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ADENOSINETRIPHOSPHATASE ACTIVITY IN SYNAPTIC VESICLES OF RAT BRAIN

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Abstract—Synaptic vesicles were found to possess a characteristic type of adenosinetriphosphatase which is stimulated by either magnesium or calcium but is not affected by sodium and potassium, dinitrophenol, or sodium azide. Maximal stimulation by magnesium and calcium, obtained with concentrations of 1 to 2 mM, falls rapidly by storage at −20°. The possibility is suggested that this type of adenosinetriphosphatase might intervene in the storage and release of acetylcholine.

IT HAS been widely postulated that most of the acetylcholine (ACh) of brain is stored in vesicles located in the presynaptic nerve endings. In recent years the isolation of these organelles has become possible and it has been shown that they do, in fact possess most of the bound ACh of brain.[1, 2] Early isolation procedures by De Robertis *et al.*[2] produced a vesicle fraction which was particularly enriched with both ACh and choline acetyltransferase. Whittaker *et al.*,[1] using a modified procedure, obtained a yet purer fraction which was devoid of this enzyme. Current criticisms support the idea that these organelles might form, artifically, from mitochondria and would consist of lipoprotein-like micelle structures capable of binding ACh nonenzymatically.[1] However, Whittaker *et al.* were unable to isolate a vesicle fraction when their procedure was applied to liver which contains mitochondria of a much more fragile nature than those of brain.[1] Although the ability of lipoprotein micelle structures to bind ACh has not been tested, micelles, formed with lecithin in water, are unable to bind ACh.[1] Among other lipids tested, phosphatidic acid was found to bind ACh via salt linkage, but only in the absence of more strongly positively-charged metal ions.[3]

The presence of cations is known to influence greatly either the amount of bound ACh recovered in a tissue[4] or the amount of free ACh found in a suspending medium or in a perfusate of this tissue.[6, 7] In recent years a wide variety of cationically-stimulated ATPases has been described. The Na–K–Mg type, present in endoplasmic membranes, has been assigned a governing role in cation transport.[8] Its activation also seems to intervene in the uptake of certain amino acids such as γ-aminobutyrate (GABA).[5] In the latter case, however, it was found that calcium had a role quite similar to sodium in promoting the binding of GABA and other amino acids.[9] All of these ions have some influence on the levels of bound and free ACh, and it is conceivable that their effects proceed through the activation of a cation-stimulated ATPase. Although such ATPase action might be functional at more than one subcellular level, as was reported by Albers *et al.*[10] and Hosie,[11] one of the most conspicous locations needing further investigation was the vesicle fraction. The present

paper reports on the presence and on the cationic specificity of an isolated ATPase present in the vesicle fraction.

METHODS

Synaptic vesicles were isolated from rat brain by the methods of De Robertis *et al.*[2] or Whittaker *et al.*[1] In the latter procedure, the D fraction, containing the vesicles suspended in a sucrose density layer, also contained much of the soluble protein derived from hypotonic treatment of the mitochondrial fraction. This resulted in a great diminution of the specific ATPase activity of the vesicle fraction. The specific activity was very much increased (approximately tenfold) when the D fraction was sedimented at 100,000 *g* for 1 hr. The isolated vesicle fractions were then suspended in 0·32 M sucrose or in water such that the final suspension contained 1 to 2 mg protein/ml. The fractions were stored at −20° for no longer than 2 days.

Proteins were determined by a biuret method;[12] succinic dehydrogenase activity was determined by the method of Slater and Bonner.[13] The vesicle fraction contained 4 to 5% of the total protein and 0·8 to 1·5% of the total succinic dehydrogenase activity of the homogenate. These values are similar to those reported.[2]

ATPase assay was performed by the method of Schwartz *et al.*[14] The incubation mixture of a 1 ml-volume consisted of water as a diluent, 100 mM Tris buffer (pH 7·4), 3 mM Tris ATP, various amounts of metal chlorides, and 0·1 ml of vesicle suspension. Inorganic phosphate liberated by the enzyme was measured by the Fiske–SubbaRow method.[15]

RESULTS AND DISCUSSION

It can be seen from Table 1 that vesicles isolated by the method of either De Robertis *et al.*[2] or Whittaker *et al.*[1] possess ATPase activity that is stimulated by magnesium. The fraction of Whittaker *et al.*, that has been assessed as a purer fraction, had in most cases a higher specific activity both in the presence and absence of divalent cations and was stimulated to a larger extent by magnesium. Figure 1 illustrates the optimal concentrations of magnesium and calcium for the activation of the ATPase present in the fraction of Whittaker *et al.*[1] Increasing the divalent cation concentration above 1·6 to 2 mM resulted in a slight decrease in activity.

