Electrophoretic Separation of Different Phophosproteins Associated with Ca-ATPase and Na,K-ATPase in Human Red Cell Ghosts

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ABSTRACT Ca has been found to increase the quantity of ^{32}P incorporated into red cell ghosts from [γ-^{32}P]ATP over the levels obtained by incubation with Mg alone or with Mg + Na, in correlation with the effect of Ca on the associated ATPase activities. When the ^{32}P-labeled ghosts were solubilized in sodium dodecyl sulfate (SDS) and electrophoresed on acrylamide gels only two bands could be detected either by autoradiography or by counting the sliced gels. The faster moving band (P-2) had the same mobility and the same molecular weight (103,000) as the phosphoprotein found either with Mg alone or with Mg + Na. The slower moving band (P-1) was not found in extensively washed ghosts labeled in the absence of Ca. The molecular weight of P-1 is approximately 150,000. P-1 like P-2 was not affected by pretreatment of intact cells with Pronase before labeling indicating that neither the phosphorylating mechanism nor the phosphoprotein are accessible to externally applied Pronase. The demonstration that a Ca-phosphoprotein is separable from the Na-stimulated phosphoprotein suggests that the Ca-ATPase is distinct from and independent of the Na,K-ATPase. The fact that Ca blocks the dephosphorylation by K of the Na-phosphoprotein indicates that caution is required in interpreting results when the activities of the different phosphoproteins have not been separately determined.

INTRODUCTION

The internal Ca concentration of the red cell is maintaied at a very low level (15) most probably to prevent the acceleration of potassium leakage (5, 6, 11, 16, 23) and inhibition of the Na:K pump (10) which accompany high intracellular Ca. Isolated red cell membranes contain a (Mg + Ca)-dependent ATPase activity (8, 10) which functions even at very low Ca levels (25, 26).

It is believed to be responsible for the active extrusion of Ca from resealed red cell ghosts (21, 24, 26) and for the uptake of Ca into inside out red cell membrane vesicles (27). Thus it appears that Ca must be present at the cyto plasmic surface of the membrane in order to stimulate this ATPase activity.

In the sarcoplasmic reticulum it has been shown that a phosphorylated protein is formed (12, 19, 20, 28, 29) in the course of a similar (Ca + Mg)-ATPase reaction. This (Ca + Mg)-ATPase is associated with a Ca pump which maintains a low Ca concentration inside the muscle. Phosphorylation of this protein is stimulated by Ca (half-maximal activation at about 10^{-7} M free Ca [12]) while Mg appears to stimulate both phosphorylation and dephosphorylation with the result being a net increase in the steady-state level of phosphorylated protein (12). The phosphorylated protein appears to be the ATPase itself and has a molecular weight of 102,000 as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (18). The present experiments demonstrate the presence of a similar phosphoprotein in human red cell ghosts separate from other membrane associated phosphorylated proteins.

To determine the means whereby internal Ca inhibits the Na,K-ATPase and ouabain-sensitive Na efflux (10) we have also investigated the effects of Ca on the phosphorylated protein which appears to be associated with the Na,K-ATPase (2, 3, 4). A preliminary report of these experiments has been previously presented (13).

METHODS

Except as noted methods and sources of materials were as described in the preceding paper (14). As previously stated, Mg-phosphoprotein and Na-phosphoprotein refer to the ^{32}P-labeled protein formed in the presence of Mg or the increment resulting from addition of Na, respectively. The additional phosphorylated protein formed when 0.5 mM Ca is added to the phosphorylation mixture will be referred to as Ca-phosphoprotein.

