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Purification of the Ca^{2+}- and Mg^{2+}-requiring ATPase from rat brain synaptic plasma membrane

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A Ca^{2+}-ATPase (Ca^{2+}- and Mg^{2+}-requiring ATPase) was purified from a synaptic plasma-membrane fraction of rat brain. This enzyme had properties similar to those of plasma-membrane Ca^{2+}-ATPases from other organs: its splitting of ATP was dependent on both Ca^{2+} and Mg^{2+}, it bound in a Ca^{2+}-dependent fashion to calmodulin–Sepharose and it cross-reacted with specific antibodies raised against human erythrocyte-membrane Ca^{2+}-ATPase. It had an apparent M_r of 138000, similar to those of plasma-membrane ATPases from human erythrocyte and from dog heart sarcolemma. Previous high-Ca^{2+}-affinity ATPases observed in brain had M_r 100000; in at least one case, such an ATPase probably represented a different type of enzyme, derived from coated vesicles.

It is widely believed that movement of Ca^{2+} ions across the plasma membrane of the presynaptic portion of nerve is crucial to transmission of the nerve impulse across the synaptic cleft. Depolarization of the presynaptic membrane is believed to open Ca^{2+} channels; this allows extracellular Ca^{2+} to enter and raise the Ca^{2+} concentration inside. This in turn is believed to stimulate exocytosis of neurotransmitter from storage vesicles near the presynaptic membrane. Finally, the intracellular free Ca^{2+} must be decreased to low concentrations by sequestration of the Ca^{2+} into organelles or pumping of Ca^{2+} back out across the membrane. The literature pertinent to this view has been summarized by Gill *et al.* (1981) and Sorenson & Mahler (1981).

The mechanism by which the intracellular free Ca^{2+} is disposed of is controversial; although it is well established that mitochondria are involved in synaptosomal Ca^{2+} uptake, the extent of their involvement is uncertain. A non-mitochondrial component is also definitely present, but its localization and the nature of the activity are unclear. Coated vesicles (Blitz *et al.*, 1977), endoplasmic reticulum (Blaustein *et al.*, 1978*a*,*b*; Kendrick *et al.*, 1977) and plasma membranes (Gill *et al.*, 1981; Itano & Penniston, 1980; Sobue *et al.*, 1979) have all been invoked to explain this activity. In order to distinguish between the contributions of various subcellular organelles of Ca^{2+} management, it will be necessary to define the role of each Ca^{2+} pump more accurately. As a step toward that goal, we report here our purification from synaptosomes of an ATPase with a high affinity for Ca^{2+}, which several criteria indicate is of plasma-membrane origin.

Abbreviations used: Ca^{2+}-ATPase, Ca^{2+}- and Mg^{2+}-requiring ATPase; SDS, sodium dodecyl sulphate.

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Materials and methods

All reagents were of highest purity available. [γ-^{32}P]ATP was obtained from New England Nuclear, Boston, MA, U.S.A. Phosphatidylcholine (type III-S) was obtained from Sigma, St. Louis, MO, U.S.A. CNBr-activated Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden. YM-30 ultrafilters were from Amicon, Lexington, MA, U.S.A.

Membrane preparation

Synaptic plasma membranes were prepared by the method of Jones & Matus (1974) from rat brain. All the isolation procedures were performed at 0–4°C. In a typical preparation, 12 male Sprague–Dawley rats of 100–150g body wt. were decapitated, the brains were rapidly removed, washed with 0.32M-sucrose in 10mM-Tris/citrate buffer, pH7.4, and homogenized in 10vol. of the same medium by a Potter–Elvehjem Teflon/glass homogenizer at 400rev./min (eight passes up and down). The homogenate was centrifuged (20min, 3000*g*), the pellet discarded and the supernatant further centrifuged (30min, 10000*g*). This pellet was lysed by

