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Properties of a microsomal adenosine triphosphatase from the adrenal medulla

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IN CONNECTION with studies of the ability of calcium to release catecholamine from granules of the adrenal medulla, a microsomal fraction from the medulla was investigated from the standpoint of its ATPase activity, the effects of ions and drugs on the activity, and the ability of this fraction to remove calcium from the medium.

Calf adrenal medullae were first forced through a tissue press and then homogenized in a glass–Teflon homogenizer in a 0·3 M sucrose plus 100 mM Tris (pH 6·8) solution. Five ml of this solution was used for every gram of adrenal medullary pulp to be homogenized. The supernatant from a centrifugation at 12,000 *g* for 20 min was further centrifuged at 100,000 *g* for 60 min. The sediment was resuspended in 0·3 M sucrose and designated the microsomal fraction. The suspension was stored at −20° until used.

Incubations were carried out at 37° for 15 or 20 min. The incubation volume of 1·0 ml contained 120 mM Tris, pH 7·4, 3 mM Tris-ATP (Sigma Chemical Co.), microsomal fraction (0·15–0·2 mg protein) plus appropriate metal ions and drugs. The incubations were initiated by addition of ATP and terminated by placing the tubes in ice and adding 0·5 ml of ice-cold 10 per cent trichloroacetic acid. Phosphate was determined by the method of Fiske and Subbarow[1] and protein by the method of Lowry *et al.*[2]

Experiments on calcium uptake by the microsomes were performed at room temperature under conditions found to give good calcium uptake by a microsomal fraction isolated from heart.[3] In one group of experiments the incubation contained, in addition to 1 mg microsomal protein, 10 mM Tris-maleate buffer, pH 6·8; 120 mM KCl; 3 mM $MgCl_2$; 3 mM Tris-ATP; and an ATP generating system of 6 mM creatine phosphate and 0·1 mg of creatinephosphokinase/ml (Sigma Chemical Co.). Two concentrations of calcium were used, 10^{-7} M and 10^{-6} M. Calcium-45 (Iso/Serve, Inc.) was added in sufficient quantity to give control values of 5 to 10 min counting time for 1000 counts. Total incubation volume was 2·5 ml. The reaction was started by addition together of ATP and creatine phosphate. Aliquots were removed at 0, 2, 5, and 10 min from each incubation. The microsomes were filtered out by suction filtration through a nitrocellulose filter with a pore size of either 0·3 or 0·45 μ diameter (Millipore Filter Corp.). After filtration, 0·2 ml of the filtrate from each time period was counted on a gas-flow counter (Nuclear Chicago). In another group of experiments, sodium phosphate concentration was changed from 1 to 10 mM. All other conditions remained the same.

Calcium activated the ATPase of the microsomal fraction, as shown in a typical experiment in Fig. 1. Maximal activation occurred at about 2 mM calcium. Experiments varying the pH showed a steadily increasing ATPase activity in the presence of calcium from pH 6·5 to 9.4. No apparent leveling off of activity occurred at the highest pH tried, 9·4. A study with 2 mM calcium at 37° and pH 7·4 showed that with the usual concentration of microsomal fraction, the rate of ATP splitting was constant at least up to 35 min.

Magnesium incubated under the same conditions as calcium also activated microsomal ATPase, but to a lesser degree (Fig. 1). In the several microsomal preparations tested, maximal activation occurred at 3–4 mM. In contrast to the effect found with calcium, variation of pH in the range of 6·5—8·6 had little if any effect on ATPase activation by magnesium.

There appears to be an interaction of calcium and magnesium relative to activation of microsomal ATPase. In three experiments in which the ATPase activity with varying calcium concentrations alone was compared to the ATPase activity with the same calcium concentrations plus 3 mM magnesium, the amount of phosphate liberated was in all cases equivalent to that found with only 3 mM magnesium present (Fig. 2). Thus, 3 mM magnesium was able to completely suppress activation due to calcium. Moreover, in three other experiments in which only 1 mM magnesium was used, the calcium activation curve was definitely depressed relative to the activation curve of calcium alone (Fig. 2). In Fig. 3 the effect of increasing concentrations of magnesium on the activation by 2 mM calcium is shown. Noticeable inhibition of the calcium activation of ATPase occurred at a magnesium concentration of about 300 μM.

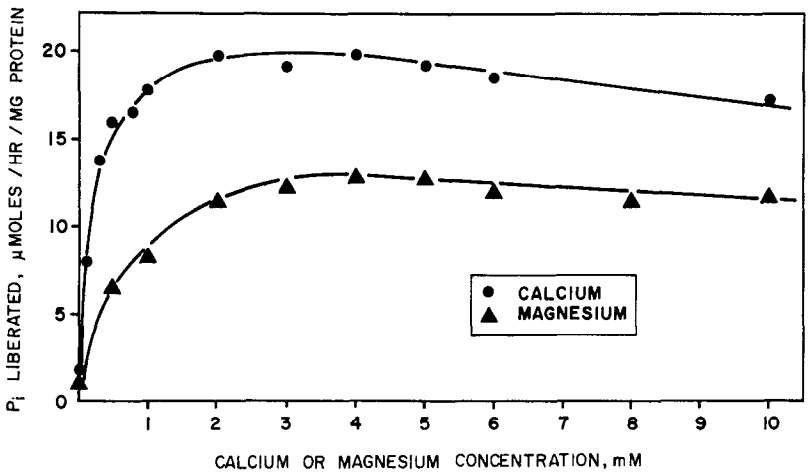

FIG. 1. Calcium and magnesium activation of microsomal ATPase. Microsomal fraction (0·15–0·2 mg protein) was incubated for 15 min at 37° with 125 mM Tris buffer, pH 7·4, 3 mM Tris-ATP, and indicated concentrations of calcium or magnesium.

