Comparative Data of Ca$^{2+}$ Transport in Brain and Skeletal Muscle Microsomes*

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SUMMARY

A vesicular membrane system (microsomes) was prepared from rabbit brain. Ca$^{2+}$ uptake was observed in this preparation upon incubation with ATP and Mg$^{2+}$. Tris-oxalate did not increase Ca$^{2+}$ uptake. A decrease in adsorbed Ca$^{2+}$, coupled to an increase in Ca$^{2+}$ transport, was observed upon addition of 4 mM Pi, to the incubating media. Ca$^{2+}$ adsorption was higher in presence of 120 mM KCl than in a medium with 120 mM NaCl. Ca$^{2+}$ transport was lower in a medium without supporting concentrations of monovalent cations.

Acetyl phosphate as substrate would not support Ca$^{2+}$ uptake in brain microsomes.

Brain microsomal ATPase was activated by Ca$^{2+}$ and Mg$^{2+}$ similarly to that in skeletal muscle microsomes. Ca$^{2+}$-dependent ATPase activity was higher in media containing 120 mM KCl than 120 mM NaCl. Ouabain did not inhibit this activity.

The amount of Ca$^{2+}$ bound by skeletal muscle microsomes in an oxalate-free medium was 2- to 4-fold higher than in brain microsomes. In these experiments, the Ca$^{2+}$ P$_i$ ratio was 0.15 to 0.30 both in brain and muscle microsomes. Ca$^{2+}$ P$_i$ ratios between 1.0 and 1.2 were obtained in skeletal muscle microsomes incubated in media containing 4 mM Tris-oxalate.

A highly efficient ATP-dependent system for Ca$^{2+}$ transport has been described in muscle microsomes (sarcoplasmic reticulum). This system plays a key role in the process of excitation-contraction coupling in muscle cells (1-3).

Evidence that Ca$^{2+}$ interferes with the electrical properties of nerve cell membranes has been presented by different authors (4-7). The presence of a Ca$^{2+}$ transport system in nerve and brain membranes has been studied by various authors with differing results (8-10). Ca$^{2+}$ uptake and ATP hydrolysis in dog brain microsomes was observed by Otsuka, Ohtsuki, and Ebashi (8). Brain microsomes differ from muscle microsomes in that their uptake of Ca$^{2+}$ is not increased by oxalate; however, ATP-dependent Ca$^{2+}$ transport activated by oxalate was reported in crustacean peripheral nerve microsomes by Lieberman et al. (9). They also reported a lower total amount of Ca$^{2+}$ bound when KCl (115 mM) was replaced by NaCl in the assay medium.

Alonso and Walser (10), with rat brain microsomes and a rapid perfusion incubation procedure, did not observe ATP-dependent Ca$^{2+}$ uptake. If KCl was substituted by NaCl in the perfusion medium, there was some Ca$^{2+}$ release from microsomes.

In this paper, the effect of monovalent cations in Ca$^{2+}$ uptake and ATPase activity of rabbit brain microsomes has been studied. Most of these experiments were designed to compare Ca$^{2+}$ transport and ATPase activity in brain and muscle microsomal fractions.

METHODS

Preparation of Microsomes—All operations were performed at 4°. Brains of two rabbits were homogenized with 3 volumes of ice-cold 120 mM KCl in a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 1,500 $\times$ g for 10 min and the pellet was discarded. The supernatant fraction was centrifuged in a Sorvall RC2-B centrifuge at 10,000 $\times$ g for 10 min to remove mitochondria. The supernatant containing microsomes and soluble protein was then centrifuged at 41,000 $\times$ g for 1 hour. The supernatant was discarded and the pellet was washed once with 20 ml of either 120 mM KCl or 240 mM sucrose. The material was dispersed in a Potter-Elvehjem tissue grinder and centrifuged at 41,000 $\times$ g for 45 min. The pellet was suspended at a protein concentration of 10 mg per ml in the solution used for washing. Muscle microsomes were prepared by essentially the same procedure. Protein was estimated by the biuret method. All preparations used were less than 2 hours old.

