CALCIUM BINDING OF ISOLATED SYNAPTIC MEMBRANES
FROM RAT CEREBRAL CORTEX

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When nerve cells are stimulated, influx of Na<sup>+</sup> and efflux of K<sup>+</sup> increase and the action potential thus produced is propagated as a nerve impulse. On the other hand, a transmitter must be released from synaptic terminals to transmit nerve impulses to the next cell and the presence of extracellular Ca<sup>++</sup> is required for this release. It is therefore considered that the concentration of intracellular free Ca<sup>++</sup> increases with change in the membrane potential and release of the transmitter (1, 2).

In relation to the Ca<sup>++</sup> movement in synaptic area, Ca<sup>++</sup>-uptake by brain microsomes (3-7), and synaptosomes (3, 8, 9) were studied and the regulatory function was proposed. Ca<sup>++</sup> uptake by synaptic membrane was not studied herein.

Whittaker et al. (10, 11) isolated a nerve ending fraction (synaptosomes) by discontinuous sucrose density gradient centrifugation of rat brain crude mitochondrial fraction. Synaptic membranes having high Na-K ATPase (12-14) and Ca<sup>++</sup>-stimulated ATPase (7, 14) activities were then prepared from this fraction by treatment under hypotonic conditions and sucrose gradient centrifugation. In this work, Ca<sup>++</sup>-binding by synaptic membranes was studied and it was found that the ATP-dependent Ca<sup>++</sup>-binding had a specific character.

MATERIALS AND METHODS

Preparation of rat brain synaptic membranes:

Synaptic membranes were prepared from the cerebral cortex of Sprague-Dawley rats weighing 200-250 g, by a modification of the method of Whittaker et al. (11), as shown in Fig. 1. The cerebral cortex was homogenized in 0.32 M-sucrose and a crude mitochondrial fraction (P<sub>2</sub>) was separated. P<sub>2</sub> was then resuspended in 0.32 M-sucrose and layered on a discontinuous density gradient consisting of 0.8 M- and 1.2 M-sucrose. The gradient was centrifuged at 53,500 × g for 1 hr, and then the intermediate phase between 0.8 M- and 1.2 M-sucrose was diluted with 2 volumes of water and centrifuged at 105,000 × g for 30 min. The resultant pellet (synaptosomes) was disrupted by adding 2.5 ml of water per g of the original tissue. The preparation was then layered on a sucrose density gradient (0.6 M and 1.0 M) and centrifuged at 53,000 × g for 1 hr. The intermediate zone was diluted with an equal volume of water, and the synaptic membranes were obtained by
centrifuging the mixture at 105,000 × g for 30 min. In the second sucrose gradient, the upper zone consisted mainly of synaptic vesicles and small membranes while the pellet at the bottom consisted of intrasynaptosomal mitochondria (ISM) and unbroken synaptosomes. The precipitate obtained by centrifugation of S₂ at 105,000 × g for 30 min was used as the microsomal fraction.

**Electron microscopy**

Samples were fixed with 2% OsO₄, embedded in propylene resin and examined under a Hitachi 7S electron microscope after staining with 1% uranyl-acetate and lead citrate.

**Assay of Na-K ATPase and Ca²⁺-stimulated ATPase activity**

Na-K ATPase activity was calculated by subtraction of Mg²⁺-ATPase activity, assayed in the presence of 0.1 mM ouabain and absence of NaCl and KCl from the total ATPase activity, assayed in medium containing 40 mM tris-HCl buffer (pH 7.4), 100 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 2 mM ATP. Mg²⁺-ATPase activity was assayed in medium containing 40 mM tris-HCl buffer (pH 7.4), 100 mM KCl, 2 mM MgCl₂, 0.1 mM EGTA, 0.1 mM ouabain and 2 mM ATP, and the difference between ATP hydrolysis in the presence and absence of 9 × 10⁻⁵ M CaCl₂ was taken as Ca²⁺-stimulated ATPase activity. Na-K ATPase and Ca²⁺-stimulated ATPase activities were both estimated in a final volume of 2.0 ml and the reaction was terminated with 0.5 ml of 50% TCA. After centrifugation, inorganic phosphate in the supernatant was assayed by Takahashi's method (15).