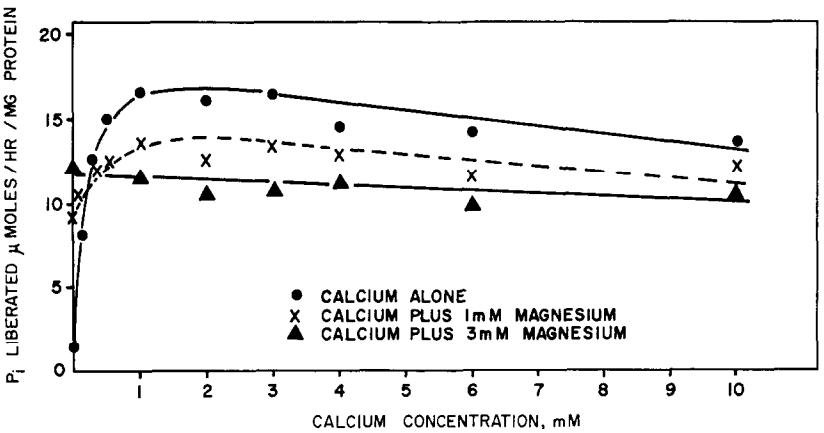

FIG. 2. Calcium activation of microsomal ATPase with and without 1 or 3 mM magnesium. Results are from a typical experiment. Microsomal fraction (0·15–0·2 mg protein) was incubated for 15 min at 37° with 125 mM Tris buffer, pH 7·4, 3 mM Tris-ATP, and indicated concentrations of calcium either alone or with 1 or 3 mM magnesium.

There are two possible interpretations of this interaction. It may be that there is one enzyme for which magnesium has a greater affinity but a lesser activation than calcium. Or it may be that there are two enzymes, one of which is activated by magnesium and the other by calcium. In the latter case, it may be that magnesium, although not able to activate the calcium-activated enzyme, can prevent calcium from acting. At present there is no way to choose between the two possibilities. The magnesium inhibition does not appear to be competitive, since increasing the calcium concentration in the presence of 3 mM magnesium to give five times that needed for maximal activation in the absence of magnesium did not alter the ATPase activation (Fig. 2). Further, even when 1 mM magnesium was present, which gives partial inhibition of calcium activation, increments in calcium concentration did not yield the maximum possible activation (Fig. 2). In the presence of 1 mM magnesium, only about 25 per cent of maximal calcium activation was obtained. Finally, it should be emphasized that, in other tissues, calcium activates ATPase much less than does magnesium. Moreover, in the other tissues calcium inhibits magnesium activation.[4]

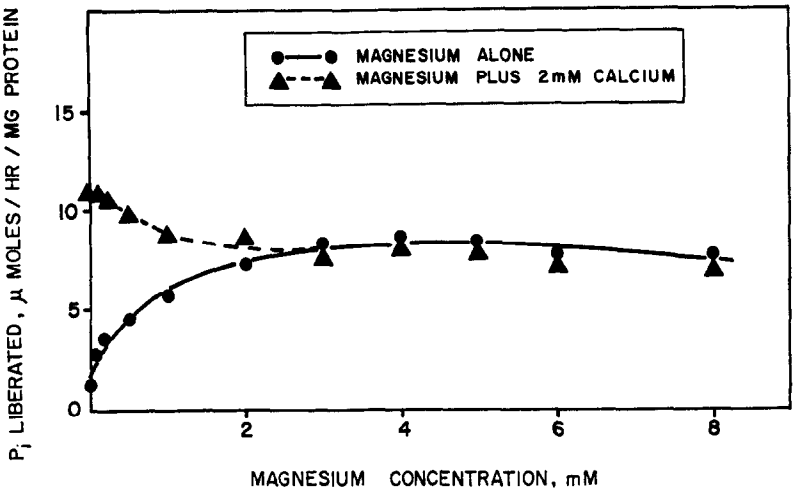

FIG. 3. Effect of increasing concentrations of magnesium on calcium activation of microsomal ATPase. Microsomal fraction (0·15–0·2 mg protein) was incubated for 15 min with 125 mM Tris buffer, pH 7·4, 3 mM Tris-ATP, and indicated concentrations of magnesium with or without 2mM calcium.

