# CALCIUM-STIMULATED PHOSPHORYLATION OF A BRAIN (Ca + Mg)-ATPase PREPARATION

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Received 5 December 1977
Revised version received 19 January 1978

## 1. Introduction

Regulation of the cytoplasmic concentration of calcium ions is particularly important in the nervous system, for the level of free calcium affects both axonal conduction and synaptic transmission. In addition to systems for extruding calcium from the cell by Na/Ca exchange [1] and for sequestering it within mitochondria [2], there also appears to be a calcium transport system in membrane fractions from brain linked to (Ca + Mg)-ATPase activity [3–5]. This ATPase activity, defined operationally as the increment in activity when $CaCl_2$ is added in the presence of an otherwise optimal $MgCl_2$ concentration, has been little studied, but it would seem analogous to (Ca + Mg)-ATPases of muscle sarcoplasmic reticulum [6] and red blood cell plasma membrane [7]. These latter ATPases are both associated with calcium transport, and both enzymes appear to undergo phosphorylation by ATP as a step in their reaction sequences [6,8–10], as does the sodium pump (Na + K)-ATPase [6].

The experiments described here indicate that a (Ca + Mg)-ATPase preparation from rat brain is also phosphorylated by ATP and sodium dodecyl sulfate (SDS)–gel electrophoresis of the phosphorylated preparation shows that calcium stimulates incorporation into a single major fraction with an approx. app. mol. wt 100 000. The calcium-dependent phosphorylation is rapid, as is the spontaneous dephosphorylation, consistent with a reaction intermediate. In addition, the calcium-dependent phosphorylation is sensitive to hydroxylamine, as are the acyl phosphate intermediates of the (Ca + Mg)-ATPases and the (Na + K)-ATPase [6,9,10].

## 2. Methods and materials

The ATPase preparation was obtained from rat brain microsomes [5] by treatment with the detergent Lubrol WX: 0.2 mg microsomal protein was incubated at room temp. in 0.55 ml medium containing 0.07% (w/v) Lubrol-WX, 0.07 M KCl, 0.01 M Tris–HCl (pH 7), and 40% (v/v) dimethyl sulfoxide. The incubation was terminated by adding 0.1 ml 0.03 M $MgCl_2$, 0.1 ml 1% (w/v) lecithin suspension, and 1.0 ml 1.2 M sucrose, all at 0°C; this mixture was then diluted with 25 ml 0.25 M sucrose, and centrifuged for 30 min at 30 000 × *g*. The resulting pellet, containing the ATPase activity, was resuspended in 0.25 M sucrose.

ATPase activity was measured at 37°C in terms of the release of inorganic phosphate, measured colorimetrically [6], or as the release of $^{32}P_i$ from incubation with [$\gamma$-$^{32}$P]ATP [11]. The standard medium contained 30 mM histidine·HCl–Tris (pH 7.0), 3 mM ATP, 3 mM $MgCl_2$, 100 mM KCl, and either 0.1 mM $CaCl_2$ or 0.1 mM ethyleneglycerol-bis-($\beta$-amino-ethyl ether)-*N,N'*-tetraacetate (EGTA). The difference in activity between incubation in the absence of calcium (presence of calcium chelator EGTA) and in the presence of calcium is taken to be the (Ca + Mg)-ATPase activity.

Phosphorylation of the preparation was measured

at 0°C in terms of the incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]ATP. The standard medium contained 30 mM histidine·HCl–Tris (pH 7.0), 3 $\mu$M ATP with tracer quantities of [$^{32}P$]ATP, 0.5 mM $MgCl_2$, 100 mM KCl, and either 0.1 mM $CaCl_2$ or 0.1 mM EGTA. The reaction was terminated by adding 70 vol. 5% (w/v) tricholoracetic acid containing unlabeled ATP, and this mixture was filtered through Gelman membrane filters (0.45 $\mu$m pore size) coated with Celite; the filters were then washed twice with 5% tricholoracetic acid containing unlabeled inorganic phosphate [12]. Radioactivity remaining on the filter was measured by liquid scintillation counting. The difference in incorporation between incubation in the absence and presence of $CaCl_2$ is taken to be the Ca-dependent phosphorylation.

Gel electrophoresis of the phosphorylated preparation, solubilized by SDS [13], was performed at 4°C on Bio-Rad 7.5% polyacrylamide gels using Bio-Rad BioPhore SDS buffer. For determining radioactivity by liquid scintillation counting, the gels were sliced into 1.6 mm thick discs. Molecular weights were estimated by using British Drug House (BDH) molecular weight markers for gel electrophoresis (these consist of proteins crosslinked with diethylpyrocarbonate to give a series of oligomers, monomer to pentamer, with mol. wt 53 000–265 000). Bands were stained with Coomassie blue.