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suspension to a final volume of 26.2ml in 10mM-Tris/citrate, pH7.4 (buffer I), followed by homogenization and sonication in the homogenizer tube (Heat Systems Ultrasonic Sonifier, setting at 40W, micro-tip, 15s in ice bath). To the lysate was added 63.8ml of 48% (w/w) sucrose, which made the solution 34% (w/w) in sucrose. A discontinuous gradient had been previously prepared by layering 10ml of 28.5% (w/w) sucrose in buffer I under 5ml of 10% (w/w) sucrose in the same buffer. In each tube 15ml of the lysate (made 34% in sucrose) was placed under the other sucrose layers, by using a syringe. The gradient thus created was spun at 60000*g* for 2h. Material equilibrated at the 28.5%–34%-sucrose interface was removed (a measured amount of 10ml from each of six Beckman SW 27 centrifuge tubes), diluted with 120ml of 15mM-Tris/citrate (pH7.4)/1.5mM-EGTA and re-centrifuged (30min, 100000*g*); the pellet was suspended in buffer I and washed by centrifugation (30min, 100000*g*).

Solubilization of membranes

This was done by using Triton X-100 (1mg/mg of protein) in 50mM-imidazole/HCl (pH6.8)/6mM-$MgCl_2$/100mM-KCl/20mM-NaCl/1% glycerol/1% Tween-20/10μM-phenylmethanesulphonyl fluoride/0.1mM-ouabain (buffer A) containing 0.1mM-$CaCl_2$. The mixture was kept on ice with stirring for 20min and the insoluble material was separated by centrifugation at 100000*g* for 30min. Phosphatidylcholine was then added to the supernatant to a final concentration of 0.05%

Calmodulin affinity chromatography

This was done for purification of Ca^{2+}-ATPase by the method of Niggli *et al.* (1979). The calmodulin–Sepharose column was equilibrated with buffer A containing 0.3M-KCl instead of 0.1M, 0·05% phosphatidylcholine, 2mM-EGTA and 1.8mM-$CaCl_2$ (1.0μM free Ca^{2+}); the solubilized extract was then applied to the column, which was subsequently washed with 10–12 bed vol. of the same buffer. Ca^{2+}-ATPase was eluted by buffer A from which ouabain and $MgCl_2$ were omitted and which contained 0.05% phosphatidylcholine and 2mM-EGTA. The ATPase was eluted from the column at between 1 and 2 column vol. after EGTA was introduced; it was usually necessary to locate the ATPase peak by assay of the column fractions.

If 6, instead of 12, vol. of Ca^{2+}-containing buffer was used to wash the column, other proteins in addition to the ATPase were eluted by EGTA. These proteins were of lower molecular weight and were eluted from the column ahead of the ATPase. In this case, pooling the tail of the protein peak could still yield a reasonably pure ATPase. Although the ATPase assayed immediately after the elution was devoid of activity in the presence of Mg^{2+} alone (without Ca^{2+}), such a Mg^{2+}-ATPase activity appeared during storage. Activity was assayed immediately after column chromatography.

Ca^{2+}-ATPase assay

ATPase activity was quantified by measuring the liberation of P_i from [γ-^{32}P]ATP. The assays were done by incubating the enzyme for 30min at 37°C in a medium containing 50mM-imidazole/HCl, pH6.8, 0.1M-KCl, 20mM-NaCl, 6mM-$MgCl_2$, 0.1mM-ouabain, 2mM-EGTA, Tween 20 (10mg/ml), 6mM-[γ-^{32}P]ATP and different concentrations of $CaCl_2$, in the presence and in the absence of calmodulin. P_i was extracted as described previously (Jarrett & Penniston, 1977).

For $Na^+ + K^+$-ATPase, ouabain was omitted. Where $Na^+ + K^+$- or Ca^{2+}-ATPase is reported, the ATPase activity in the presence of Mg^{2+} and ouabain was subtracted from the activity in the complete mixture.

Protein determination

Protein content was determined by the method of Lowry *et al.* (1951). To avoid interferences, the protein was precipitated with deoxycholate and trichloroacetic acid (Bensadoun & Weinstein, 1976) before the assay.

