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ATP-DEPENDENT CALCIUM ACCUMULATION IN BRAIN MICROSOMES ENHANCEMENT BY PHOSPHATE AND OXALATE

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SUMMARY

1. ATP-dependent calcium uptake by a rabbit brain vesicular fraction (microsomes) was studied in the presence of phosphate or oxalate. These anions, which are known to form insoluble calcium salts, increased the rate of calcium uptake and the capacity of the vesicles for calcium accumulation.

2. The degree of activation depended on the concentration of phosphate or oxalate. Under optimal conditions, phosphate promoted a 5-fold increase in the amount of calcium stored at steady state. This level was 200–250 nmol Ca^{2+}/mg protein.

3. Inital rate of calcium uptake followed Michaelis-Menten kinetics with an apparent K_m for calcium of $6.7 \cdot 10^{-5}$ M and a V of 44 nmol/min per mg protein. Optimal pH was 7.0. With 2 mM ATP, optimal Mg^{2+} concentration was 2 mM.

4. Dinitrophenol and NaN_3 inhibited calcium uptake in a mitochondria-enriched fraction but not in the microsomal fraction.

5. Calcium uptake activity was compared in the six subfractions prepared from the whole microsomal fraction by means of a sucrose density gradient fractionation.

6. The Mg^{2+}-dependent ATPase activity of brain microsomes was activated by calcium. Maximal activation was attained with 100 μM $CaCl_2$. Greater calcium concentrations caused a progressive inhibition.

7. The data suggest that the ATP-dependent calcium uptake in brain microsomes, as in muscle microsomes, is brought about by an active transport process, calcium being accumulated as a free ion inside the vesicles.

INTRODUCTION

Calcium uptake by skeletal muscle microsomes is now an extensively studied phenomenon [1–3], that has been identified as an active transport process which creates a calcium concentration gradient at the expense of hydrolysis of ATP.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

Activation of calcium uptake by phosphate or oxalate provides evidence that calcium is accumulated as a free ion in the inner space of the vesicles in a concentration higher than in the extravesicular medium [1–6]. These anions act as precipitating agents, providing a sink for calcium by forming calcium phosphate or calcium oxalate deposits inside the vesicles [7]. ATP-dependent calcium uptake enhanced by oxalate was also observed in subcellular particles of crustacean peripheral nerve [8].

ATP-dependent calcium binding by brain microsomes was first reported by Otsuka et al. [9] and Yoshida et al. [10], and was later confirmed by other authors [11–17]. This process, however, was different from calcium uptake in muscle microsomes in that activation by those calcium-precipitating agents could not be observed [9–14], although Ohtsuki [12] mentions an effect on aged preparations. The lack of this observation has hindered the recognition of an active calcium transporting system in brain microsomes and favoured the proposition of other mechanisms, such as an ATP-activated calcium binding to membrane specific sites [16, 17].

Using concentrations of phosphate or oxalate higher than those of the previous investigators, we have observed a strong activation of calcium uptake in brain microsomes. These findings suggest that, as in muscle microsomes, this calcium uptake is brought about by a transport through the membrane, calcium being accumulated inside the vesicles in a concentration higher than in the extravesicular medium. A general characterization of the phenomenon under these conditions is presented.

MATERIALS AND METHODS

Preparations of microsomes

Adult rabbits were killed by dislocation of the neck and the brain was removed from the skull immediately. After rapid removal of meninges and big blood clots with tweezers, each brain was minced with scissors, dropped into 7 vols of ice-cold 120 mM KCl containing 0.1 % bovine serum albumin and gently homogenized by hand in a Potter-Elvehjem homogenizer. All operations were performed at 1–4 °C. The whole brain homogenate was then centrifuged at $2\,700 \times g$ for 12 min. The pellet was resuspended in 120 mMKCl and washed once. The supernatants were combined and centrifuged at $15\,000 \times g$ for 15 min. In some experiments, this pellet was resuspended in 120 mM KCl and centrifuged at $8000 \times g$ for 12 min to give a second pellet which was resuspended in a smal vollume of 120 mM KCl and used as the mitochondrial fraction in tests of the effect of dinitrophenol and NaN_3. The supernatant of the $15\,000 \times g$ run was then centrifuged at $40\,000 \times g$ for 45 min. This pellet was resuspended in a small volume of 120 mM KCl, kept at 0 °C and used for the experiments as the microsomal fraction. Protein was estimated by the biuret method. The average yield was 20 mg of microsomal protein for 8 g brain. All of the preparations used for the experiments were less than 5 h old, since the calcium uptake activity decreased rapidly with ageing. As in previous reports [11, 13, 14], electron micrographs of the microsomal fraction showed heterogeneous membranous elements with numerous vesicles of different diameters.