Standard Assay—Unless otherwise stated, the incubation medium consisted of 8 mM Tris-maleate buffer, pH 6.8; 4 mM MgSO$_4$; 2 mM ATP; and the specified amounts of NaCl, KCl, and CaCl$_2$. The total volume was usually 1.0 ml. The reaction was started by the addition of microsomal protein, and carried out at 37°. The incubation time was usually 5 min. When ATPase activity was measured, the reaction was stopped by the addition of 0.1 ml of a 10% solution of trichloroacetic acid. When Ca$^{2+}$ uptake was measured, the reaction was stopped by removal of particles with Millipore filters, type HA, with 0.45 μ average pore size, as previously described (11). In all experiments, controls both with and without microsomes and without ATP were performed. The pH of the filtrates was measured.
FIGS. 1 to 3
Fig. 4. Brain microsomes; Ca\(^{2+}\) uptake, ATP dependence. Incubation medium as described under "Methods," plus KC\(_1\), 120 mm, 44CaCl\(_2\), 0.025 mm, and 1 mg per ml of microsomal protein. Incubation time, 5 min.

Table I

Brain microsomes: effect of NaCl and KCl on Ca\(^{2+}\) bound

| Ion in incubation medium | Adsorbed Ca\(^{2+}\) | Total Ca\(^{2+}\) | Transformed Ca\(^{2+}\) | Probability\(^a\) |
|--------------------------|--------------------|-----------------|------------------------|-----------------|
| None added               | 7.2 ± 0.79         | 13.2 ± 0.66     | 5.9 ± 0.87             |                 |
| KCl, 120 mm              | 6.0 ± 0.75         | 13.7 ± 0.66     | 7.7 ± 1.00             | <0.005          |
| NaCl, 120 mm             | 4.0 ± 0.40         | 12.2 ± 0.50     | 8.3 ± 0.60             | <0.001 <0.01    |

\(^a\) Student t test; significant levels of change.

with a microelectrode pH meter (Radiometer E-5021A, Copenhagen) and remained within the limits 6.75 to 6.90. Each set of data reported is representative of replicate experiments showing essential agreement.

Measurement of Ca\(^{2+}\) Uptake—Two 0.05-ml aliquots of each filtrate were dried on planchets, counted in a Nuclear-Chicago gas flow counter, and averaged. The percentage of Ca\(^{2+}\) bound to microsomes was calculated from the radioactivity of the microsome-free incubation medium and compared with that of the samples containing microsomes.

ATPase Activity—This enzyme was assayed by measuring the P\(_i\) content of the sample by the method of Fiske and SubbaRow (12).

Electron Microscopy—After incubation, microsomes were centrifuged at 80,000 \(\times g\) for 30 min. The pellet obtained was fixed overnight at 5\(^\circ\) C in a 2% solution of OsO\(_4\) in 120 mm KCl. After washing in distilled water, it was dehydrated in ethanol, stained with uranyl acetate, carefully removed from the centrifuge tube, and embedded in Epon. The pellets were cut to have

Fig. 1. Skeletal muscle microsomes. Incubation medium as described under "Methods," plus KCl, 120 mm, 44CaCl\(_2\), 0.1 mm, and 1 mg of microsomal protein. Final volume was 10 ml. Other conditions as described under "Methods." Similar electron micrographs were obtained whether the microsomes were incubated with or without ATP. When incubated with ATP, 100% Ca\(^{2+}\) uptake by the vesicles was observed. Magnification \(\times 28,500\). The horizontal bar at the lower left corner of Fig. 3 indicates 1 \(\mu\).

Fig. 2. Precipitation of calcium oxalate in skeletal muscle microsomes. Conditions and incubation medium as in Fig. 1, except for the addition of 2 mm ATP and 4 mm Tris-oxalate in the incubation medium. Arrows point to electron-opaque material in the vesicles. When small amounts of microsomal protein were used, the micrograph was difficult to interpret, because of the presence of large areas of electron-opaque material. Thus, protein concentrations were 1 to 2 mg per ml.

Fig. 3. Brain microsomes. Incubation medium as described under "Methods," plus 44CaCl\(_2\), 0.05 mm, KCl 120 mm, and 1 mg of microsomal protein per ml. Other conditions as described under "Methods." Similar electron micrographs were obtained in incubation media with or without ATP and with or without 4 mm Tris-oxalate. Magnification \(\times 28,500\).
**TABLE III**

