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Mg^{2+} and Ca^{2+} Activated ATPase Activities of Bovine Aortic Microsomes[1] (40430)

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Calcium and magnesium stimulated ATPase activities of microsomes from vascular smooth muscle are not as well defined as they are for microsomes from cardiac and skeletal muscle (1–3). Such information is of interest because microsomal hydrolysis of ATP is purportedly involved in modulating cytosolic concentrations of Ca^{2+} and subsequent interactions between the contractile proteins actin and myosin.

Rabbit aortic microsomes exhibit an ATPase which is activated by magnesium (4, 5). Hydrolysis of ATP can also be activated by calcium independently of magnesium activation. Whether these ATPase activities represent different enzyme moieties or a single moiety which is activated by either magnesium or calcium has not been elucidated.

Additionally, aortic microsomes from rats (6), cattle (7), and rabbits (8), reportedly contain a calcium stimulated but magnesium dependent ATPase which is associated with microsomal accumulation of calcium. This activity is analagous to the classic "extra" ATPase activity of striated muscle sarcoplasmic reticulum (1, 9). However, several investigators could not detect "extra" ATPase in smooth muscle microsomes even when accumulation of calcium was demonstrable (10–12).

In this study we provide evidence suggesting that magnesium activated and calcium activated hydrolysis of ATP by bovine aortic microsomes is probably ascribable to a single ATPase. We also show that "extra" ATPase is not demonstrable over a wide range of calcium concentrations.

Materials and Methods. Bovine aortic microsomes were prepared according to procedures adapted from Ford and Hess (13). Briefly, 100 g of muscularis freed of intima and surrounding adventia was minced in a commercial meat grinder, and homogenized in 500 ml of a solution containing 0.1 m*M* NaN_3, 10 m*M* $NaHCO_3$, and 10 m*M* imidazole pH 7.0. The homogenate was centrifuged for 15 min at 1500*g*. The resulting supernatant was centrifuged at 40,000*g* for 60 min and the sedimented microsomal pellet was washed once in 0.6 *M* KCl, 10 m*M* $NaHCO_3$ and 10 m*M* imidazole pH 7.0, and washed again in 80 m*M* KCl and 10 m*M* imidazole pH 7.0. Washed microsomes were suspended in 0.3 *M* sucrose and 10 m*M* imidazole pH 7.0. The suspension was frozen and studies with each preparation were completed within 18–24 hr. All preparative procedures were performed at 4°.

Microsomal protein was determined according to the method of Lowry *et al.* (14). ATPase activities were determined from time course assays similar to those employed for actomyosin ATPase (15). Reaction mixtures (8 mls) consisted of 100 m*M* KCl, 10 m*M* NaN_3, 20 m*M* imidazole pH 7.0, and various concentrations of ATP (disodium salt), Mg^{2+}, and Ca^{2+}. Additional reagents were included as needed (see results and Figure Legends). Assays were performed at 37° with constant stirring. Except where indicated the reaction was initiated by addition of 0.2 mg/ml of microsomal protein and terminated by transfer of timed samples to 10% trichloroacetic acid kept at 4°. Liberated inorganic phosphate was determined by the method of Rockstein and Herron (16).

Results. The yield of microsomal protein for 15 preparations studied ranged from 4–7 mg/g of aortic muscularis. A magnesium activated as well as a calcium activated ATPase activity was demonstrable in all of the preparations studied.

The time course for Mg^{2+} activation shows that a high rate of ATP hydrolysis was attained (Fig. 1A). An early burst of hydrolysis was always evident within the first 30 sec of the reaction. Accordingly, steady state rates of hydrolysis were calculated from the later well defined linear portion of the time course curves. No extra ATPase was detected when