SDS/polyacrylamide-gel electrophoresis

The protein was precipitated with 8% (w/v) trichloroacetic acid, and washed once with water. The pellet was resuspended in a buffer containing 60mM-Tris/HCl, pH8, 2% (v/v) SDS, 3% mercaptoethanol, 0.002% Bromophenol Blue and 10% (v/v) glycerol. The mixture was heated for 4min in a boiling-water bath and then applied to polyacrylamide gels (5.6% or 7.5%, w/v). Electrophoresis was performed by the method of Laemmli (1970).

Formation of a [^{32}P]phosphoprotein

Eluted fractions were concentrated to about one-fifth of the original volume in an Amicon ultrafiltration chamber with a YM-30 filter. The incubation medium contained 50mM-imidazole/HCl, pH6.8, 100mM-KCl, 2μM-[γ-^{32}P]ATP (sp. radioactivity, 5–7μCi/nmol), about 10μg of purified enzyme and, where indicated, 0.1mM-$CaCl_2$ and 15μM-$MgCl_2$. The reaction (at 0°C) was started by the addition of the radioactive ATP and stopped after 1min with 1vol. of an ice-cold solution containing 14% trichloroacetic acid, 10mM-H_3PO_4 and 4mM-ATP (stopping solution). In ATP-chase experiments, after 1min an excess of non-radioactive ATP (60mM) was added, and precipitation by trichloroacetic acid solution followed after 15s.

After centrifugation for 20min at 2400*g* at 4°C, the pellet was washed twice with stopping solution and once with water, and then resuspended for gel

electrophoresis in 100mM-sodium phosphate, pH6, containing 1% SDS, 10% glycerol, 20mM-dithiothreitol and 0.002% Bromophenol Blue as a tracking dye. SDS/polyacrylamide-gel electrophoresis of the ^{32}P-labelled enzyme on 5% gels at pH6.0 was done as described by Niggli *et al.* (1979). The gels were then frozen on solid CO_2, and 2mm slices were incubated overnight with 0.6ml of NCS solubilizer at 37°C. After neutralization of the digest with acetic acid, 4ml of scintillation liquid was added and the vials were counted for radioactivity in a Beckman LS-100C scintillation counter. The scintillation liquid was made up as follows: 2 litres of toluene, 1kg of Triton X-100, 16.5g of 2,5-diphenyloxazole and 300mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene.

Competitive-inhibition radioimmunoassay

This was performed as described by Verma *et al.* (1982).

Other methods

Calmodulin was prepared from bovine brain, by a modification of the method used by Jarrett & Penniston (1978) for erythrocyte calmodulin. Free Ca^{2+} concentration was calculated by means of a computer program (Graf & Penniston, 1981) which took into account all complexes involving Mg^{2+}, Ca^{2+}, EGTA and ATP. Different calcium concentrations were checked with a Ca^{2+}-sensitive electrode standardized against optimally buffered solutions of EDTA and Ca^{2+}, in which Ca^{2+} was varied by setting the pH at different values.

Results

Purification procedure

Table 1 summarizes the changes in specific activity of three plasma-membrane-associated ATPases, at various stages of purification. Solubilization was achieved at a Triton X-100/protein ratio of 1:1, by this procedure Mg^{2+}-ATPase and Ca^{2+}-ATPase were preferentially solubilized. After being loaded on a calmodulin–Sepharose 4B column, most proteins passed through the column and were eluted with the washing buffer; this fraction contained Mg^{2+}-ATPase and some calmodulin-independent Ca^{2+}-ATPase. The Ca^{2+}-ATPase retained was free of Mg^{2+}- or $Na^+ + K^+$-ATPase, and could be eluted only after a buffer containing 2mM-EGTA was applied to the column.

Characterization

If the column was washed extensively before the elution with EGTA, the purified Ca^{2+}-ATPase showed a single major band by SDS/polyacrylamide-gel electrophoresis. Fig. 1 shows an SDS/

Table 1. *ATPase at various stages of purification*
Purification separated the Ca^{2+}-ATPase from the $Na^+ + K^+$- and Mg^{2+}-ATPases.