## 3. Results and discussion

Addition of low concentrations of $CaCl_2$ to the incubation medium markedly increased the incorporation of radioactivity from [$\gamma$-$^{32}P$]ATP into the ATPase preparation. With 3 $\mu$M ATP and 500 $\mu$M $MgCl_2$, the increment in labeling due to the addition of 100 $\mu$M $CaCl_2$ averaged 6 pmol/mg protein (table 1). Moreover, SDS–gel electrophoresis of the labeled preparation showed that the calcium-dependent incorporation was confined largely to a single fraction, with approx. app. mol. wt 100 000 (fig.1). For both the (Na + K)-

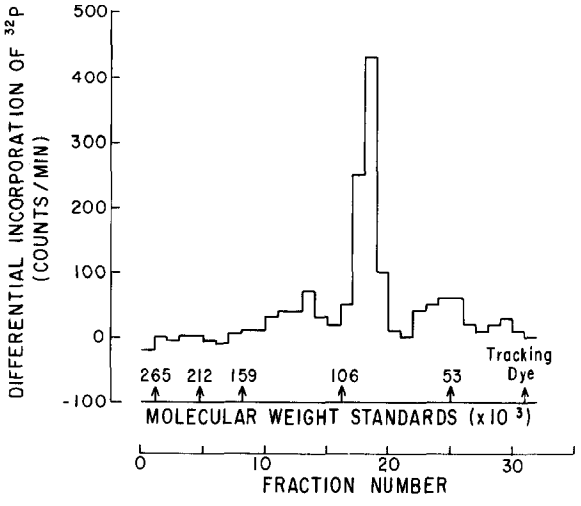


Fig.1. Electrophoretic pattern of calcium-dependent incorporation of $^{32}P$ from [$^{32}P$]ATP. The ATPase preparation was incubated at 0°C in the standard phosphorylation medium in the presence and absence of calcium (see section 2). The preparation was then dissolved in SDS and subjected to SDS–gel electrophoresis. Radioactivity of sequential fractions of the gels was measured by liquid scintillation counting and is presented as the calcium-dependent incorporation. The positions of the molecular weight markers, run concurrently, are also shown.

Table 1
Characteristics of the phosphorylation and ATPase properties of the preparation

| Conditions[a] | ATPase activity ($\mu$mol $P_i$/min·mg protein) | Phosphorylation (pmol/mg protein) |
|---|---|---|
| A. With EGTA | 0.05 ± .01 | 2.2 ± .3 |
| B. With $CaCl_2$ | 0.36 ± .02 | 8.0 ± .4 |
| C. Ca-dependent (B–A) | 0.31 | 5.8 |

[a] ATPase activity was measured in the standard medium (see section 2) with 0.1 mM EGTA (A) or 0.1 mM $CaCl_2$ (B); incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]-ATP was measured in 10 s incubations in the standard medium (see section 2), also with either 0.1 mM EGTA (A) or 0.1 mM $CaCl_2$ (B). Data are averages of six determinations ± SEM

ATPase and the sarcoplasmic reticulum (Ca + Mg)-ATPase [6] the phosphorylated peptides also have approx. mol. wt 100 000, estimated from SDS–gel electrophoresis. By contrast, the red blood cell (Ca + Mg)-ATPase appears, from similar studies, to have mol. wt 150 000 [7–9]. In these three cases the polypeptide is thought to span the membrane in vivo.

(Ca + Mg)-ATPase activity of this preparation, measured at 37°C under near-optimal condition in the standard medium, averaged 0.3 μmol $P_i$ released/min·mg protein (table 1). The concentration of free calcium for half-maximal activity (estimated through the use of Ca–EGTA buffers [14] was about 1 μM. Double reciprocal Lineweaver-Burk plots of ATPase activity against ATP concentration were biphasic, with app. $K_m$ values of 0.1 mM [5] and 3 μM. (Biphasic Lineweaver-Burk plots for ATP for the sarcoplasmic reticulum (Ca + Mg)-ATPase and for the (Na + K)-ATPase are in [6].)

The concentration of free calcium for half-maximal incorporation of $^{32}P$ was also about 1 μM, and the $K_m$ for ATP 2 μM. With 100 μM $CaCl_2$ and 3 μM ATP, lowering the $MgCl_2$ concentration from 500–50 μM reduced calcium-dependent incorporation of $^{32}P$ by 25%, as did raising it to 3 mM.

Incorporation of $^{32}P$ was rapid at 0°C, attaining the steady-state level by 10 s (fig.2). Unlabeled ATP addition after 10 s, to terminate further incorporation of $^{32}P$, resulted in a rapid loss of labeling, the incorporation falling by 75% within 10 s (fig.2).