**Effect of Na⁺, K⁺, and ouabain on brain ATPase activity**

| Addition to incubation medium | Enzyme activity | Increment due to Ca²⁺ |
|------------------------------|-----------------|-----------------------|
| No ouabain                   |                 |                       |
| MgSO₄, 4 mM                  |                 |                       |
| Without monovalent cation.   | 0.619 ± 0.08    |                       |
| KCl, 120 mM                  | 0.546 ± 0.07    |                       |
| NaCl, 120 mM                 | 0.730 ± 0.07    |                       |
| MgSO₄, 4 mM + CaCl₂, 0.05 mM|                 |                       |
| Without monovalent cation.   | 0.720 ± 0.05    | 0.091 ± 0.030         |
| KCl, 120 mM                  | 0.644 ± 0.06    | 0.098 ± 0.030         |
| NaCl, 120 mM                 | 0.753 ± 0.06    | 0.086 ± 0.020         |

| Ouabain, 1 mM                |                 |                       |
| MgSO₄, 4 mM                  |                 |                       |
| Without monovalent cation.   | 0.620 ± 0.060   |                       |
| KCl, 120 mM                  | 0.547 ± 0.07    |                       |
| NaCl, 120 mM                 | 0.669 ± 0.06    |                       |
| MgSO₄, 4 mM + CaCl₂, 0.05 mM|                 |                       |
| Without monovalent cation.   | 0.664 ± 0.06    | 0.043 ± 0.001         |
| KCl, 120 mM                  | 0.641 ± 0.06    | 0.085 ± 0.020         |
| NaCl, 120 mM                 | 0.702 ± 0.05    | 0.086 ± 0.01          |

**RESULTS**

**Ca²⁺ Transport in Brain Microsomes—**Fig. 4 and Table I show that Ca²⁺ binds to brain microsomes in the absence of ATP. This fraction will be referred to as “adsorbed Ca²⁺.” Upon addition of ATP and Mg²⁺, larger amounts of Ca²⁺ are bound. This will be referred to as “total Ca²⁺.” With the several microsomal preparations tested, the maximal binding of Ca²⁺ was found between ATP concentrations of 0.20 to 0.50 mM. In the absence of Mg²⁺, the amount of Ca²⁺ bound with or without ATP was essentially the same, thus showing that Mg²⁺ is required for ATP supported Ca²⁺ binding. The differences between total Ca²⁺ and adsorbed Ca²⁺ will be referred to as “transported Ca²⁺.”

In experiments in which microsomes were incubated for different time intervals with or without ATP, it was observed that the amount of Ca²⁺ transported increased progressively with the incubation time. Fig. 5 shows one of these experiments. These data are in agreement with Otsuka et al. (8) and Lieberman et al. (9).

**Effect of Oxalate and Phosphate Ions on Ca²⁺ Uptake—**It has been shown that the addition of oxalate or P;i to the incubation medium activated Ca²⁺ uptake in muscle microsomess through the precipitation of Ca²⁺ within the vesicles (1, 14, 15). Figs. 1 and 2 show that after oxalate addition to muscle microsomal preparations an electron-opaque material can be detected in
several vesicles. As mentioned previously, opposite results with oxalate have been reported by Otsuka et al. (8), and Lieberman et al. (9), in dog brain and crustacean peripheral nerve microsomes. Species differences could account for this discrepancy. Thus, the effects of Tris-oxalate and K$_2$HPO$_4$ were tested with brain microsomes. Addition of Tris-oxalate in concentrations varying from 1 to 4 mM did not modify Ca$^{2+}$ adsorbed or Ca$^{2+}$ transported. No accumulation of electron-opaque material could be detected in the brain preparation after incubation with oxalate. This could be due to the impermeability of brain microsomes to oxalate. The difference in activity in KCl, as compared to NaCl, is shown (18, 19) that skeletal muscle microsomes can use acetyl phosphate as a substrate for Ca$^{2+}$ transport. Although brain microsomes hydrolyze acetyl phosphate (20-22), no Ca$^{2+}$ transport was observed when acetyl phosphate was used as substrate under various experimental conditions. Thus, the acetyl phosphate found in brain microsomes seems not to be an integral part of the Ca$^{2+}$ transport system.

