BOUND FORMS OF Ca TAKEN UP BY THE SYNAPTIC PLASMA MEMBRANE

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Abstract—Temperature dependent Ca-binding by the synaptic plasma membrane was increased in the presence of ATP and Mg++. Apparent Km for ATP was about $2.8 \times 10^{-5}$ M and optimal concentration of Mg++ was 2 mM in the presence of 2 mM ATP. After preincubation with nonradioactive Ca++, ATP and Mg++ to attain a steady state, addition of $^{45}$Ca resulted in remarkable labelling of the membrane, indicating rapid turnover of most of the membrane bound Ca. The presence of oxalate (60 mM) greatly increased Ca uptake on prolonged incubation. The Ca uptake in presence and absence of oxalate had similar substrate specificity and was similarly influenced by various monovalent cations. Furthermore, activities for Ca-uptake in the presence and absence of oxalate could not be separated by sucrose density gradient centrifugation of the synaptic plasma membrane fraction. Accordingly, it was considered that Ca++ in the medium was taken up by surface of the membrane, ATP- and temperature-dependently and then transferred into a cavity where the Ca-oxalate complex is formed.

The uptake of Ca** by the synaptic plasma membrane is interesting for the following reasons. 1) This uptake may be a partial reaction of energy dependent Ca-efflux, that is the so-called Ca-pump. 2) Even if such is not a partial reaction of Ca-efflux, the Ca-uptake may control the concentration of free Ca++ inside the nerve terminal. 3) This uptake may function as a regulator of the structure of excitable membranes.

Previously we reported that a low concentration of Ca++ was taken up by the synaptic plasma membrane in the presence of ATP at 37°C and properties of the Ca-uptake by the synaptic plasma membranes were different from those by mitochondria, microsomes and by sarcoplasmic reticulums (1, 2). In this work, the state of Ca taken up in the synaptic plasma membrane was examined and the following results were obtained.

MATERIALS AND METHODS

Rat cerebral synaptic plasma membranes were prepared from the nerve ending fraction by hypotonic shock and discontinuous sucrose gradient centrifugation following the method of Whittaker et al. (3) with slight modification (1, 4). Characters of the synaptic plasma membrane thus obtained have already been reported (1, 4). The synaptic plasma membranes were suspended in water and stored in a deep freezer at $-20°C$ until use. The ability of the membranes to take up Ca++ was labile and was lost in a few days, even with

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The reaction medium used for measuring Ca-uptake consisted of 50 mM Tris-HCl buffer (pH 7.4), 100 mM KCl, 2 mM MgCl₂, 2 mM ATP, 10⁻⁴ M EGTA, 9 × 10⁻⁵ M CaCl₂, and about 0.2 μCi ⁴⁵Ca in a total volume of 2 ml. Final concentration of free Ca²⁺ in this reaction mixture was calculated to be about 3 × 10⁻² M and this concentration was optimal for the Ca-uptake. The reaction was started by addition of 200–300 μg protein of synaptic plasma membranes to the prewarmed medium. After incubation at 37°C for 2 min or a certain period, the reaction was stopped by addition of 5 ml of ice-cold mixture containing 50 mM Tris-HCl buffer, 100 mM KCl and 2 mM MgCl₂. The cold reaction mixture was then passed through a Millipore filter (HA 0.45 μm pore size) and the membranes on the filter were washed twice with the same cold mixture and their radioactivity was estimated in a liquid scintillation counter as described previously (2). Without ATP, the amount of membrane bound ⁴⁵Ca was about 25–30% of that of ATP-containing system under these experimental conditions, and this value was subtracted to estimate the amount of Ca-uptake.

Succinic dehydrogenase was determined by the method of Kirshner et al. (5), and protein was estimated by the method of Lowry et al. (6)

RESULTS

Requirements of ATP and Mg²⁺ for Ca-uptake by the synaptic plasma membranes

As shown in Fig. 1, ATP was required for Ca-uptake by the synaptic plasma membranes. The apparent Km for ATP was 2.8 × 10⁻⁵ M in the presence of 2 mM MgCl₂. Mg²⁺ had a stimulatory effect and the optimal concentration of Mg²⁺ was 2 mM in the presence of 2 mM ATP (Fig. 2). The maximum amount of Ca taken up by the membranes in the presence of both ATP and Mg²⁺ was 7.8 μmoles/mg of membrane protein.