Sucrose density gradient

The whole microsomal fraction was subfractionated in a discontinuous sucrose density gradient (0.3–1.2 M sucrose). The suspension of microsomes in 120 mM KCl

was carefully layered on the top of the gradient and centrifuged at 100 000 × *g* for 90 min. The material sedimented into five layers and a pellet. Most of the material was recovered in two fractions which equilibrated in the interphase between the sucrose concentrations 0.3 and 0.6 M and 1.0 and 1.2 M.

The subfractions were separated by aspiration from above downward, under visual control, slowly diluted 10-fold with 120 mM KCl and centrifuged at 40 000 × *g* for 70 min. Each pellet was resuspended in a small volume of 120 mM KCl and used for the experiments. Protein was estimated by the method of Lowry et al. [18].

Calcium uptake

Unless otherwise stated, the incubation mixture for calcium uptake assay contained 20 mM Tris/maleate buffer, pH 7.0, 3 mM $MgCl_2$, 2.5 mM ATP, 0.4 mg/ml of microsomal protein and the specified concentrations of $^{45}CaCl_2$ and potassium oxalate or potassium phosphate buffer, pH 7.0. All of the experiments were carried out at 37 °C for times of incubation stated in the legends. The reaction was started by the addition of microsomes and stopped by Millipore filtration (type HA, 0.45 μm pore size) as described previously [4]. The amount of calcium taken up by the microsomes was calculated from the difference between the radioactivity of the filtrate and the initial radioactivity of the reaction mixture.

The mean specific activity of the $^{45}CaCl_2$ was 5 Ci/mol. The results of the single experiments shown in Figs. 1, 3, 4 and 6 were confirmed in each case by at least three similar experiments.

(Mg^{2+}-Ca^{2+})-ATPase activity

The ATPase activity was assayed by measuring the liberation of P_i by the method of Fiske and SubbaRow [19]. The incubation mixture contained 20 mM Tris/maleate buffer, pH 7.0, 3 mM $MgCl_2$, 2.5 mM ATP, 0.1 mM ouabain, 0.25 mg/ml of microsomal protein and 1 mM ethyleneglycol-bis(β-aminoethyl ether) -N,N^1-tetraacetic acid (EGTA) or the specified concentrations of $CaCl_2$. Time of incubation was 15 min and the temperature was 37 °C. The reaction was started by the addition of microsomes and stopped by addition of ice-cold trichloroacetic acid to a final concentration of 8 % (w/v).

RESULTS

Activaton of calcium uptake by phosphate. Phosphate concentrations of about 5 mM markedly increase calcium uptake in muscle microsomes [4]. Similar P_i concentrations had been tested in brain microsomes without any significant effect on total calcium incorporation [12, 13]. Figs 1 and 2 show that, in the case of brain microsomes, the concentrations of P_i required for activation of calcium uptake are higher.

Different P_i concentrations increased the rate of calcium uptake and the calcium storing capacity of the vesicles, its effect being more pronounced at longer incubation intervals (Fig. 1). The passive calcium binding, measured in the absence of ATP, was not modified significantly by the P_i concentrations tested (Fig. 1). At any initial calcium concentration, increases in P_i concentration produced a linear enhancement of the calcium storing capacity of the microsomes (Fig. 2), until reaching a plateau, in which the external calcium becomes the limiting factor for further uptake.

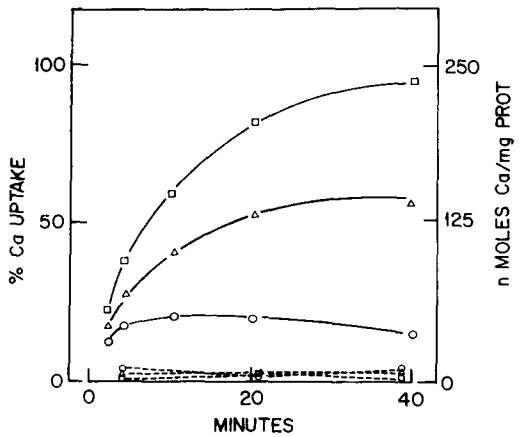

Fig. 1. Time course of ATP-dependent calcium uptake activation by P_i. The $CaCl_2$ concentration was 100 μM. (—), with 2.5 mM ATP. (- - -), without ATP. ○, in absence of P_i; △, with 40 mM P_i; □, with 80 mM P_i. Further details are given under Materials and Methods.