**Estimation of succinate dehydrogenase (SDH) activity**

SDH activity was measured by the method of Kirshner et al. (16). Samples were incubated in medium containing 0.5 ml of 0.2 M phosphate buffer (pH 7.4), 0.05 ml of
0.1 mM cytochrome c, 0.05 ml neotetrazolium chloride (5 mg/ml) and 0.1 ml of 0.2 M Na succinate (pH 7.4) at 37 °C for 30 min. The reaction was stopped by adding 3 ml of acetone and after centrifugation, the optical density was measured at 500 mλ. The enzyme activity was expressed as O.D. per mg protein per 30 min.

Assay of ACh

ACh was assayed using frog abdominal muscle following the method of Chang and Gaddum (17).

Ca**-binding and measurement of Ca

Ca**-binding was estimated under similar conditions to those used for assay of Ca**-stimulated ATPase activity. The reaction was stopped by adding 4 ml of the same medium, cooled with ice, and the membranes were removed by centrifugation at 105,000 × g for 30 min. Total Ca was measured with a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Samples were ashed by treatment with nitric acid and evaporated to dryness. The ash was dissolved in hydrochloric acid and lanthanum chloride was added to a final concentration of 0.5%. When ^{45}Ca was used for binding experiments, bound ^{45}Ca was extracted with 10% TCA and radioactivity was measured in a liquid scintillation counter with Bray's solution (18).

Calculation of the free calcium concentration [Ca++]

The concentration of free calcium was calculated by the method of Portzehl et al. (19) using medium containing 20 mM tris-maleate buffer (pH 7.0), 100 mM KCl, 2 mM MgCl, and 0.1 mM ouabain with or without 2 mM ATP. The concentration of Ca-EGTA plus EGTA was fixed at 2 mM.

Release of ^{45}Ca

Synaptic membranes, microsomes and ISM preloaded with ^{45}Ca by the method described above were suspended at 0 °C in medium containing 40 mM tris-HCl buffer (pH 7.4), 100 mM KCl and 2 mM MgCl. The suspensions were then incubated at 37 °C to a final volume 2.0 ml with or without ATP. The reaction was stopped by adding 4 ml of cold medium as in binding experiments. After centrifugation at 105,000 × g for 30 min, the amount of ^{45}Ca remaining in the precipitate was measured.

Protein assay

Protein was determined by the method of Lowry et al. (20) with bovine serum albumin as a standard.

RESULTS

1. Electron microscopy and distribution of ACh and SDH, Na-K ATPase, and Ca**-stimulated ATPase activities

Electron micrographs of the preparation of synaptosomes and synaptic membranes used in this experiment are shown in Fig. 2A and 2B. Synaptosomes contained synaptic vesicles and ISM (Fig. 2A), while the synaptic membranal fraction did not (Fig. 2B).

The ACh contents and SDH, Na-K ATPase and Ca**-stimulated ATPase activities of these fractions are shown in Table 1.
The synaptosomal fraction contained much ACh. SDH, an enzyme used as a mitochondrial marker, was localized predominantly in the bottom fraction both in the first and the second sucrose density gradient and was found in synaptic membranal fraction to some degree.

Na-K ATPase and Ca**-stimulated ATPase activities were found in the synaptosomal fraction.

Fig. 2A. Electron micrograph of synaptosomal fraction (×25,000).

Fig. 2B. Electron micrograph of synaptic membranes (×14,000).
fraction and further fractionation of this, resulted in high specific activities in the synaptic membranal fraction. These results are similar to those obtained previously (7, 14).

Ca²⁺-stimulated ATPase activity was maximal at a concentration of 9 × 10⁻⁵ M CaCl₂ in the presence of 10⁻³ M EGTA.