Type of ATPase	ATPase (μmol·min^{-1}·mg^{-1}) Homogenate	Synaptic plasma membrane	Purified ATPase
Mg^{2+}	0.052	0.049	-0.1 ± 0.2
$Na^+ + K^+$	0.186	0.221	*
Ca^{2+}	0.015	0.058	1.8 ± 0.2

* Addition of ouabain raised the amount of P_i in the control; this gave a negative number for the $Na^+ + K^+$-ATPase. The values reported for $Na^+ + K^+$-ATPase and for Ca^{2+}-ATPase were obtained by subtracting the ATPase activity in the presence of Mg^{2+} and ouabain from the activity in the presence of the complete mixture.

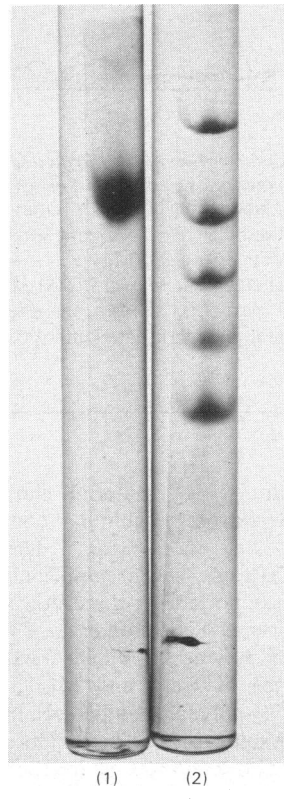

Fig. 1. *Electrophoresis of purified synaptic plasma-membrane Ca^{2+}-ATPase*
Gel (1), ATPase; gel (2), standards of molecular weight (from the top to the bottom; myosin 200000; β-galactosidase, 116000; phosphorylase *b*, 92500; bovine serum albumin, 66000; ovalbumin, 45000). Electrophoresis on 5.6% polyacrylamide gels was performed as described in the Materials and methods section. The gels were stained with Coomassie Blue; the anodic end of the gel appears at the bottom.

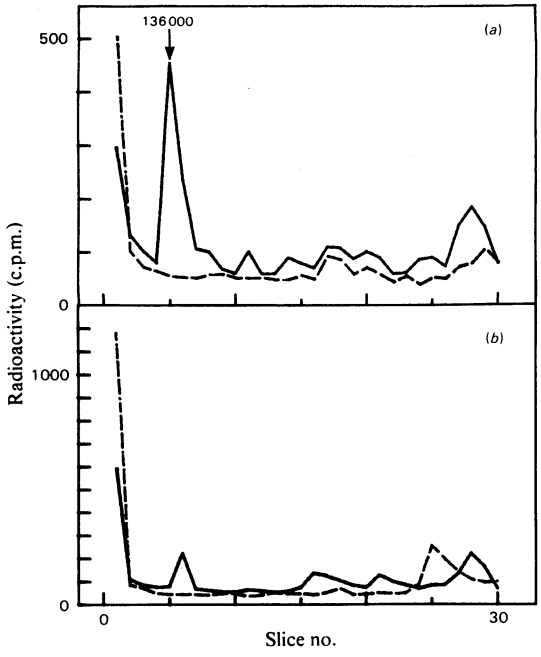

Fig. 2. *Phosphorylation of the purified Ca^{2+}-ATPase*
The figure shows the distribution of ^{32}P radioactivity after phosphorylation for 1 min at 0°C. (*a*) ——, Mg^{2+} and Ca^{2+}; ----, same after chase with unlabelled ATP. (*b*) ——, Mg^{2+}; ----, Mg^{2+}, but no protein. The peak at $M_r = 136000$, shown by the solid line in part (*a*), showed the presence of a Ca^{2+}-dependent phosphorylated intermediate.