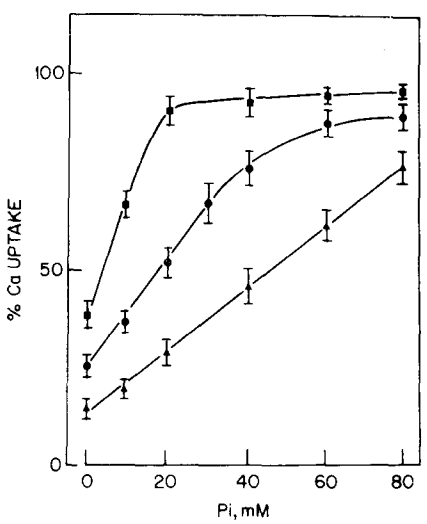

Fig. 2. Enhancement of the Ca^{2+} storing capacity by P_i. Initial $CaCl_2$ concentrations were: 10 μM (■), 50 μM (●), and 100 μM (▲). Time of reaction was 25 min. Further details are given under Materials and Methods. Each point represents the mean +S.E. of four experiments.

As shown in Fig. 3, vesicles that had removed nearly 95 % of the external calcium showed further uptake upon a subsequent addition of calcium to the assay medium.

Activation of calcium uptake by oxalate. Fig. 4 (left) shows the time course of calcium uptake in the absence and presence of three different oxalate concentrations. Like P_i, oxalate enhanced the rate of calcium uptake and the Ca^{2+} filling capacity of microsomes. The passive binding was not modified significantly. Again, the same marked activation at prolonged incubation intervals and a less noticeable effect at shorter incubation times (Fig. 4, right) are observed. The concentrations of oxalate

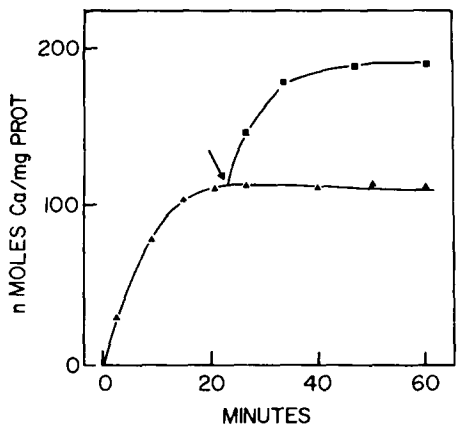

Fig. 3. Calcium uptake by preloaded vesicles. The initial $CaCl_2$ concentration was 50 μM and P_i was 80 mM. At the time indicated by the arrow, half of the reaction mixture was transferred to another tube, (■) containing $CaCl_2$, at the same specific activity, in a very small volume to give a final concentration of 50 μM. Further details are given under Materials and Methods.

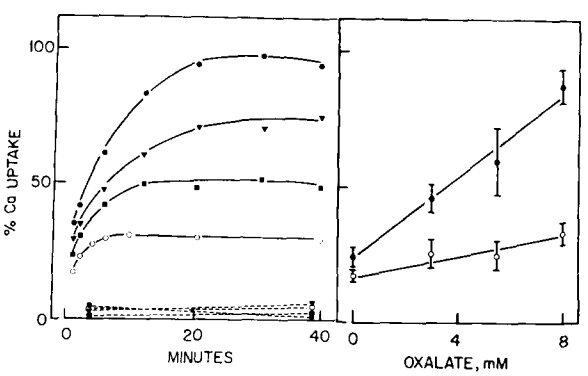

Fig. 4. Left: time course of ATP-dependent calcium uptake activation by oxalate. Initial $CaCl_2$ was 50 μM. (—), with 2.5 mM ATP. (- - -), without ATP. ○, in absence of oxalate; ■, with 3 mM; (▼), with 5.5 mM; ●, with 8 mM oxalate. Right: effect of oxalate on the initial rate and on the steady-state levels of calcium uptake. Initial $CaCl_2$ was 50 μM. Time of reaction was 2 min (○) or 25 min (●). Each point represents the mean ±S.E. of four experiments. Further details are given under Materials and Methods. 100 % Ca^{2+}-uptake corresponds to Ca^{2+} incorporation of 125 nmol/mg protein.

required for activation were also higher than those reported to activate calcium uptake in muscle microsomes [1–6].

The maximal calcium-incorporating capacity commonly reported for brain microsomes (10–80 nmol/mg protein) [9–17] is consistent with our levels in the absence of precipitating agents, as shown in Figs 1 and 4 (left).