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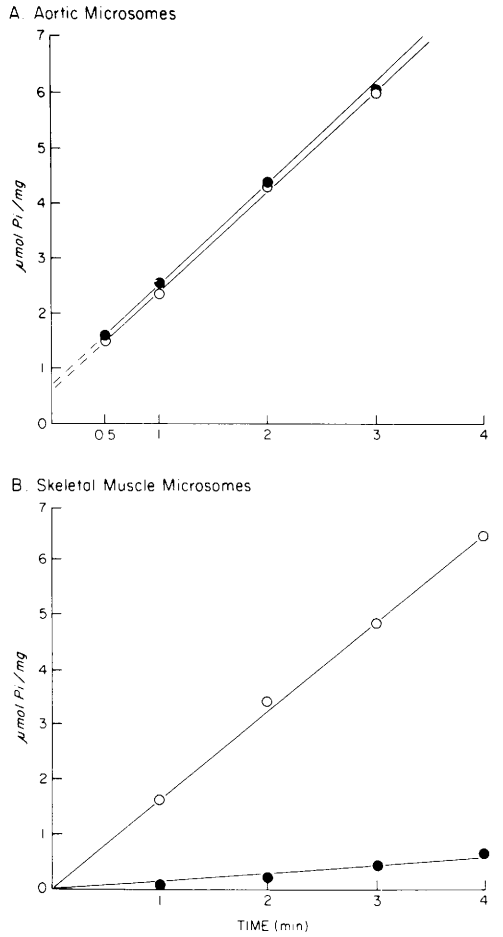

FIG. 1. ATP hydrolysis as a function of time by microsomes from bovine aorta (A) and rabbit skeletal muscle (B). ATPase activity of bovine aortic microsomes was assayed in standard medium with 0.2 mg/ml protein and the following reagents: dark circles, 5 m*M* $MgCl_2$, 5 m*M* ATP and 1 m*M* EGTA; clear circles, 5 m*M* $MgCl_2$, 5 m*M* ATP, 1 m*M* EGTA and 1.046 m*M* $CaCl_2$ (50 μM free Ca^{2+}). In B, ATPase activity of skeletal muscle microsomes was assayed with 0.2 mg/ml protein and the same reagents given in A.

50 μM Ca^{2+} was included in the reaction mixture (Fig. 1A, clear circles).

In sharp contrast, the Mg^{2+} activated ATPase of skeletal microsomes prepared in identical fashion was markedly lower than the corresponding activity of aortic microsomes (Fig. 1B, dark circles). However, the expected Ca^{2+} + Mg^{2+} "extra" ATPase was easily demonstrable in the skeletal muscle preparations. Thus in such preparations the activity increased 10-fold in the presence of 50 μM Ca^{2+} (Fig. 1B, clear circles).

Figure 2 shows that no "extra" ATPase could be detected with aortic microsomes even when the concentration of Ca^{2+} was varied from 5×10^{-7} to 5×10^{-3} *M*. In fact, the basic Mg^{2+} activity was not altered at any concentration of Ca^{2+} tested. Similarly, varying the concentration of Mg ATP (0.25–2.5 m*M*) in the presence of 50 μM Ca^{2+} did not reveal "extra" ATPase activity in three different preparations tested. In the absence of calcium and magnesium (1 m*M* EDTA) no ATPase activity was manifest.

Additionally, aortic microsomes exhibited an ATPase activity that was activated by Ca^{2+} alone, i.e., not dependent on Mg^{2+}. Like the Mg ATPase, this Ca ATPase (5 m*M* Ca^{2+}) was of a high specific activity and was characterized by an initial rapid burst of ATP hydrolysis that occurred within the first 30 sec of the reaction. Double reciprocal plots of both the Mg ATPase and Ca ATPase activities as a function of divalent cation concentration in the presence of 5 m*M* ATP are shown in Fig. 3A. The apparent K_m (0.37 m*M*) and V_{max} (2.1 μmol Pi/mg/min) of the Ca ATPase were identical to those of the Mg ATPase. When ATP was varied keeping the ratio of divalent cation to ATP at 2:1 throughout, the apparent K_m and V_{max} values for the respective activities were again identical (Fig. 3B). Furthermore, these values were not different from those shown in Fig. 3B. These