RESULTS

Table I shows the effect of 0.5 mM Ca on the phosphorylation of human red blood cell ghosts incubated under standard conditions. Without Ca the results were the same as those previously reported by Blostein (3, 4) and in the preceding paper (14): Na stimulated the phosphorylation and addition of K reduced the labeling to the same level as when Na was absent. When Ca was included in the phosphorylation medium, as shown in the middle column, the total amount of phosphorylation increased in all cases, with the greatest increase when both Na and K were present together with Ca. The amount of additional phosphorylation caused by Ca in the presence of Mg varied with different ghost preparations, the minimum being 0.4 pmol/mg protein and the mean of five separate ghost preparations being 0.93 ± 0.25 SEM. In

TABLE I

EFFECT OF CA ON THE INCORPORATION OF ^{32}P FROM [γ-^{32}P]ATP INTO RED CELL GHOSTS

Washed red cell ghosts were phosphorylated for 15 s at 0°C in a medium containing 2 μM [γ-^{32}P]ATP, 12 μM Mg, 50 mM Na, 10 mM K, 0.5 mM Ca, 10 mM Tris, pH 7.5. Choline chloride was substituted for Na and K when they were absent. The reaction was stopped by addition of 5% TCA containing 1 mM orthophosphate and 0.1 mM ATP as described in the preceding paper (14). Two other experiments gave similar results.

Condition	−Ca	+Ca	ΔCa
	pmol P_i/mg protein		
Mg	0.78	2.05	0.27
Mg+Na	1.31	2.71	1.40
Mg+Na+K	0.84	2.47	1.63

separate experiments it was shown that the concentration of Mg used (12 μM) was sufficient to provide for maximum incorporation of ^{32}P since the levels of phosphoprotein remained the same when the Mg concentration was increased to 100 or 1,000 μM.

Blostein (3) has previously shown the lability or turnover of the ^{32}P incorporated under the various conditions presented in Table I in the absence of Ca. To determine if the Ca phosphoprotein was similarly labile the following experiment was performed: After incubation for 15 s in the presence of Ca + Mg under the conditions as outlined in Table I sufficient EDTA or MgATP was added to give a final concentration of either 10 mM EDTA or 100 μM MgATP and the incubation continued for 30 s before the level of ^{32}P incorporation was determined in the usual way. There was a 36% decrease in incorporated ^{32}P brought about by EDTA and an 80% decrease induced by the addition of the MgATP compared to the control where no additions were made and where the level of ^{32}P incorporation remained at its 15-s value. This result implies that the phosphoprotein represents a labile intermediate in the reaction.

ATPase activities measured under the same conditions as given in Table I are presented in Table II. In this situation Ca caused a 47% increase in ATPase activity in the presence of Mg, indicating a Ca-ATPase activity with which the Ca-phosphoprotein might be associated. When Na or Na + K were added, the ATPase activity was increased, as shown by Bond and Green (7), but there was less stimulation of total ATPase activity by Ca probably due to inhibition of the Na-ATPase (see below).

To gain further information about the chemical nature of the protein-phosphate product formed in the presence of Ca, phosphorylated ghosts were treated with 0.6 N hydroxylamine, a procedure which hydrolyzes acylphosphate bonds (17). When ghosts labeled in the presence of either Mg or Mg + Ca were treated with hydroxylamine (Table III) almost all of the phosphate

TABLE II

EFFECT OF CA ON THE ATPase ACTIVITIES OF RED CELL GHOSTS

The ATPase reaction was carried out for 20 min at 0°C. Incubation medium was the same as described in the legend of Table I. ATP hydrolysis was measured as described in (14). The results represent duplicate determinations. Two other experiments gave similar results.

Condition	ATP hydrolysis	
	−Ca	+Ca
	nmol P/mg protein × h	
Mg	0.19	0.28
Mg+Na	0.28	0.33
Mg+Na+K	0.28	0.34

TABLE III

EFFECT OF HYDROXYLAMINE TREATMENT ON MG- AND CA-PHOSPHOPROTEINS

Ghosts phosphorylated in the presence of Mg or Mg + Ca (see legend to Table I and reference (14)) were exposed to 0.6 N hydroxylamine in 0.08 N acetate at pH 5.3 for 10 min at 23°C. Other ghosts were exposed to 0.6 N NaCl under identical conditions. Control ghosts were phosphorylated but were not incubated at 23°C.