TABLE 1. THE EFFECT OF CALCIUM AND MAGNESIUM ON ATPASE OF VESICLES

Ca	Mg	De Robertis[2] (μmoles P_i/mg ptn*/hr)	Whittaker[1] (μmoles P_i/mg ptn/hr)
0		2·63 (13)†	3·91 (6)
0·2	0	4·59 (7)	5·65 (3)
0·4	0	4·81 (8)	6·28 (3)
0·8	0	5·84 (11)	7·06 (5)
2·0	0	6·65 (13)	7·05 (6)
0	0·2		
0	0·4	4·38 (7)	7·12 (6)
0	0·8	5·91 (7)	9·54 (5)
0	1·6		10·74 (5)
0	2·0	6·19 (7)	9·84 (6)

*Protein
† Figures in parenthesis indicate numbers of experiments.

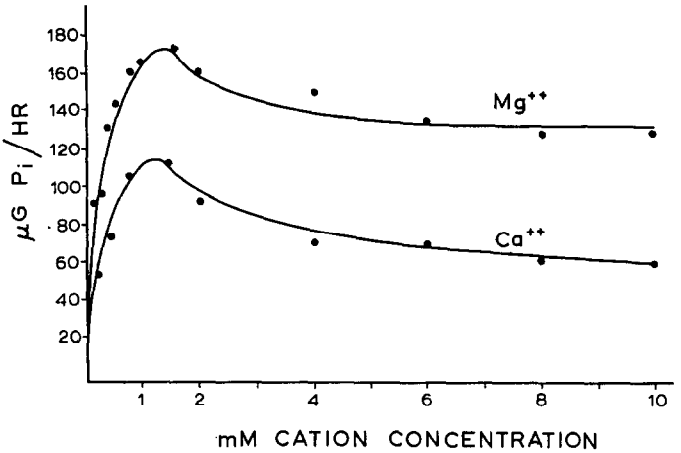

FIG. 1. The effect of varying the concentration of calcium and magnesium on the ATPase activity of the vesicle fraction of Whittaker *et al.*[1] Conditions: the reaction mixture of 1 ml volume contained water as diluent, 100 mM Tris—HCl buffer (pH 7·4), 3 mM Tris ATP added after 5-min preincubation of the vesicles with buffer and divalent ions at 37°. After ATP addition, the reaction proceeded 15 min and was stopped with 1 ml of 10% TCA and cooled to 0°.

Table 2 shows the effect of sodium or potassium alone as well as various combinations of sodium, potassium, and magnesium on the ATPase activity of both fractions. The vesicles of De Robertis *et al.* possess a second type of ATPase that is activated by mixtures of Na, K, Mg and inhibited by ouabain. Since the latter ATPase is quite characteristic of the endoplasmic reticulum, it would appear that the De

TABLE 2. THE EFFECT OF MAGNESIUM, SODIUM, AND POTASSIUM ON ATPASE OF VESICLES

Mg (mM)	Na (mM)	K (mM)	Oubain (mM)	De Robertis[2] (μmoles P_i/mg ptn/hr)	Whittaker[1] (μmoles P_i/mg ptn/hr)
0	0	0	0	4·09 (6)	2·73 (3)
0	25	0	0	4·19 (4)	2·45 (2)
0	50	0	0	4·38 (5)	2·48 (2)
0	75	0	0	4·06 (5)	
0	100	0	0		2·78 (3)
0	0	0	0	3·75 (9)	2·73 (3)
0	0	30	0	3·68 (9)	2·60 (2)
0	0	50	0	4·43 (1)	2·89 (2)
0	25	30	0	3·69 (4)	
0	50	30	0	3·78 (4)	
0	75	30	0	3·81 (4)	
0	100	30	0	3·75 (4)	
0	0	0	0	3·06 (4)	3·91 (6)
0·2	0	0	0	5·00 (2)	7·56 (4)
0·2	100	30	0	11·09 (2)	7·00 (3)
0·2	100	30	0·15	6·66 (2)	7·12 (2)
2·0	0	30	0	8·25 (3)	10·94 (5)
2·0	50	30	0	9·63 (4)	9·90 (4)
2·0	100	30	0	16·38 (4)	10·89 (5)
2·0	100	30	0·15	11·41 (4)	10·56 (5)

Robertis fraction is heavily contaminated by microsomal structures, as the electron-micrographs of Whittaker *et al.* indicated previously. The ATPase activity was little affected by sodium or potassium alone or by mixtures of these two ions. In most instances, mixtures of K, Na, and Mg had little or no net effect on the fraction of Whittaker *et al.* as shown in Table 2 but, in a few instances, the addition of sodium and potassium together produced an inhibition of the magnesium-stimulated ATPase which was proportional to the concentration of sodium. The inconsistency of these results might be explained on the basis of a slight contamination by microsomal ATPase which, from run to run, would vary in amount and would tend to abolish the inhibitory effect.