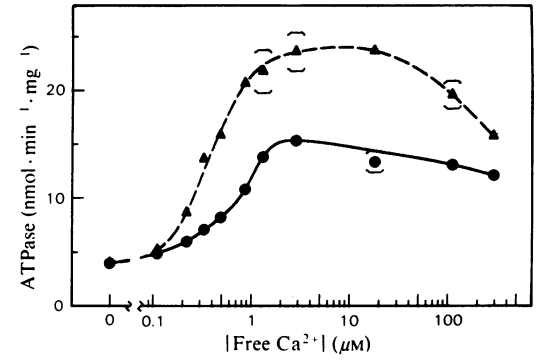

Fig. 3. *ATPase activity of erythrocyte ghosts as a function of pCa*
Ghosts were prepared in EDTA as previously described (Jarrett & Penniston, 1976), and stored frozen before assay. ▲, 300 ng of calmodulin added; ●, no calmodulin. Error bars represent the S.E.M. Three values were observed for each point; very short error bars were omitted. Calmodulin caused a significant stimulation at each pCa < 6.8; P was <0.05 for all pairs of points below pCa 6.8, except the pair at pCa 5.88, where $P < 0.1$.

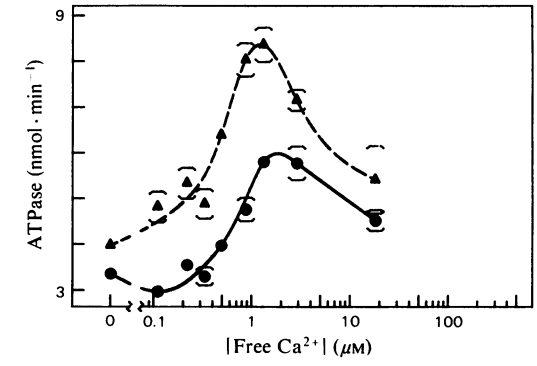

Fig. 4 *ATPase activity of EGTA-extracted synaptic plasma membranes as a function of pCa*
Symbols have the same meaning as in Fig. 3; three values were observed for each point. The activities indicated are those observed for the whole assay; the protein concentration was not determined, but was estimated to be 30 μg/assay. This estimate was based on the yield from previous preparations. Significant stimulation was observed for pCa between 7 and 5; P was <0.05 for all pairs of points in this range.

polyacrylamide gel of material eluted from the calmodulin–Sepharose column, compared with standards. The M_r was determined as 138000 ± 8000 (S.D., seven samples), in agreement with the value reported for the erythrocyte plasma-membrane enzyme (Penniston *et al.*, 1980).

The purified enzyme was labelled with [γ-^{32}P]ATP in the presence of Ca^{2+} and Mg^{2+}, and electrophoresed on 5%-polyacrylamide gels, in a phosphate buffer/SDS system, pH 6. The results are shown in Fig. 2. The major radioactivity peak corresponds to an apparent M_r of 136000. The phosphorylation was Ca^{2+}-dependent and was chased in a short time by the addition of an excess of non-radioactive ATP. The radioactivity at the origin cannot be related to ATPase phosphorylation, since it was also seen in the control gel without added protein. The smaller amount of phosphorylation observed in the absence of Ca^{2+} was probably due to the generation of some Mg^{2+}-ATPase activity during the concentration of the enzyme. Presumably this occurs because of denaturation or a slight proteolysis of the ATPase.

Effect of Ca^{2+}

Figs. 3, 4 and 5 show the ATPase activity as a function of Ca^{2+} concentration in synaptic plasma membranes, purified ATPase and erythrocyte membrane ATPase. All three preparations display a

Table 2. *Immunological cross-reaction of rat brain and human erythrocyte Ca^{2+}-ATPase*

The data shown are the mean $\pm\frac{1}{2}$ range of the assays done in duplicate. The concentration of detergents and other constituents was raised in all the assay tubes to make it equal to that in the assay tubes containing the highest concentrations. Methods were as described in the text.