2. Ca²⁺-binding to synaptic membranes

Ca²⁺-binding to synaptic membranes was investigated under conditions similar to those used to measure Ca²⁺-stimulated ATPase activity. As shown in Table 2, there was no alteration in the membranal Ca²⁺-content with 9 × 10⁻⁵ M CaCl₂ or with CaCl₂ plus 2 mM ATP at 0 °C. However, at 37 °C the Ca²⁺-content of the membranes increased by 8–10 mµmoles per mg protein in the presence of CaCl₂ and ATP. This ATP and temperature dependent Ca²⁺-binding was completed in 1–2 min (Fig. 3).

The Ca²⁺-binding of microsomes was also investigated under the same conditions. The Ca²⁺-binding of the microsomal fraction reached a maximum in 1–2 min but amounted to only 2–3 mµmoles of Ca²⁺-binding per mg protein (Table 3). The Ca²⁺-content of ISM could not be determined from the atomic absorption spectrophotometer because of

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**TABLE 1. Distribution of ACh and SDH, Na-K ATPase and Ca²⁺-stimulated ATPase activities.**

| Microsomes | ACh content (mµmoles/mg protein) | SDH activity (O.D. mg protein 30 min) | Na-K ATPase activity (mµoles Pi/mg protein 30 min) | Ca²⁺-stimulated ATPase activity (mµoles Pi/mg protein 30 min) |
|------------|---------------------------------|--------------------------------------|-----------------------------------------------|-----------------------------------------------------------|
| P₁         | 0.147                           | 0.32                                 | 1.5                                           | 0.4                                                       |
| P₃         | 0.039                           | 0.06                                 | 3.6                                           | 0.7                                                       |
| P₅         | 0.204                           | 0.29                                 | 0.6                                           | 1.2                                                       |
| P₆         | 0.025                           | 1.29                                 | 3.6                                           | 1.7                                                       |
| P₇         | 0.21                            | 4.3                                  | 4.3                                           | 1.0                                                       |
| P₈         | 0.90                            |                                       | 14.4                                          | 2.8                                                       |

**TABLE 2. Ca²⁺-binding to synaptic membranes.**

| Temperature | mµmoles Ca²⁺/mg protein |
|-------------|-------------------------|
| No addition | 22.4 ± 3.1              |
| 0 °C        | 19.2 ± 1.5              |
| 17.9 ± 4.0  | 21.1 ± 3.2              |
| 19.9 ± 2.3  | 30.9 ± 2.1              |
| 19.5 ± 1.8  | 16.2 ± 2.5              |

Synaptic membranes were incubated in medium containing 40 mM tris-HCl buffer (pH 7.4), 100 mM KCl, 2 mM MgCl₂, 0.1 mM EGTA and 0.1 mM ouabain with or without 2 mM ATP and 9 × 10⁻⁵ M CaCl₂. Values are averages of 3 experiments ± S.D.
Fig. 3. Time course of Ca\textsuperscript{2+}-binding to synaptic membranes.
Synaptic membranes were incubated at 37°C in the presence of CaCl\textsubscript{2} and ATP. Points represent means ± S.D. of 3 experiments.

| Table 3. Ca\textsuperscript{2+}-binding to microsomal fraction. |
|---------------------------------------------------------------|
| my\textsuperscript{moles} Ca\textsuperscript{2+}/mg protein |
| No addition | 22.7 ± 7.2 |
| Ca            | 21.7 ± 4.4 |
| Ca, ATP       | 24.9 ± 3.7 |
| ATP           | 25.2 ± 5.7 |

The microsomal fraction was incubated at 37°C for 2 min in medium containing 40 mM tris-HCl buffer (pH 7.4), 100 mM KCl, 2 mM MgCl\textsubscript{2}, 0.1 mM EGTA, 0.1 mM ouabain with or without 2 mM ATP and 9 × 10\textsuperscript{-4} CaCl\textsubscript{2}. Values are averages of 3 experiments ± S.D.

interference.