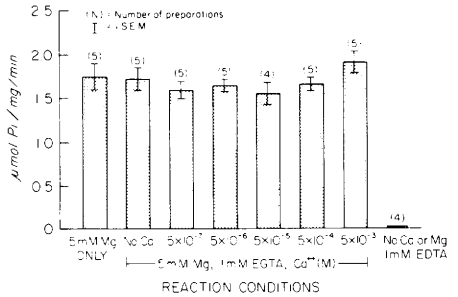

FIG. 2. ATPase activity of aortic microsomes in the presence of magnesium at various concentrations of free Ca^{2+}. ATPase activity was assayed in standard reaction medium with 0.2 mg/ml microsomal protein, 5 m*M* ATP, and reagents as indicated in the figure. Calcium chloride was added in amounts to achieve the desired free Ca^{2+} concentration indicated taking the concentration of EGTA and the Ca EGTA binding constant (5.22×10^6 M^{-1}) at pH 7.0 (Ref. 22). ATPase activity at any concentration of free Ca^{2+} was not significantly different from activity in the presence of Mg alone ($p > 0.5$).

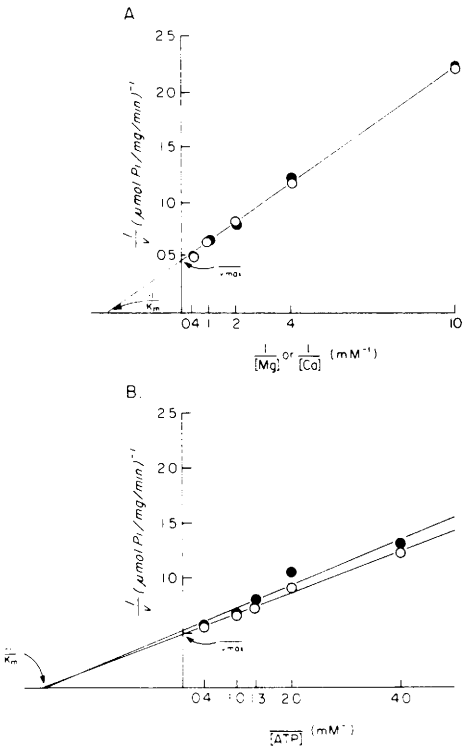

FIG. 3. Double reciprocal plots of magnesium basic ATPase and calcium ATPase activities. (A) ATPase activity was assayed in standard reaction medium with 0.2 mg/ml microsomal protein and the following reagents: dark circles, 5 m*M* ATP, 0.1 m*M* EGTA and various concentrations of $MgCl_2$: clear circles, 5 m*M* ATP and various concentrations of $CaCl_2$. Points represent means of three to four preparations. (B) ATPase was assayed in standard reaction medium with 0.125 mg/ml microsomal protein and the following reagents: dark circles, 0.1 m*M* EGTA and varying concentrations of total ATP with total magnesium present at a 2:1 ratio at each concentration of ATP: clear circles, varying concentrations of ATP with total calcium present at a 2:1 ratio at each concentration of ATP. Points represent means of three preparations. Slope and V_{max} for magnesium basic ATPase were not significantly different from those of calcium ATPase ($p > 0.5$ for slopes, and $p > 0.2$ for V_{max}).

observations suggest that Mg ATP and Ca ATP are the true substrates for their respective activities.

Preincubation of microsomes with 0.5 m*M* mersalyl, a sulfhydryl reagent (9), for 5 mins in standard medium with either 5 m*M* Mg^{2+} or 5 m*M* Ca^{2+} resulted in inhibition of both the Mg ATPase and Ca ATPase to the same degree. Thus in five different preparations tested Mg ATPase was inhibited by 34 ± 4% and Ca ATPase was inhibited by 29 ± 3%. In these experiments, the reaction was started by the addition of ATP (5 m*M*).

Figure 4 shows the results of experiments in which ATPase activity was measured when both magnesium and calcium was varied in the presence of 5 m*M* ATP. At each Mg concentration, the Ca concentration was increased from 0.1 to 1 m*M*. Note that the resultant ATPase activity at any concentration combination was related to the total divalent cation concentration, and not to the sum of activities for each magnesium concentration and calcium concentration alone. At high concentrations of either cation the total ATPase activity reached a maximal level, i.e., the ATPase was saturated.