Condition	Mg	Mg + Ca
	pmol/mg ghost protein	
Control	0.97	1.44
NH_2OH	0.07	0.09
NaCl	0.43	0.90

was released. Incubation under the same conditions in a medium without hydroxylamine led to the release of a substantial part of the Mg-stimulated labeling but the Ca-phosphoprotein appeared to be less readily hydrolyzed under these conditions.

In order to characterize further the Ca-phosphoprotein and to separate it from the Mg-phosphoprotein and Na-phosphoprotein, the phosphorylated ghosts were solubilized and electrophoresed on acrylamide gels. The results are shown in Fig. 1.

At the top is shown a photograph of a gel stained with Coomassie Blue which shows the typical protein pattern of red cell ghosts. The top graph is a densitometer trace of an autoradiogram from a gel containing protein phosphorylated in the presence of Mg + Ca. Two ^{32}P-labeled peaks are seen. The larger left-hand peak (hereafter referred to as P-1) represents the new phosphorylated protein formed in the presence of Ca. The smaller right-hand peak (P-2) is the Mg-phosphoprotein described in the preceding paper (14). The Ca-phosphoprotein (P-1) has a considerably higher molecular weight than P-2: about 150,000 as compared to 103,000. Addition of Na or Na + K (middle and bottom graphs, respectively) to the phosphorylating medium seems to have little effect on P-1, in contrast to P-2 where an unexpected

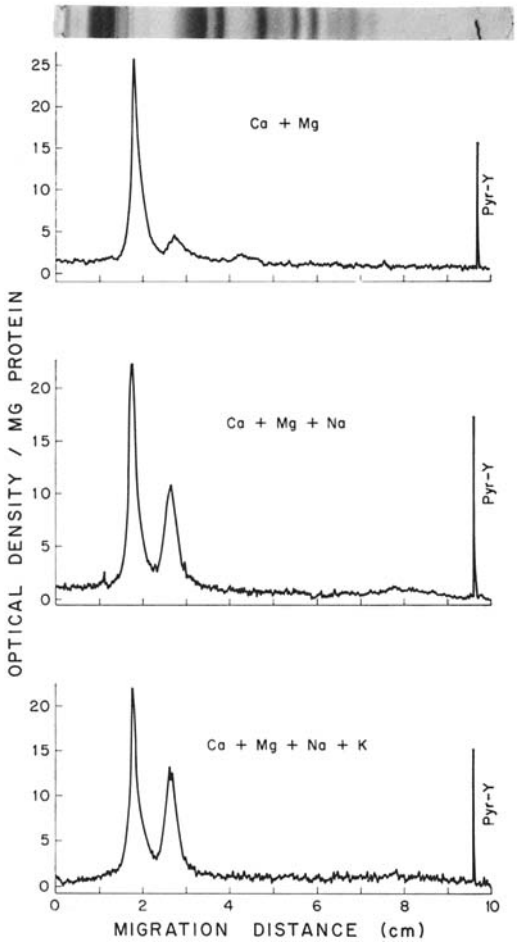

FIGURE 1. Gel electrophoresis of ghosts phosphorylated in the presence of 0.5 mM Ca. At the top is a photograph of a disk gel stained with Coomassie Brilliant Blue to show the electrophoretic pattern of the ghost proteins. The three graphs represent densitometric scans of autoradiograms of $\frac{1}{16}$-inch slab gels prepared as described in Fig. 2. The ordinate, optical density/milligram protein, is proportional to the amount of ^{32}P per unit length of gel per milligram protein added to the gel. The sharp peaks to the right of each trace show the position of the Pyronin-Y marker. Labeling conditions were as follows: 2 μM ATP, 12 μM $MgCl_2$, 10 mM Tris, 0.5 mM $CaCl_2$. When present the concentration of NaCl was 50 mM and KCl, 10 mM. When Na was absent, choline chloride was substituted.

