Na^+ media may be better interpreted as events at the synaptosomal outer membrane. The effects of electrical stimulation, though quantitatively not great, were consistent. De Belleroche and Bradford [13] have discussed the actions of electrical pulses on synaptosomes and conclude that the responses are primarily due to depolarization.

The effects of low Na^+ media on ^{45}Ca uptake were quantitatively much greater than the effects of electrical stimulation and were of particular interest in being demonstrable even in the presence of rotenone, which depresses Ca^{2+} uptake in mitochondria and in synaptosomes (Fig. 3). If the effects of low Na^+ are due to events at the outer synaptosomal membrane, this may mean that as long as an inward Na^+ gradient is present, the internal Ca^{2+} concentration can be kept at a low level. The possibility has not, however, been excluded that the effects of altering medium Na^+ are also due to direct effects at the mitochondrion itself. Experiments on purified cerebral mitochondria are underway in these laboratories and suggest that cerebral mitochondria, when incubated in low K^+, do indeed take up considerably less Ca^{2+} when Na^+ is present [28]. Interpretation of synaptosomal data must, therefore, be limited by the knowledge that mitochondrial uptake may have a major influence on these observations.

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