Table 3 shows the lack of effect of DNP and sodium azide on the ATPase of vesicles isolated by the method of Whittaker *et al.* It would appear that the sodium- and magnesium-stimulated ATPase of vesicles is quite different from that of aging mitochondria, in which case the activation due to magnesium becomes increasingly predominant and the calcium–DNP effect becomes suppressed. On the other hand, aging of vesicles (Fig. 2) resulted in a rapid and simultaneous decrease in both the Mg- and Ca-activated ATPase. The magnesium-stimulated ATPase of vesicles again differs from that of microsomes in that it is not apparently inhibited by sodium azide,

TABLE 3. THE EFFECT OF SODIUM AZIDE AND 2 4-DINITROPHENOL ON ATPASE OF VESICLE FRACTION OF WHITTAKER *ET AL.*[1]

Mg (mM)	DNP (mM)	Na azide (mM)	(μmoles P_i/mg ptn/hr)
0	0	0	3·11 (3)
0	0·25	0	3·06 (2)
0	0·50	0	3·15 (3)
0	0·75	0	3·01 (2)
0·8	0	0	10·94 (5)
0·8	0	0·10	11·20 (2)
0·8	0	0·30	10·64 (3)
0·8	0	0·70	9·98 (2)

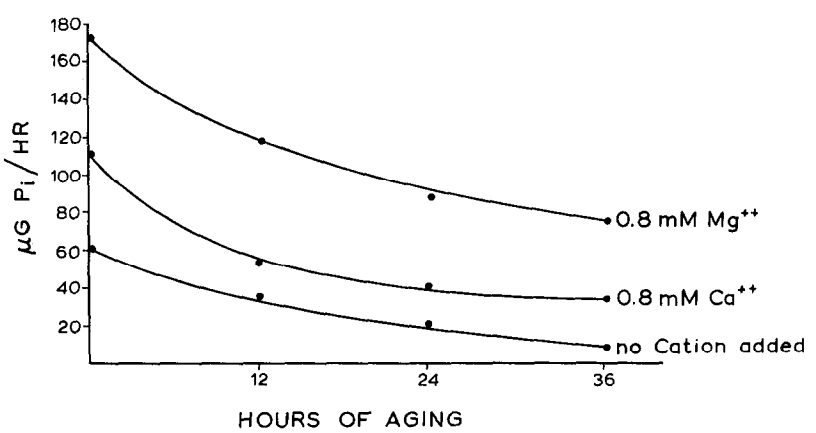

FIG. 2. The effect of storage at −20° on ATPase activity of vesicle fraction of Whittaker *et al.* Conditions were similar to those described for Fig. 1.

Our overall results show that the vesicles do possess a distinctive type of ATPase activity which is dependent on magnesium and calcium. The results also show that the Na–K–Mg activated ATPase is not characteristic of the vesicle but is likely due to the presence of contaminating microsomes. One can therefore dismiss the possibility that the release of ACh promoted by sodium and potassium is due to an activation of an Na–K–mg activated ATPase present in the vesicles. Such an ATPase might well intervene at some other subcellular level. Further refinements in the isolation technique for vesicles and purification of the enzyme itself will be necessary before one can ascertain the inhibitory effect of sodium and potassium on vesicle ATPase and show whether or not calcium and magnesium activate different enzymes.

The presence of an ATPase in the vesicle fraction suggests that ACh storage and release might well proceed through an ATP-dependent mechanism of transport. Before this point can be further clarified, more information is needed concerning the effect of calcium and magnesium on the behaviour of vesicles with respect to release and uptake of ACh.

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REFERENCES

1. V. P. Whittaker, I. A. Michaelson and R. J. Kirkland, *Biochem. J.* **90**, 293 (1964).
2. E. de Robertis, G. Rodriguez de Lores Arnaiz, L. Salganicoff, A. Pellegriono de Iraldi and L. M. Zieehers, *J. Neurochem.* **10**, 225 (1963).
3. W. Vogt, *Hoppe-Seyler's Z. physiol. Chem.* **331**, 239 (1963).
4. J. H. Quastel, *Neurochemistry*, p. 437. Thomas, Springfield, Ill. (1962).
5. H. Weinstein, S. Varon, D. R. Muhlman and E. Roberts, *Biochem. Pharmac.* **14**, 273 (1965).
6. R. Birk and F. C. MacIntôsh, *Can. J. Biochem.* **39**, 387 (1961).
7. D. M. Quastel and R. Birk, *Proc. Can. Fed. biol. Soc.* **5**, 64 (1962).
8. L. E. Hokin and M. R. Hokin, *Ann. Rev. Biochem.* p. 553 (1965).
9. K. A. C. Elliot, R. Tariq Khan, F. Bilodeau and R. A. Lovell, *Can. J. Biochem.* **43**, 407 (1965).
10. R. W. Albers, G. Rodriguez de Lorez Arnaiz and E. de Robertis, *Proc. Nat. Acad. Sci. (Wash.)* **53**, 557 (1965).
11. R. J. A. Hosie, *Biochem. J.* **93**, 13P (1964).
12. E. Zomenhof, in *Methods of Enzymology* **3**, 702 (1957).
13. E. C. Slater and W. D. Bonner, *Biochem. J.* **52**, 85 (1952).
14. A. Schwartz, H. J. Bachelard and H. McIlwain, *Biochem. J.* **89**, 626 (1962).
15. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).