effect may be observed. In the middle graph, addition of Na causes the usual stimulation of P-2 phosphorylation (1, 3, 14). When K is added, however, as in the bottom trace the size of the peak remains the same as with Na + Mg. This is very different from the result obtained in the absence of Ca (see 14) where potassium causes a reduction in the amount of phosphorylation to that

seen with Mg alone. It appears that Ca prevents potassium from decreasing the amount of Na-stimulated phosphorylation, perhaps by blocking a potassium-stimulated dephosphorylation step. The Ca-stimulated increase in phosphorylation in the presence of Mg, Na, and K (Table I) thus actually consists of two components: a Ca-stimulated increase in phosphorylation of P-1, similar in amount to that seen with Mg present, and Ca-inhibition of the K-stimulated dephosphorylation of P-2.

As Table IV demonstrates, the recovery of the Ca-phosphoprotein was somewhat better than that of the Na- or Mg-phosphoprotein; especially if the gels were not cooled during electrophoresis. These results are consistent with those of Table III, where the Ca-phosphoprotein was found to be more re-

TABLE IV

RECOVERY OF ^{32}P-LABELED PROTEIN UNDER DIFFERENT ELECTROPHORESIS CONDITIONS

Ghosts were phosphorylated in the presence of various ions as described in the legend to Table I. Electrophoresis was carried out in exp A by the Standard method (14): current was 4 mA/gel and water at 10–15°C was circulated through the electrophoresis apparatus. In B, the current was also 4 mA/gel but there was no water cooling. In C, the current was 8 mA/gel without cooling. Percent recovery is the percent of the ^{32}P radioactivity in the original TCA precipitate remaining in peaks 1 and 2 after electrophoresis (determined by counting transversely sliced gels).

	A		B		C	
Condition	−Ca	+Ca	−Ca	+Ca	−Ca	+Ca
			% recovery in peaks			
Mg	42.9	47.0	25.6	31.1	17.5	—
Mg+Na	42.8	45.3	25.6	30.9	15.1	—
Mg+Na+K	39.5	44.7	24.8	30.0	12.5	28.0

sistant to spontaneous hydrolysis at room temperature than the Mg- or Na-phosphoproteins.

Even wide variations in the recovery of phosphorylated protein in the peaks P-1 and P-2 had no significant effect on the results of addition of various ions to the phosphorylation medium (Table V). With regard to P-1, the additional phosphorylation with Ca was roughly the same despite a difference in the recovery of 15% from exp A to B (Table IV). The slight decrease in P-1 phosphorylation upon the addition of Na, and especially with Na + K, was a consistent finding.

The effects of ions on P-2 phosphorylation were also independent of the electrophoretic conditions. In most experiments Ca caused a slight decrease in phosphorylation with Mg alone present and a slight increase in phosphorylation with Mg + Na but this was not always observed. Essentially complete inhibition by Ca of the K-dependent dephosphorylation was seen in every experiment.

TABLE V

RELATIVE AMOUNTS OF P-1 AND P-2 PHOSPHOPROTEIN FORMED UNDER DIFFERENT CONDITIONS OF PHOSPHORYLATION AND ELECTROPHORESIS

Electrophoresis conditions for Expts. A, B and C were as described in Table IV; phosphorylation conditions as in Table I. Gels were sliced transversely and prepared for counting as described in the preceding paper (14). The slice midway between peaks 1 and 2 was apportioned to these peaks according to the ratio of the counts in the slices adjacent to it on either side. In general the number of counts in this intermediate slice was small and so the manner of apportionment did not significantly affect the results.

Phosphorylation conditions	P-1 (% relative to Ca + Mg)				P-2 (% relative to Mg)					
	−Ca		+Ca		−Ca			+Ca		
	A	B	A	B	A	B	C	A	B	C
Mg	14	18	100	100	100	100	100	71	71	—
Mg+Na	11	14	91	86	175	188	166	198	199	—
Mg+Na+K	9	8	86	66	99	96	80	190	188	158