	Protein (μg)	Specific binding (% of maximum)	P
Purified human erythrocyte Ca^{2+}-ATPase	0	100 ± 0.8	
	0.05	83.6 ± 1.3	0.0010
	0.1	63.9 ± 0.9	0.0000
Purified rat brain synaptic plasma-membrane Ca^{2+}-ATPase	1	95.7 ± 0.8	0.0200
	2	82.5 ± 0.1	0.0013

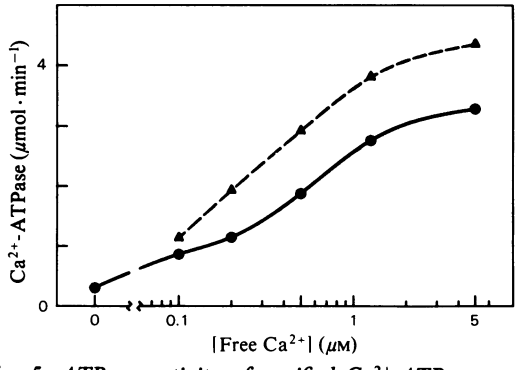

Fig. 5. *ATPase activity of purified Ca^{2+}-ATPase as a function of pCa*

Conditions were the same as for Figs. 3 and 4, and symbols also have the same meaning; three values were observed for each point. Significant stimulation by calmodulin was observed for each pair of points. In order of increasing free Ca^{2+}, P was 0.046, 0.0004, 0.0002, 0.0001 and 0.0016 for the pairs of points. All of the errors were less than 4% of full scale, and error bars are therefore omitted.

similar high affinity for Ca^{2+}, and calmodulin increases the affinity of each preparation for Ca^{2+}, as well as increasing the $V_{max.}$ for ATP splitting. However, for synaptic plasma membranes, the optimal calmodulin activation was reached at lower Ca^{2+} concentrations and the degree of stimulation by calmodulin was less.

The plasma-membrane origin of this ATPase is further supported by its cross-reactivity with antibodies raised against the human erythrocyte-membrane Ca^{2+} pump. This is shown in Table 2, which shows that our purified rat brain Ca^{2+}-ATPase gives an easily detectable cross-reaction in the radioimmunoassay for human erythrocyte Ca^{2+}-ATPase.

Discussion

As mentioned in the introduction, several organelles have been proposed as being responsible for non-mitochondrial Ca^{2+} sequestration in the nerve terminal. We report here a Ca^{2+}-ATPase resembling the Ca^{2+}-pumping ATPase from erythrocyte membranes. The known types of Ca^{2+} pumps are those from sarcoplasmic or endoplasmic reticulum, mitochondria, and plasma membranes. There is little disagreement about the existence or localization of the mitochondrial pump because of its easy inhibition by specific inhibitors of mitochondrial ATPase. The main problem, therefore, is making a distinction between Ca^{2+}-pumping ATPase of endoplasmic-reticulum origin and those of plasma-membrane origin. There are four lines of evidence which indicate that the ATPase purified here originates from plasma membrane.

(1) The enzyme comes from a synaptic plasma-membrane preparation which contains relatively small amounts of mitochondria and reticular membranes (Sorenson & Mahler, 1981; Sobue *et al.*, 1979; Jones & Matus, 1974). The high-affinity Ca^{2+}-ATPase is preferentially enriched along with other plasma-membrane enzymes (Sobue *et al.*, 1979).

(2) The enzyme studied here interacts directly with calmodulin, a type of interaction which has not been reported for the endoplasmic- or sarcoplasmic-reticulum Ca^{2+}-pumping ATPases. In cardiac tissue only, the sarcoplasmic-reticulum ATPase has been reported to be modulated by calmodulin, but this occurs indirectly via a phosphorylation of lamban (LePeuch *et al.*, 1979). Direct regulation by calmodulin is characteristic of the Ca^{2+}-pumping ATPase from erythrocyte ghosts (Niggli *et al.*, 1981) and the Ca^{2+}-ATPase from sarcolemma (Caroni & Carafoli, 1981). That the rat brain enzyme described here interacts directly with calmodulin is shown by its specific binding to a calmodulin–Sepharose column.