Brain microsomes are reported to bind much Ca\textsuperscript{2+} (3–5, 21, 22), so the Ca\textsuperscript{2+}-bindings of synaptic membranes and microsomes were compared under the same conditions used in earlier work (3).

In medium containing 40 mM tris-HCl buffer (pH 7.4), 0.25 M sucrose, 2.5 mM MgCl\textsubscript{2}, 0.01 mM CaCl\textsubscript{2}, and 2 mM ATP, the microsomal fraction bound 5–6 my\textsuperscript{moles} of Ca\textsuperscript{2+} per mg protein, while the synaptic membranes bound only about 2 my\textsuperscript{moles} of Ca\textsuperscript{2+} per mg protein. This difference in the amounts of Ca\textsuperscript{2+} bound to synaptic membranes and microsomes according to the experimental conditions seems to be explained mainly by the difference in the Ca\textsuperscript{2+} concentration of the reaction mixture, as described in detail later (Fig. 5).

3. Binding of radioactive Ca\textsuperscript{2+}(\textsuperscript{45}Ca) to synaptic membranes, microsomes and ISM

Next, we used \textsuperscript{45}Ca to examine the Ca\textsuperscript{2+}-binding reactions of synaptic membranes, microsomes and ISM. Using unlabeled Ca\textsuperscript{2+}, the amount of Ca\textsuperscript{2+} bound to synaptic
FIG. 4. Effect of CaCl₂ concentration on ATP-dependent Ca²⁺-binding.

Synaptic membranes containing 2.5 mg protein were incubated for 2 min in the same medium as for Table 2 with various concentrations of added CaCl₂. 4 x 10⁵ c.p.m. of ⁴⁵Ca (3.05 mCi/mg) was added to the media and the amount of Ca²⁺ bound was calculated by the equation:

\[
\text{Ca²⁺ bound/mg protein} = \frac{\text{Concentration of CaCl₂ in medium (m pmoles) x c.p.m. in membranes}}{4 \times 10^5 \times \text{mg protein}}.
\]

membranes was expressed in terms of the amount of protein (in mg) precipitated by centrifugation at 105,000 xg after incubation. Using ⁴⁵Ca, the amount of ⁴⁵Ca bound was calculated in terms of the amount of protein incubated. A considerable amount of protein (about 35 %) was not precipitated by centrifugation after incubation, so when ⁴⁵Ca was incubated with synaptic membranes the amount of ⁴⁵Ca bound to synaptic membranes was calculated to be less than the value shown in Table 2.

The relation between the CaCl₂ concentration and Ca²⁺-binding was investigated. The ATP-dependent Ca²⁺-binding, calculated from the difference between the amounts bound in the presence and absence of ATP, was maximal at a concentration of 9 x 10⁻⁵ M CaCl₂, like Ca²⁺-stimulated ATPase, in the presence of 10⁻⁴ M EGTA (Fig. 4). The ISM fraction bound twice as much CaCl₂ as the synaptic membranes, but required 5-10 min for maximum binding. Maximum binding was observed at a concentration of 10⁻⁴ M-2 x 10⁻⁴ M CaCl₂ and was similar at 5 x 10⁻⁴ M CaCl₂. Oligomycin (2.5 μg/ml) inhibited the Ca—binding of ISM by 85-90 %.

In Fig. 5, the ATP-dependent Ca²⁺-binding is shown as a function of the concentration of free calcium ion [Ca²⁺] to compare the sensitivities of the synaptic membranes, microsomes and ISM to calcium. The concentration of free calcium at pH 7.0 was adjusted by the ratio of [Ca-EGTA] to [EGTA]. Results showed that synaptic membranes bound much Ca²⁺ at a low concentration of Ca²⁺ and maximum binding was found at a concentration of 3.3 x 10⁻⁷ M Ca²⁺. On the other hand, the Ca²⁺-binding of the microsomal
FIG. 5. Effect of Ca\(^{++}\) concentration on ATP-dependent Ca\(^{++}\)-binding to synaptic membranes, microsomes and ISM at \(r=2.0\).