Affinity for calcium. The initial rate of calcium uptake, measured at various initial calcium concentrations in the presence of P_i as precipitating agent, followed Michaelis-Menten kinetics with an apparent K_m for calcium of $6.7 \cdot 10^{-5}$M and a V of 44 nmol Ca^{2+}/min per mg protein (Fig. 5). Therefore, the affinity for calcium of

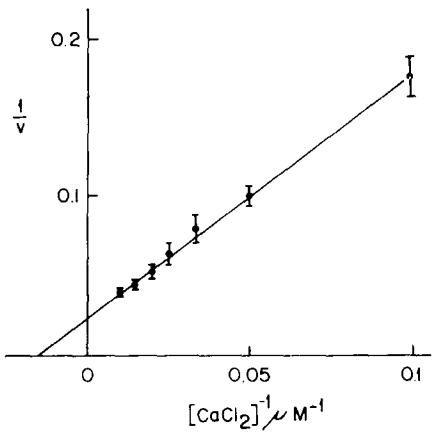

Fig. 5. Lineweaver-Burk plot of calcium uptake. Ordinate units are (nmol Ca^{2+}/mg protein per min)$^{-1}$. Time of reaction was 1 min. P_i was 50 mM. Further details are given under Materials and Methods. Each point represents the mean ±S. E. of four experiments. The line was fitted by means of a weighted linear regression.

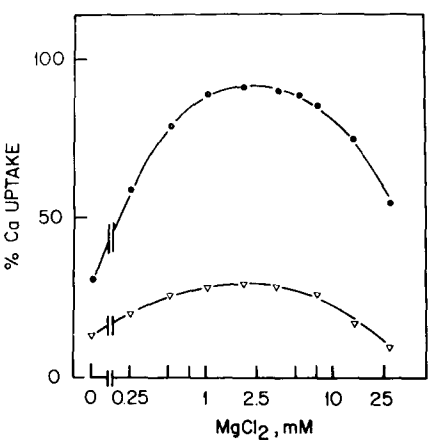

Fig. 6. Dependence of calcium uptake on Mg^{2+}. The reaction mixture contained 100 μM $CaCl_2$, 80 mM P_i and 2 mM ATP. Time of reaction was 2 min (▽) or 35 min (●). Further details are given under Materials and Methods.

brain microsomes is about 50-fold lower than that reported for muscle microsomes [2].

Mg^{2+} dependence. In the presence of 2 mM ATP, the activating effect of Mg^{2+} reached a maximum in the concentration range of 1–2 mM and started to decrease at concentrations above 8 mM (Fig. 6). In the absence of added Mg^{2+} we still obtained some calcium incorporation. Similar observations were made in the absence of precipitating agents by Ohtsuki [12] and, as proposed by that author may be attributed to traces of Mg^{2+} in the reaction mixture.

pH dependence. The pH profile for calcium uptake showed an optimum at pH 7.0. In single experiments in the presence of either P_i or oxalate as precipitating agent,

the optimal pH ranged from 6.8 to 7.2. A 70 % inhibition of the calcium uptake was observed when the pH of the assay medium was raised to 8.5 or decreased to 5.5. In the absence of precipitating agents, Robinson and Lust [11] found an optimum at pH 7.5 and Nakamaru and Schwartz [14] one at pH 6.2.

Effect of mitochondrial inhibitors. Mitochondria and submitochondrial particles from several tissues, including brain, are known to accumulate large amounts of calcium in an ATP-dependent or in a respiratory substrate-dependent process [20, 21]. The ATP-dependent calcium incorporation by liver mitochondria is inhibited by dinitrophenol or NaN_3 [22]. The effects of these inhibitors were tested in our microsomal fraction and in a mitochondria-enriched fraction obtained from the same brain homogenate as described under Materials and Methods. Fig. 7 shows that a significant inhibition was observed only in the latter, thus excluding significant interference from mitochondria in our calcium uptake measurements.

Calcium uptake by microsomal subfractions. Calcium uptake activity in presence of 100 μM $CaCl_2$ and 70 mM phosphate buffer was compared in six subfractions prepared from the whole microsomal fraction by means of a sucrose density gradient. ATP-dependent calcium uptake was observed in all subfractions obtained, being 20–40 % higher in the subfractions which equilibrated between 0.6 and 0.8 M sucrose than in those which sedimented between 1.0 and 1.2 M sucrose. The pellet which sedimented in the bottom of the gradient showed little calcium-accumulating capacity. Similar data were reported by Ohtsuki [12] in the absence of calcium-precipitating agents.