Discussion. The present findings suggest that Mg^{2+} activated and Ca^{2+} activated aortic microsomal ATPase activities are probably ascribable to a single enzyme. This conclusion is supported by the following observations. Firstly, the kinetic parameters of the Mg ATPase activity with respect to maximal specific activity and concentration requirements for divalent cation and ATP were identical to those of the Ca ATPase activity (Fig. 3). Secondly, both activities were characterized by an early burst of ATP hydrolysis (Fig. 1A). Thirdly, both were inhibited to the same degree by 0.5 m*M* mersalyl suggesting that both activities require sulfhydryl groups. Finally, when both magnesium and calcium were varied together, the resultant total ATPase activity was related to the total di-

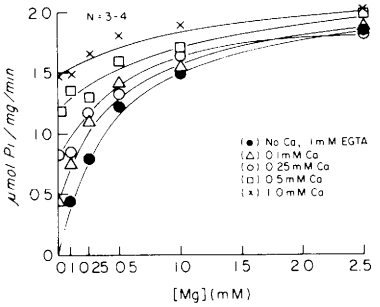

FIG. 4. ATPase activity at varying concentrations of magnesium and calcium. ATPase activity was assayed in standard medium with 0.2 mg/ml of microsomal protein, 5 m*M* ATP and varying concentrations of magnesium and calcium as indicated on the graph. In the absence of calcium but in the presence of magnesium, 0.1 m*M* EGTA was also present in the reaction mixture. Points represent means of three to four preparations.

valent cation concentration rather than the sum of activities for each concentration of magnesium alone and calcium alone (Fig. 4). The observations also suggest that the affinity of this microsomal ATPase is the same for calcium and magnesium and is not selective for either magnesium or calcium, or more appropriately, Mg ATP and Ca ATP.

Calcium activated microsomal ATPase that is independent of magnesium has been demonstrated in membrane preparations from smooth muscle (4, 5), skeletal muscle and cardiac muscle (1, 9, 17–19). However, the relationship between this Ca ATPase and the Mg ATPase in these preparations is not clear. Inasmuch as the Ca ATPase in these and our preparations is activated by concentrations of Ca^{2+} that are well above normal intracellular levels, its physiological relevance remains obscure (20). Nevertheless, Hui *et al.* (19) have suggested that the enzyme might be involved in calcium transport.

Ca^{2+} + Mg^{2+} "extra" ATPase was not demonstrable in aortic microsomes (Fig. 1A). This observation is in accord with findings reported from some laboratories (10–12), but at variance with other reports (6–8). The reasons for this disparity are not clear. Nonetheless, it should be noted that no "extra" ATPase could be detected in the present study even when the concentration of Ca^{2+} was varied over a wide range (Fig. 2), or when the concentration of Mg ATP was also varied. It is possible that the high rate of ATP hydrolysis manifest by the basic Mg^{2+} activated ATPase might compromise detection of "extra" hydrolysis attributable to added Ca^{2+}.

Expectedly, in skeletal muscle microsomes that were prepared identically to aortic microsomes, "extra" ATPase was easily demonstrable (Fig. 1B). The values for the specific activity of the "extra" and basic Mg ATPase are in good agreement with reported values (1, 9, 17). This reduces the likelihood that the lack of aortic microsomal "extra" ATPase might be due to functional alterations occurring during preparative procedures.

The apparent absence of Ca^{2+} + Mg^{2+} "extra" ATPase in bovine aortic microsomes is of particular concern because such activity present in striated muscle preparations (sarcoplasmic reticulum), is coupled to Ca^{2+} transport. We have shown previously that our aortic preparations accumulate Ca^{2+} by an ATP dependent mechanism (21). However, further interpretation requires additional studies directed toward elucidating functional relations between microsomal ATPase activities and uptake of Ca^{2+}. In this context, we must also reckon with the question of the physiologic significance of the high rate of Mg^{2+} activated hydrolysis of ATP by aortic microsomes.

Summary. Microsomes from bovine aorta contained a magnesium activated or "basic" ATPase activity and a calcium activated ATPase activity that was independent of magnesium. Ca^{2+} stimulated Mg^{2+} dependent "extra" ATPase was not demonstrable. Kinetic analyses of the magnesium basic ATPase and calcium ATPase activities suggested that the magnesium activation and calcium activation of ATP hydrolysis probably represent a single enzyme moiety. The ATPase has equal affinity for magnesium and calcium and does not distinguish between them.

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