**Activation of Microsomal ATPase Activity by Mg$^{2+}$ and Ca$^{2+}$**

The following experiments were designed to study a possible correlation between brain microsomal ATPase and Ca$^{2+}$ transport. Table III and Figs. 6 and 7 show that Mg$^{2+}$ enhances microsomal ATPase. With 2 mM ATP maximum activation was observed at MgSO$_4$ concentrations between 2.0 and 2.5 mM. If Ca$^{2+}$ was added to a medium containing 4 mM Mg$^{2+}$, further activation of ATPase was observed in all the microsomal preparations tested. However, the amount of increase was subject to marked variability (see Standard error, Table III). Maximum activation was observed with 0.05 and 0.10 mM CaCl$_2$. No ATPase activation was observed with 0.10 mM CaCl$_2$ in the absence of Mg$^{2+}$. These data are essentially the same as those described for skeletal muscle microsomes (1, 17, 18, 23). Subsequently, ATPase activity in the presence of MgSO$_4$ will be referred to as “Mg$^{2+}$-dependent” ATPase; that observed in the absence of Mg$^{2+}$, as “Ca$^{2+}$-activated” ATPase; or without monovalent cations as “total” ATPase. The difference in activity in KCl, as compared to NaCl, was statistically significant at the level (p < 0.001). Ca$^{2+}$-activated ATPase is higher in the presence of 120 mM KCl than in 120 mM NaCl (p < 0.05). Ouabain (1 mM) failed to inhibit either Mg$^{2+}$- or Ca$^{2+}$-activated ATPase, with or without the high salt media. In order to study a possible correlation between (Na$^{+}$ + K$^{+}$)-ATPase (9, 17, 24) and Ca$^{2+}$ transport, ATPase activity and Ca$^{2+}$ uptake were simultaneously measured in media containing different proportions of NaCl and KCl at a fixed concentration of 120 mM, the Ca$^{2+}$ transported was progressively higher as the proportion of NaCl increased. Addition of 1 mM ouabain in a medium containing either NaCl or KCl did not modify the Mg$^{2+}$ adsorbed or total Ca$^{2+}$ bound.

**Activation of Microsomal ATPase Activity by Mg$^{2+}$ and Ca$^{2+}$**

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**Ca^{2+}** Uptake and ATPase Activity in Brain Microsomes—In order to ascertain the efficiency of Ca^{2+} transport in brain and skeletal muscle, Ca^{2+} uptake and ATPase activity were measured simultaneously in media containing different concentrations of 45CaCl_{2}. Oxalate was not added to the assay medium since, as shown previously, this ion activated Ca^{2+} uptake only in muscle microsomes. The amount of microsomal protein added to the assay medium was adjusted to prevent Ca^{2+} exhaustion, i.e. 100% Ca^{2+} bound. Fig. 10 shows one of these experiments. In 10 different experiments, with protein concentrations of 0.4 to 0.8 mg per ml, maximum Ca^{2+} uptake was obtained with CaCl_{2} concentrations varying between 0.1 and 0.2 mM for brain microsomes and 0.2 to 0.3 mM for muscle microsomes. In terms of micromoles of Ca^{2+} per mg of protein, muscle microsomes bound 2 to 4 times more Ca^{2+} than brain microsomes. Different reports have correlated Ca^{2+} transport and Ca^{2+}-activated ATPase activity in skeletal muscle microsomes. As shown in Fig. 10, Ca^{2+}-bound and Ca^{2+}-activated ATPase reached a maximum at different Ca^{2+} concentrations. Ca^{2+}-dependent ATPase activity was inhibited at higher Ca^{2+} concentrations. This inhibition is in agreement with other reports (11, 23). Consequently, different ratios between the amounts of Ca^{2+} bound and of ATP hydrolyzed (Ca^{2+}:P_{i} ratios) can be obtained. This ratio varied between 0.15 and 0.30 at Ca^{2+}-saturating concentrations both in muscle and brain microsomes. In control experiments with skeletal muscle microsomes, with the use of 4 mM Tris-oxalate, 120 mM KCl, 4 mM ATP and Mg^{2+}, 0.1 mM 4CaCl_{2}, and microsomal protein concentrations to ensure complete Ca^{2+} removal, Ca^{2+}:P_{i} ratios of 1.0 to 1.2 were obtained. This agrees with the values reported by Hasselbach (1) and Ebashi and Yamanouchi (25).