(Mg^{2+}-Ca^{2+})-ATPase activity. The existence of a calcium-transporting system that depends on Mg^{2+} and works at the expense of ATP implies the existence of a (Mg^{2+}-Ca^{2+})-ATPase activity [2, 3]. This enzymatic activity has been demonstrated in native brain microsomes by de Meis et al. [13] and in solubilized microsomes by Nakamaru and Schwartz [14]. However, it was not found in recent studies by Nakamura and Konishi [16]. Fig. 8 shows that a calcium concentration of 100 μM activated the ATPase activity of brain microsomes by 16 % in the presence of 3 mM $MgCl_2$. Greater calcium concentrations promoted a progressive decrease with a 27 % inhibition at 5 mM.

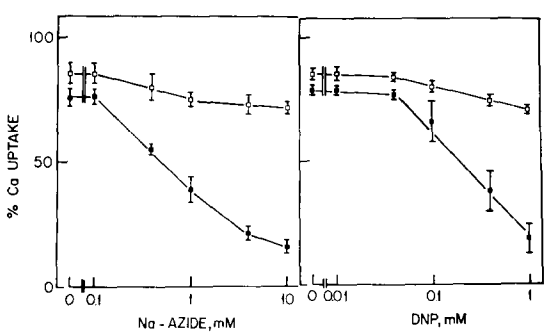

Fig. 7. Effect of NaN_3 (left) and dinitrophenol (right) on calcium uptake by microsomal (□) or mitochondria-enriched (■) fractions. Initial $CaCl_2$ was 50 μM and P_i was 70 mM. Time of incubation was 25 min. Each point represents the mean ±S.E. of three experiments. Further details are given under Materials and Methods.

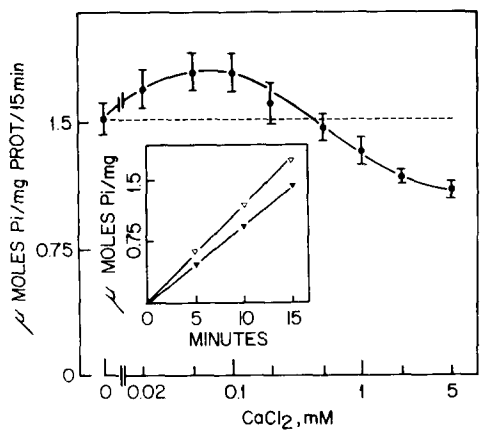

Fig. 8. Effect of $CaCl_2$ on ATPase activity of microsomal fraction. Each point represents the mean ±S.E. of four experiments. Dashed line indicates the level of the Mg^{2+}-dependent ATPase in the presence of 1 mM EGTA. Student's paired *t*-test gave a significance coefficient of $P < 0.005$ between the points at 0 and 100 μM $CaCl_2$. Inset plot is typical of four experiments showing the time course of ATP hydrolysis in the presence of 1 mM EGTA (▼) or 50 μM $CaCl_2$ (▽). Further details are given under Materials and Methods.

The inset plot shows the time course of ATP hydrolysis in the absence and presence of 50 μM calcium.

DISCUSSION

Using suitable oxalate concentrations we were able to observe activation of calcium uptake in all brain microsome preparations tested, independently of storage time. Ohtsuki [12] mentions an effect of oxalate only on aged preparations, but does not state the concentrations used.

The requirement of relatively high P_i or oxalate concentrations for calcium uptake activation in brain microsomes may be interpreted by assuming that the permeability of brain microsomal membranes to these anions is lower than that of muscle microsomes. The more marked activation after more prolonged incubations would also be explained by this assumption.

The heterogeneity of brain microsomal fractions [11, 13, 14] has made it difficult to establish a connection between a specific cellular organelle and a biochemical phenomenon such as calcium uptake studied in vitro. Furthermore, it is not possible to ascertain whether calcium uptake activity is derived from glial or neuronal vesicles [23].

As has been proposed [11], the calcium uptake activity present in brain microsomal fraction might be attributed to endoplasmic reticulum fragments.

It is known that calcium is not distributed according to its electrochemical gradient in nerve cells [24]. Thus, these cells must possess mechanisms to control their internal calcium levels, since increases in cytoplasmic free calcium are known to interfere with excitability [25] or transmitter release [26, 27].

Vesicles with properties such as those found in the present investigation might

also accumulate Ca^{2+} in vivo and thereby exert some regulatory function on Ca^{2+} concentration in the living cell.

The activation of calcium uptake by P_i and oxalate represents, as has been proposed for muscle microsomes [1–6], an argument for calcium being transported through the membrane and accumulated as a free ion inside the vesicles in a concentration higher than in the extravesicular medium.

On the other hand, by providing an easy way of increasing the calcium incorporation capacity of the vesicles, these calcium-precipitating agents may be used as a valuable tool in further studies of Ca^{2+} uptake in brain microsomes.

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