Sodium or potassium, separately, together, or in combination with magnesium, calcium, or with both did not stimulate ATPase. In fact, at high concentrations of 60 and 100 mM potassium there was inhibition of both calcium and magnesium activation of ATPase. Similar results obtained with preparations used without prior storage at −20°.

Strontium activated phosphate cleavage to a level of 65 per cent of that found with calcium, but a concentration of 30 mM strontium was required. In addition, when 100 mM strontium was added with 2 mM calcium, there was 20 per cent inhibition of the calcium activation of ATPase.

Barium appeared to stimulate ATPase, but to a lesser extent even than strontium. Concentrations higher than 2 mM could not be tested, since they interfered with the Fiske-Subbarow analysis for phosphate.

Ethyleneglycol bis (β-aminoethylether)-N,N′-tetraacetic acid (EGTA), 10^{-4}M, produced a small decrease in ATPase activity. The decrease was probably due to chelation by the EGTA of endogenous calcium. EGTA binds calcium preferentially to magnesium.

Sodium desoxycholate was used from 0·25 to 2 mM in an attempt to demonstrate a "hidden" sodium–potassium-activated ATPase,[4] and also to find out whether a differential inhibition of either magnesium- or calcium-activated ATPase could be found. With all combinations of ions used (i.e. 2 mM calcium, 3 mM magnesium, 2 mM calcium plus 100 mM sodium and 10 mM potassium, 3 mM magnesium plus 100 mM sodium and 10 mM potassium) there was an increasing inhibition of ATPase concomitant with increasing desoxycholate concentrations. For example, with 2 mM calcium present, concentrations of 0·25, 1, and 2 mM desoxycholate inhibited ATPase activity by 15, 32, and 72 per cent respectively. Further, there was no apparent differential inhibition or stimulation of any one combination of ions as compared to any other.

$HgCl_2$ at 10^{-5}M inhibited both calcium and magnesium activation of ATPase by about 75 per cent. A concentration of 10^{-6} M had no observable effect.

The following drugs had no effects on calcium- or magnesium-activated ATPase at the given concentrations: ouabain, 10^{-11}, 10^{-10}, 10^{-6}, 10^{-5} g/ml; tetracaine, 10^{-5}, 10^{-4} M; acetylcholine, 10^{-4} M; epinephrine 10^{-4}, 2×10^{-4}, 5×10^{-4} g/ml. (Epinephrine was tried only on calcium activation.)

Studies of possible calcium accumulation by the microsomal fraction, by means of ^{45}Ca, showed no calcium uptake. The absence of uptake was observed at calcium concentrations of 10^{-7} and 10^{-6} M and for all the incubation periods (2, 5, and 10 min). In the initial studies, 1 mM sodium-phosphate was used. In another group of experiments 10 mM sodium-phosphate was used. This procedure, which markedly increased calcium uptake by a sarcoplasmic reticulum fraction from

heart (H. Ladinsky, personal communication), did not alter the absence of observable calcium uptake by the microsomal fraction from the medulla.

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Dependence of rat serum lactonase upon calcium

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RECENTLY we reported that rat plasma and liver contain an enzyme that very rapidly catalyzes the hydrolysis of γ-butyrolactone (GBL) to γ-hydroxybutyric acid (GHB).[1] The fact the diisopropylfluorophosphate, prostigmine, and physostigmine did not markedly inhibit this hydrolysis suggested that the enzyme might be more specifically a lactonase than an esterase. This was further borne out by the ability of rat plasma and serum to hydrolyze the homologue, γ-valerolactone, but at a slower rate than GBL. This paper presents further evidence that supports this contention.

When simple attempts were made to purify the lactonase in rat serum, it was observed that dialysis and Sephadex treatment completely destroyed all the enzymatic activity. These observations together with our finding that EDTA completely blocks GBL hydrolysis by rat plasma suggested that one or more reversibly bound metal ions might be involved in the function of this enzyme. Therefore, the dependence of the lactonase on various cations was investigated.

Since we had established that plasma could hydrolyze GBL to GHB and that further metabolism by this tissue was negligible, a simpler method than measuring the disappearance of GBL and appearance of GHB by gas chromatography was sought to follow the rate of hydrolysis. It is known that GBL yields the corresponding hydroxy acid on hydrolysis;[2] therefore, it was considered feasible to utilize a titrimetric assay, based on the amount of acid formed during hydrolysis, to follow the lactonase activity. Similar types of titrimetric assays have been employed to measure activity of esterases.[3] Titration of the GHB formed in the reaction under consideration was found to be a very simple and reliable method for our purposes. The details of the method as well as some preliminary findings are presented below.

All incubations in these studies were carried out at 37° by means of a constant-temperature bath coupled to a jacketed incubation vessel. The internal temperature of the reaction mixture was monitored by means of a YSI model 4ZSC telethermometer and did not deviate from 37° by more than 0·2° All reactions were followed with a Radiometer Titrigraph type SBR23SBUI. Sodium hydroxide (*ca.* 0·3 M) was prepared from carbon dioxide-free distilled water and standardized with potassium acid phthalate. The concentration of GBL routinely used in this study was $1{\cdot}3 \times 10^{-2}$ M, which