(3) The rat brain enzyme has a M_r similar to those of the Ca^{2+}-ATPases from erythrocyte plasma membrane and heart sarcolemma. The M_r values observed by SDS/polyacrylamide-gel electrophoresis for these three enzymes are between 138000 and 150000, which is within the experi-

mental error of the method (Penniston *et al.*, 1980; Caroni & Carafoli, 1981). This contrasts with the M_r of 90000–105000 observed for the Ca^{2+}-pumping ATPase of reticular origin (Tada *et al.*, 1978).

(4) The final evidence indicating a plasma-membrane origin of this ATPase is its cross-reactivity with antibodies raised against the erythrocyte plasma-membrane Ca^{2+}-pumping ATPase. These antibodies are quite selective; Ca^{2+}-pumping ATPases from erythrocyte membranes of a species other than human are recognized by the antibody, but will compete for its sites only about one-tenth as well as human Ca^{2+}-ATPase. Ca^{2+}-pumping ATPases from other plasma membranes compete about one-twentieth to one-hundredth as well, and the pure rabbit skeletal-muscle sarcoplasmic reticulum does not give any detectable competition even at very high concentrations (Verma *et al.*, 1982). The rat brain enzyme discussed in the present paper shows a degree of competition consistent with its identification as coming from a plasma membrane of both a different organ and a different species. Clearly its degree of recognition by the antibody is much greater than that characteristic of the sarcoplasmic-reticulum ATPase.

The brain ATPase is less strongly activated by calmodulin than is the erythrocyte enzyme (compare Figs. 3, 4 and 5). This could be due to a variety of causes, since the enzyme is regulated by other effectors in addition to calmodulin. Acidic phospholipid, in particular, mimics the effects of calmodulin (Niggli *et al.*, 1981). A small amount of acidic phospholipid, tightly bound to the brain ATPase, would account for its lesser stimulation by calmodulin.

Previous reports of Ca^{2+}-stimulated ATPases from mammalian brains gave a M_r of 100000 for the Ca^{2+}-stimulated phosphorylation observed (Blitz *et al.*, 1977; Robinson, 1978). Blitz *et al.* (1977) studied a morphologically well-defined preparation of coated vesicles, and showed immunological reaction of these vesicles with antibodies against carcoplasmic reticulum. Robinson's (1978) preparation was less well defined; he observed Ca^{2+}-ATPase in a crude preparation derived from rat brain microsomal fraction, and applied no immunological or morphological tests to identify the source of the ATPase.

Our experience shows that a difference in M_r does not necessarily indicate a difference in the origin of the enzyme. Our early preparations of the present enzyme showed a lower apparent M_r, but as we improved our preparative methods, higher values were obtained. These changes could be due to the occurrence of proteolysis in samples which were processed too slowly. Thus it is not clear whether the lower M_r observed by Robinson (1978, 1981) is due to proteolysis or to a different origin of his enzyme. Because the ATPase of Blitz *et al.* (1977) was observed in coated vesicles and was immunologically similar to sarcoplasmic-reticulum ATPase, it seems probable that it represents an enzyme distinct from that studied here.

The enzyme purified here is presumably responsible for the high-Ca^{2+}-affinity ATPase activity observed in synaptic membranes by Sobue *et al.* (1979), Sorenson & Mahler (1981) and Gill *et al.* (1981). Their preparations were all rich in plasma membranes and their high-Ca^{2+}-affinity ATPase showed properties consistent with its being a plasma-membrane enzyme. Particularly convincing was the demonstration by Gill *et al.* (1981) that the Ca^{2+} taken into membrane vesicles could be relased by extracellular Na^+; this indicated that their Ca^{2+}-ATPase was in the same membrane with the Na^+–Ca^{2+} antiporter, a recognized plasma-membrane enzyme.

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