Exchange of membrane-bound Ca in the steady state

We have already shown (1, 2) that Ca-uptake by synaptic plasma membrane in the

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**FIG. 1.** Ca-uptake with various concentrations of ATP. Incubations of the synaptic plasma membranes were carried out at 37°C for 2 min in standard reaction mixture (See Methods) with or without various concentrations of ATP. Percent increase in amount of Ca bound to the membranes by addition of ATP is indicated in the ordinate. Number of experiments is shown in parentheses and standard errors are indicated by bars.
Ca-uptake with various concentrations of Mg++. Reaction mixture contained 50 mM Tris-HCl buffer (pH 7.4), 100 mM KCl, $10^{-4}$ M EGTA-Tris, $9 \times 10^{-5}$ M CaCl$_2$, $^{45}$Ca (about 0.2 $\mu$C), 2 mM ATP and various concentrations of Mg++. Incubations were carried out for 2 min at 37°C. Number of experiments is shown in parentheses and standard errors are indicated by bars.

Fig. 3. $^{45}$Ca-exchange in the steady state. - - - - Time courses of $^{45}$Ca-uptake by the synaptic plasma membrane in standard medium (See Methods). - - - - - After incubation of the membrane in standard medium except radioactive Ca($^{45}$Ca) for 2 min, small amount of $^{45}$Ca (0.2 $\mu$C) was added. Thereafter, labelling of the membrane was measured. Incubation temperature was 30°C. Number of experiments is shown in parentheses and standard errors are indicated by bars.

Presence of ATP is rapid and reaches a plateau level within 2 min and this level is maintained thereafter as shown in Figs. 3 and 4. To examine the state of bound Ca in the membranes in the steady state, $^{45}$Ca was added after incubation of the membranes with ATP and non-radioactive Ca$^{++}$ for 2 min.

In Fig. 3 the experimental results at 30°C are shown, but similar results were also obtained at 37°C. The membranes were rapidly labelled with $^{45}$Ca$^{++}$, indicating that the membrane bound Ca was rapidly exchanging with free Ca$^{++}$ in the medium. The initial velocity of labelling of the membranes by $^{45}$Ca$^{++}$ added in the steady state (2 min after starting the reaction) was slightly higher than that observed on adding $^{45}$Ca at the start of the reaction. As the plateau level was slightly but significantly lower when $^{45}$Ca was added at 2 min after the start of the reaction than when it was added at the start, a small part of
the membrane-bound Ca may not easily be exchanged with Ca\(^{++}\) in the medium, although most of it is rapidly exchanged.

**Effect of oxalate on Ca uptake by the synaptic plasma membranes**

Oxalate is known to increase the amount of Ca taken up by the sarcoplasmic reticulum (7-9). However, 1 mM oxalate did not show any significant effect on Ca-uptake by synaptic plasma membranes which had been incubated for 2 min, as described previously (1). But with longer incubation with high concentrations of oxalate, accumulation of Ca in the synaptic plasma membranes increased with time, though the initial velocity of uptake was slightly reduced (Fig. 4). The reduction in initial velocity seems to be due to decrease in free Ca\(^{++}\) concentration in the medium by oxalate. An increase in Ca-accumulation by brain microsomes in the presence of high concentrations of oxalate has recently been reported (10, 11). The optimal concentration of oxalate for Ca-accumulation by synaptic plasma membranes was 60 mM, as shown in Fig. 5. With this concentration of oxalate, the amount of Ca accumulated in the membrane reached 51.5 m\(\mu\)moles per mg of the membrane protein after incubation for 30 min. The concentration of oxalate had no influence on amount of Ca trapped on the filter without addition of the membrane.

**Fractionation of the synaptic plasma membrane preparation**

Electron microscopy showed that the preparation of synaptic plasma membranes was contaminated with mitochondria and tissue fragments. Accordingly, the preparation of
FIG. 6. Distribution of activities of Ca-uptake in the presence and absence of oxalate and succinic dehydrogenase in subfractions of the membrane preparation on sucrose density gradient. The membrane suspension in water (about 8 mg protein) was overlaid on continuous sucrose gradient from 0.6 to 1.2 M and centrifuged at 53,500 g for 60 min in a rotor RPS 25A of a Hitachi ultracentrifuge at 0°C. 1.5 ml portions from the bottom were then taken with a fraction collector. Activities of Ca-uptake in the presence and absence of oxalate and succinic dehydrogenase were determined in each fraction. Activity of Ca-uptake in presence of oxalate (60 mM) was estimated by 30 min incubation and that in absence of oxalate was measured by 2 min incubation at 37°C. The amount of membrane bound Ca by incubation in ATP free medium was subtracted to estimate the Ca-uptake.