In these experiments the phosphorylated preparations were routinely washed with 5% trichloroacetic acid. Raising the washing solution to pH 6.0 (using 0.8 M sodium acetate buffer) reduced the final content of $^{32}P$ to 50% control values. When the phosphorylated preparation was allowed to react for 5 min with 0.6 M hydroxylamine (pH 6.0, in 0.8 M sodium acetate), the calcium-dependent incorporation fell to 6% control values. This sensitivity to hydroxylamine, as well as the acid-stability, are consistent with the presence of an acyl phosphate intermediate, and also with observations on $^{32}P$ incorporation into both (Ca + Mg)-ATPases [6,9,10] and into the (Na + K)-ATPase [6].

These experiments thus describe a calcium-dependent phosphorylation of a 100 000 mol. wt peptide, with a rapid turnover, linked to the (Ca + Mg)-ATPase of the preparation by a similar concentration dependence on calcium and ATP. The stability of the phosphorylated product is consistent with an acyl phosphate intermediate. These properties are analogous to those of other transport ATPases, although this enzyme differs from the red blood cell (Ca + Mg)-ATPase in molecular weight. The similar distribution in subcellular fractionation of this (Ca + Mg)-ATPase with the (Na + K)-ATPase [3,5] suggests a localization in the plasma membrane. If calcium transport by the membrane fraction as measured in vitro [3–5] is into everted sacs of the plasma membrane [15], then this enzyme may function in vivo to extrude calcium from the cell.

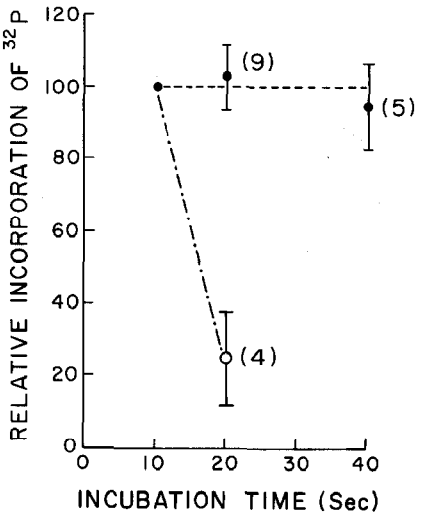


Fig.2. Time course of calcium-dependent incorporation of $^{32}P$. The ATPase preparation was incubated at 0°C for the times indicated in the standard medium, in the presence and absence of calcium, and the calcium-dependent incorporation of radioactivity measured (•) (see section 2). In other experiments, after incubation for 10 s in the standard medium unlabeled ATP was added (final conc.: 10 mM) to halt further incorporation of $^{32}P$, and the amounts of radioactivity remaining after 10 s further incubation measured (○). Data are presented relative to the incorporation at 10 s, defined as 100 ± SEM for the no. experiments indicated within the brackets.

**Acknowledgement**

This work was supported by US Public Health Service grant NS-12193.

## References

[1] Baker, P. F., Blaustein, M. P., Hodgkin, A. L. and Steinhardt, R. A. (1967) J. Physiol. 192, 43–44.
[2] Lust, W. D. and Robinson, J. D. (1970) J. Neurobiol. 1, 303–316.
[3] Nakamaru, Y. and Schwartz, A. (1971) Arch. Biochem. Biophys. 144, 16–29.
[4] deMeis, L., Rubin-Altschul, B. M. and Machado, R. D. (1970) J. Biol. Chem. 245, 1883–1889.
[5] Robinson, J. D. (1976) Arch. Biochem. Biophys. 176, 366–374.
[6] Robinson, J. D. (1976) in: Chemical Mechanisms in Bioenergetics (Sanadi, D. R. ed) pp. 78–122, ACS Monograph Series, Washington.
[7] Schatzman, H. J. and Rossi, G. L. (1971) Biochim. Biophys. Acta 241, 379–392.
[8] Knauf, P. A., Proverbio, F. and Hoffman, J. F. (1974) J. Gen. Physiol. 63, 324–336.
[9] Rega, A. F. and Garrahan, P. J. (1975) J. Memb. Biol. 22, 313–327.
[10] Katz, S. and Blostein, R. (1975) Biochim. Biophys. Acta 389, 314–324.
[11] Robinson, J. D. (1976) Biochim. Biophys. Acta 429, 1006–1019.
[12] Post, R. L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540.
[13] Hokin, L. E., Dahl, J. L., Deupree, J. D., Dixon, J. F., Hackney, J. F. and Perdue, J. F. (1973) J. Biol. Chem. 248, 2593–2605.
[14] Godt, R. E. (1974) J. Gen. Physiol. 63, 722–739.
[15] Logan, J. G. and Waters, G. A. (1976) J. Physiol. 256, 30.