The Ca\(^{++}\) concentration was adjusted with calcium-EGTA buffer taking the equilibrium constant as \(K=5 \times 10^{-5} \text{M}\). Synaptic membranes (1.8 mg) or microsomes (2.85 mg) were incubated for 2 min, and ISM (3.8 mg) was incubated for 6 min in medium containing 20 mM tris-maleate buffer (pH 7.0), 100 mM MgCl\(_2\), 0.1 mM ouabain, and 2 mM of EGTA plus Ca-EGTA and 2 mM ATP. 2.9 \times 10^{6} \text{c.p.m. (2.72 mCi/mg Ca)} \) of \(^{45}\text{Ca}\) was applied and the amount of Ca\(^{++}\) bound was calculated from the equation same as in FIG. 4.

a. O---O ; synaptic membranes, b. •---• ; microsomes, c. •---• ; ISM.

The above results show that the character of Ca\(^{++}\)-binding of synaptic membranes differs from that of microsomes or ISM.

4. Nucleotide specificity of Ca\(^{++}\)-binding to synaptic membranes

Ca\(^{++}\)-binding by the synaptic membranes showed nucleoside triphosphate specificity, as shown in Table 4. ADP was slightly effective, but other nucleotides were ineffective. These results were consistent with the report of Lust and Robinson (9) on binding by synaptosomes. The presence of Mg\(^{++}\) was required for the Ca\(^{++}\)-binding, and maximal binding was observed in the presence of Mg\(^{++}\) concentrations greater than 0.25 mM (not shown).

5. Effects of drugs on Ca\(^{++}\)-binding to synaptic membranes

Ca\(^{++}\)-binding to synaptic membranes unlike the binding to the sarcoplasmic reticulum
of skeletal muscle, was not stimulated by oxalate (10^{-3} M). Moreover it was not affected by ouabain (10^{-4} M), salyrgan (5 \times 10^{-5} M), or protovatrine (10^{-4} M). Oligomycin (2.5 \gamma/ml) inhibited the Ca^{++}-binding about 40%.

6. Effect of caffeine on 45Ca release

Axelsson and Thesleff (23) demonstrated that caffeine induced contracture of frog muscle without change of the membrane potential. Caffeine causes release of Ca^{++} from frog sarcoplasmic reticulum, (24–26) and so it has recently been considered that the contracture brought about by caffeine is induced by release of sarcotubular Ca^{++}. Caffeine

![Fig. 6. Effects of caffeine on retention of 45Ca.](image)

Synaptic membranes, microsomes or ISM which had been preloaded with 45Ca were resuspended at 0°C in medium containing 40 mM tris-HCl buffer (pH 7.4), 100 mM KCl, 2 mM MgCl2 and incubated at 37°C with or without ATP.

- ●●● control, ◯—◯ ATP (2 mM), ×—× caffeine (1 mM), □—□ ATP (2 mM) + caffeine (1 mM), △—△ ATP (2 mM) + caffeine (5 mM).

Data were shown as the percentage of 45Ca retention of that of zero-time incubation.
also stimulates the central nervous system. Thus it seemed interesting to investigate the effect of caffeine on Ca" in synaptic membranes.

Caffeine was found to have no effect on Ca"-binding by synaptic membranes. Next its effect on "^Ca retention in synaptic membranes, microsomes and ISM preloaded with "Ca was investigated. As shown in Fig. 6, in the absence of ATP, retention of "Ca in synaptic membranes, microsomes and ISM decreased rapidly, with caffeine having no effect. (The effect on microsomes was not investigated.) However, in the presence of ATP, "Ca was retained, and caffeine decreased its retention in synaptic membranes but not in the microsomes or ISM. Approx. 80% of radioactivity of "Ca preloaded membrane was lost by the procedure of suspending in incubation medium at 0°C. In Fig. 6, data is shown as percentage of "Ca retention at zero-time incubation. For this reason, the retention of "Ca is above 100% in some cases in Fig. 6, probably due to reuptake during the incubation at 37°C.