**DISCUSSION**

**Ca^{2+} Transport in Excitable Tissues**—It is not possible to determine whether microsomes derived from Schwann or neuronal cells are responsible for the Ca^{2+} uptake observed in brain microsomal fractions. Nevertheless, the data presented suggest that Ca^{2+} transport is a general feature of excitable tissues, regardless of their origin. Microsomal fractions obtained from nonexcitable tissues, such as liver and kidney, did not show significant Ca^{2+} uptake (8). As in skeletal muscle, brain microsomal Ca^{2+} transport may represent a system for depleting the cells of free Ca^{2+}. However, it is premature to speculate on a possible functional role of Ca^{2+} transport in neural membranes. Alonso and Walser (10) did not observe ATP-dependent Ca^{2+} uptake in rat brain microsomes. The use of deoxycholate in their procedure for microsomal preparation may have impaired the Ca^{2+} transport system.

**Effect of Na^{+} and K^{+} on Ca^{2+}-activated ATPase and Ca^{2+} Transport**—In skeletal muscle microsomes, Ca^{2+}-activated ATPase has been associated with Ca^{2+} transport. In brain microsomes, Ca^{2+} transport was higher in the presence of 120 mM NaCl or 120 mM KCl. However, Ca^{2+}-activated ATPase was higher only in a medium containing KCl. It is difficult then to correlate Ca^{2+} transport and Ca^{2+}-activated ATPase in this preparation. The presence of other phosphatases, contaminant or microsomal, cannot be ruled out.

It is interesting to note that, in contrast with skeletal muscle microsomes, acetyl phosphate cannot be used as substrate for Ca^{2+} transport in brain microsomes. This may suggest different characteristics in the Ca^{2+} transport system of these tissues.

**Efficiency of Ca^{2+} Transport in Brain and Skeletal Muscle Microsomes**—Ca^{2+} transport in brain and skeletal muscle cannot be rigorously compared in terms of specific transport capacity. More significant measurements would be obtained with purified enzyme preparations. The efficiency of the Ca^{2+} transport system is also related to the concentration of ionic Ca^{2+} in the vesicles. Even on a unit protein basis, different results can be obtained depending on the vesicular volumes. Thus, with the same amount of microsomal protein, and two systems, one of which has vesicles 4 times larger in volume than the other, the amount of Ca^{2+} bound required to achieve the same ionic Ca^{2+} concentration within the vesicles will be 4 times larger for the system with the larger vesicles. Our brain microsomal preparation was not pure, and vesicles of different diameters were observed in the electron microscope. Thus, the 2- to 4-fold increase in bound Ca^{2+} observed for muscle microsomes is only an approximate value.

**Ca^{2+}:P_{i} Ratio**—The Ca^{2+}:P_{i} ratio obtained was similar for...
brain and muscle microsomes when the vesicles reach saturation with Ca$^{2+}$. These data suggest the same energy efficiency in Ca$^{2+}$ transport in both systems. The Ca$^{2+}$:Pi ratio for muscle microsomes reported in the literature varies from 1 to 3.5 (1, 17, 25). By using oxalate and microsomal protein concentrations sufficient to ensure complete removal of Ca$^{2+}$ from the assay medium, the Ca$^{2+}$:Pi ratio found was 1.0 to 1.2, in agreement with Hasselbach (1) and Ebashi and Yamanouchi (25). However, in the absence of oxalate, and with incomplete removal of Ca$^{2+}$ from the assay medium, the value found was 0.15 to 0.30. This again raises the question about the presence of other phosphatases.

In muscle microsomes, actomyosin is the most likely contaminant phosphatase (26). Several muscle microsomal preparations show no decrease in Ca$^{2+}$-dependent ATPase activity when washing for 90 min in 20, 120, or 600 mm KCl. This seems to exclude the possibility of actomyosin contamination. The low Ca$^{2+}$:Pi ratios found could also result from the occurrence of a rapid turnover of Ca$^{2+}$. When equilibrium is reached, part of the Ca$^{2+}$ may leak out of the vesicles by diffusion, so that pumping of corresponding amount would be needed in order to maintain a stable Ca$^{2+}$ level within the microsomes. However, different authors (1, 23) have shown that muscle microsomes previously filled with Ca$^{2+}$ have no Ca$^{2+}$-dependent ATPase activity when incubated in a medium with ATP and Mg$^{2+}$, thus excluding this possibility. On the other hand, Ebashi and Yamanouchi (25) observed in rabbit skeletal muscle that Ca$^{2+}$-dependent ATPase activity varied upon the presence or absence of oxalate in the assay medium. A possible explanation for the low Ca$^{2+}$:Pi ratios we obtained with brain microsomes could be related to the ineffectiveness of oxalate in the assay medium and different free Ca$^{2+}$ concentrations within the vesicles.

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