The synaptic plasma membranes was fractionated by centrifugation on a continuous density gradient of 0.6-1.4 M sucrose, and the activities of Ca-uptake in presence and absence of oxalate in each fraction were measured. Their succinic dehydrogenase activities were also estimated as a marker of mitochondria. As shown in Fig. 6, the activities of Ca-uptake in the presence and absence of oxalate formed a single peak with maximum in the same fraction, completely separated from the peak of succinic dehydrogenase activity.

Substrate specificities of Ca-uptake by synaptic plasma membranes in presence and absence of oxalate

As shown in Table 1, ATP was a specific substrate for the Ca-uptake either in the presence or absence of oxalate. Other phosphate compounds, such as GTP, UTP, CTP, ITP, carbamylphosphate, acetylphosphate and p-nitrophenylphosphate, were ineffective as substrates for either process. Thus these membranes differ from the sarcoplasmic reticulum, where ITP, CTP, GTP, acetylphosphate and p-nitrophenylphosphate can serve as energy donors for Ca-uptake (12-16).

Influence of monovalent cations on Ca-uptake by synaptic plasma membranes in the presence and absence of oxalate

As observed previously, Ca-uptake in the absence of oxalate by synaptic plasma membranes was strongly influenced by monovalent cations, especially by Na+. Na+ inhibited the Ca-uptake and released the membrane bound Ca. This Na+ effect was increased in the
TABLE 1. Effects of various phosphate compounds on the Ca-uptake

| Phosphate Compound | Without Oxalate (100%) | With Oxalate (60 mM) |
|--------------------|------------------------|----------------------|
| ATP                | 100                    | 100**                |
| UTP                | 5.3                    | negligible (<1)      |
| ITP                | 9.3                    | negligible (<1)      |
| GTP                | 0.1                    | negligible (<1)      |
| CTP                | negligible             | negligible (<1)      |
| PNP-P              | 2.9                    | negligible (<1)      |
| Carb.-P            | 3.4                    | negligible (<1)      |
| Acetyl.-P          | negligible             | negligible (<1)      |

All phosphate compounds were added at a concentration of 2 mM. Incubations were carried out for 2 min without oxalate and 30 min with it. Mean values of different two experiments are shown.

* Amount of Ca taken up was 5.9 µmols/mg of membrane protein.
** Amount of Ca was 51.5 µmols/mg of membrane protein.

TABLE 2. Effects of monovalent cations on Ca-uptake in the presence and absence of oxalate

| Addition (100 mM) | Absence of Oxalate | Presence of Oxalate (60 mM) |
|-------------------|--------------------|-----------------------------|
|                   | Basic medium       | KCl (100 mM)                | Basic medium       | KCl (100 mM)                |
| None              | 100                | 100**                       | 100                | 100**                       |
| KCl               | 143.7±15.7 (4)     | 115.0±4.0 (4)               | 162.1±4.1 (4)      | 144.4±3.1 (4)               |
| RbCl              | 138.1±9.6 (4)      | 115.8±7.0 (4)               | 152.3±4.2 (4)      | 131.0±5.2 (4)               |
| CsCl              | 135.4±7.5 (4)      | 112.9±4.7 (4)               | 137.0±5.6 (4)      | 146.4±6.5 (4)               |
| LiCl              | 94.2±8.9 (4)       | 96.9±1.6 (4)                | 94.5±3.0 (4)       | 116.7±5.3 (4)               |
| NaCl              | 41.7±4.7 (4)       | 31.2±5.4 (4)                | 110.1±11.1 (4)     | 71.9±9.3 (4)                |

* Amount of Ca taken up was 5.9 µmols/mg of membrane protein. ** Amount of Ca was 51.5 µmols/mg of membrane protein. *** Mean value±standard error (numbers of experiments). Basic medium contained 50 mM tris-HCl buffer (pH 7.4), 2 mM MgCl₂, 2 mM ATP-tris, 10⁻⁴ M EGTA, 9·10⁻⁵ M CaCl₂ and about 0.2 µC of Ca.

presence of KCl (2). We then examined the influence of oxalate on the effects of monovalent cations on Ca-uptake by synaptic plasma membranes.

As shown in Table 2, Ca-uptake in the presence of oxalate was increased by K⁺ and Rb⁺, and decreased by Na⁺ and Li⁺ but the inhibitory effects were less remarkable compared with effects in the absence of oxalate. These results imply that Na⁺ decreases the amount of membrane bound Ca but has no significant influence on the Ca-oxalate complex.

DISCUSSION

Uptake of ⁴⁰Ca by the synaptic plasma membranes was greatly increased by ATP and Mg²⁺, and this increase was due to a net increase in the amount of Ca bound, as already reported (1).