**DISCUSSION**

In this work a crude mitochondrial fraction from rat brain cortex was fractionated by a modification of method of Whittaker et al. (11). Synaptic membranes, where active transports of Na^+ and K^+ occur, have high Na-K ATPase activity (12-14). They also have high Ca"-stimulated ATPase activity (7, 14), therefore it has been suggested that this may be related to Ca"-binding. The Ca"-binding by synaptic membranes and brain microsomes was ATP and temperature dependent. It was rapid and not enhanced by oxalate, unlike that of the sarcoplasmic reticulum of skeletal muscle. In these characters, the Ca"-binding of synaptic membranes was similar to that of brain microsomes. The two differed, however, in the amounts of the Ca"-binding according to the experimental conditions, as shown in Tables 2 and 3 and in the text. The difference between the two experimental conditions was no doubt due to the concentration of free Ca" (Fig. 5).

On the other hand, ISM bound approx. twice as much Ca" as the synaptic membranes, but required 5-10 min for maximum uptake and was inhibited by oligomycin.

From these results, it appears likely that the Ca"-binding observed in this experiment is specific for synaptic membranes. It should also be noted that the synaptic membranes were the most sensitive to a low concentration of calcium (Fig. 5).

Ohtsuki (5) suggested that Ca"-uptake by brain microsomes is important for regulation of the intracellular Ca" level. On the other hand, Lust et al. (8, 9) regarded the mitochondria as the site of Ca" regulation. Moreover, we considered that the Ca"-binding observed in this experiment to be partially involved in the regulation of intracellular concentration of Ca", especially at low Ca" concentration. Other physiological explanations should be considered on this Ca"-binding. For example, regulation of the structure of the synaptic membrane, partial reaction of Ca" pump from inside of cell to the outside, etc. These problems remain to be clarified.

Caffeine may cause muscle contracture by release of Ca" from the sarcotubular system, as caffeine affects Ca" in fragmented sarcoplasmic reticulum from skeletal muscle. Most
studies on caffeine were done however using sarcoplasmic reticulum from frog muscle (24–26), and few using material from mammals (25, 27). In our study, caffeine had no effect on \(^{45}\)Ca retention in microsomes or ISM, but decreased it in synaptic membranes, though this may be partly due to inhibition of reuptake of \(^{45}\)Ca. This also shows the specific nature of the Ca\(^{++}\) in synaptic membranes. Mechanisms of the action of caffeine on Ca\(^{++}\) in synaptic membranes are now being investigated.

**SUMMARY**

Synaptic membranes isolated from rat brain cortex bound Ca\(^{++}\) in the presence of Mg\(^{++}\) and ATP. In the presence of 2 mM MgCl\(_2\) and 10\(^{-4}\) M EGTA, the optimal concentration of CaCl\(_2\), for this ATP-dependent Ca\(^{++}\)-binding was 9 x 10\(^{-5}\) M, and maximum of 8–10 \(\mu\)moles Ca\(^{++}\)-binding per mg of protein was reached in 1–2 min.

Ca\(^{++}\)-binding by synaptic membranes was temperature dependent, was not affected by oxalate and showed nucleoside triphosphate specificity. Under the same conditions, the intrasynaptosomal mitochondrial fraction (ISM) took up approx. twice as much Ca\(^{++}\) as the synaptic membranes, but required 5–10 min for maximum binding. Ca\(^{++}\)-binding of the microsomal fraction was little, under these conditions. Of these three fractions, the synaptic membranes bound Ca\(^{++}\) at the lowest concentration of free calcium ions, when the latter was adjusted with calcium EGTA buffer.

Accumulated \(^{45}\)Ca was decreased from synaptic membranes by caffeine in the presence of ATP, but not from microsomes or ISM.

From the present results, the Ca\(^{++}\)-binding to synaptic membranes is considered to be different from that of microsome or ISM.

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