Most of the bound Ca was rapidly exchanged, as shown in Fig. 3 and the turnover of...
binding sites for Ca\(^{++}\) also appeared to be rapid. One possible binding site for Ca-uptake which has a high turnover in the membranes is Mg·Ca-ATPase, and it is known to bind Ca in the sarcoplasmic reticulum (7). Previously, we reported that the synaptic plasma membranes showed Mg·Ca-ATPase activity (4). But La\(^{+++}\), Mn\(^{++}\) and ruthenium red did not depress Ca-uptake by the synaptic plasma membranes at concentrations which caused strong, selective inhibition of Mg·Ca-ATPase activity (17). Thus it seems unlikely that Mg·Ca-ATPase activity is closely connected with the Ca-uptake. Another candidate for Ca-binding site seems to be polyphosphoinositide which has already been postulated with erythrocyte ghosts (18) and with brain microsomes (11, 19). Furthermore it was reported that enzymes involved in the incorporation of \(^{32}\)P from ATP-\(^{32}\)P into polyphosphoinositides were localized on the cytoplasmic surface of erythrocyte membranes (20), and in brain synaptosomes phosphatidylinositol kinase was mainly present in the synaptic plasma membranes (21). However, the optimal concentrations of Mg\(^{++}\) for phosphatidylinositol kinase and diphosphoinositide kinase were reported to be 40 and 20 mM, respectively (22, 23) whereas we found that the optimal concentration of Mg\(^{++}\) for Ca-uptake by the synaptic plasma membranes was 2 mM. At present the nature of Ca-binding sites in the synaptic plasma membrane is unknown.

The activities for Ca-uptake in the presence and absence of oxalate could not be separated by sucrose density gradient centrifugation. Furthermore, they showed the same substrate specificity and were similarly influenced by various monovalent cations, though to a different extent. Accordingly, the following assumptions are made on bound forms of Ca in the membrane. Ca\(^{++}\) in the medium is taken up by surface of the membrane in the presence of ATP, Mg\(^{++}\) and K\(^{+}\) and the bound Ca may then move into the hydrophilic cavity by some unknown mechanism, such as rotation of a high molecule component to which Ca\(^{++}\) is bound. In the presence of oxalate, a Ca-oxalate complex may be formed in the cavity resulting in an increase in the amount of bound Ca in the membrane. But at present it is unknown whether the Ca-oxalate complex is formed in the intramembranal cavity or the complex is present in the cavity of the membrane vesicle. The latter was considered after an electronmicroscopic finding that our membrane preparation contained a few vesicular structures, and on a report which described formation of vesicular structure by incubation of erythrocyte ghosts with ATP (24).

The Ca taken up by the membranes seems to be of physiological importance in

![Fig. 7. Effect of ionic composition of the medium on Ca-uptake by synaptic plasma membrane. The synaptic plasma membranes were incubated at 37°C for 2 min in reaction mixtures containing 50 mM Tris-HCl buffer (pH 7.4), 2 mM MgCl\(_2\), 2 mM ATP-Tris, 0.1 mM EGTA-Tris, 9×10^{-5} M CaCl\(_2\) (0.2 \text{uC} \text{45Ca}) and 150 mM monovalent cations (NaCl and KCl) in various ratios as indicated in the abscissa. Mean values and standard errors from 3 different experiments are shown.](image-url)
regulating the concentration of free Ca\(^{++}\) in the cytoplasm of the nerve endings, because the amount of Ca taken up by the membranes is reduced by Na\(^+\) and increased by K\(^+\) in isotonic media as shown in Fig. 7. If the number of synapses per gram wet weight of brain cortex is indeed \(4 \times 10^{11}\), as calculated by Whittaker (25) and the mean volume of nerve endings is 0.1 \(\mu^3\), then volume of nerve ending in 1 g of brain should be about \(0.4 \times 10^{11} \mu^3\). Accordingly, about 40 \(\mu l\) per gram of fresh brain seems to be occupied by nerve endings and about 26.6 \(\mu l\) by their cytoplasm. We obtained 3.4 mg of protein of synaptic plasma membranes from 1 g wet weight of brain. Therefore, about 20 \(m\) moles of Ca are taken up ATP-dependently by the synaptic plasma membranes in 1 g of brain. Accordingly, if 20 \(m\) moles of Ca are present in the free form in synaptosomes in 1 g of brain, the concentration of free Ca\(^{++}\) in the cytoplasm of the nerve endings is more than \(10^{-1}\) M. These calculations are based on many assumptions, but they show that the amount of Ca taken up by the membranes is quite sufficient to regulate the physiological function of Ca\(^{++}\) at the nerve endings. Accordingly, regulation of Ca-uptake by Na\(^+\) and K\(^+\) seems to be as closely related with the function of nerve endings as with the release of transmitters.

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