More detailed examination of an autoradiogram of a gel on which the phosphorylated proteins were electrophoresed (Fig. 2) reveals that the P-1 band actually consists of two components which can barely be resolved. Most of the phosphoprotein migrates in a dense, slower moving band but a small amount runs slightly faster. (This appears as a slight broadening of the leading edge of the P-1 band in the densitometer traces of Fig. 1. By using a much narrower densitometer slit width a separate peak could sometimes be resolved.) This faint band has an approximate molecular weight of 142,000 as compared with 150,000 for the dense band. Schatzmann and Rossi (25) have described rather complicated kinetic behavior for the (Ca + Mg)-ATPase of human red blood cells: Below 0.01 mM Ca the ATPase exhibits saturation kinetics but there is a further increase in activity between 0.05 and 0.5 mM. In order to determine whether the two components of P-1 might be differentially stimulated by Ca, phosphorylation was carried out at 0.01 mM Ca as well as at 0.5 mM Ca. Both components of P-1 appeared to increase symmetrically as Ca was increased but small differences might have been overlooked due to the difficulty of separating the two components.

The effect of different Ca concentrations on the total phosphorylation of P-1 and P-2 is presented in Table VI. Phosphorylation of P-1 was stimulated at least fivefold by 0.5 mM Ca as compared to 0.01 mM Ca. As before, 0.5 mM Ca almost completely prevented the K-dependent dephosphorylation of P-2. At 0.01 mM Ca the K-stimulated dephosphorylation was still inhibited by 50%. Even low Ca concentrations are thus capable of dramatically inhibiting K-stimulated dephosphorylation and may be responsible for the failure to observe dephosphorylation by K under some conditions (e.g. Table V of reference 3).

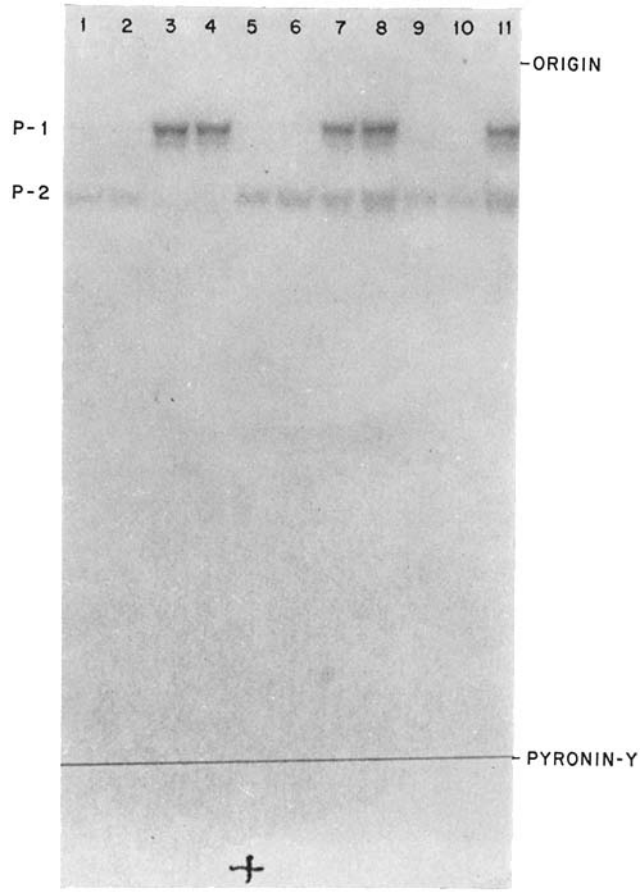

FIGURE 2. Autoradiogram of a 5.6% acrylamide slab gel. Ten μl of solubilizing mixture containing 15–20 μg protein were added to each well of a $\frac{1}{16}$-inch thick acrylamide gel, prepared in an apparatus as described by Reid and Bieleski (22). Electrophoresis was carried out at 25 mA (32 V) for 6.5 h, by which time the Pyronin-Y marker had traveled 9.5 cm from the origin. The gel was dried as described in the preceding paper (14) and autoradiographed on Kodak SB-54 X-ray film. The origin is at the top and the position of the Pyronin-Y marker is indicated by the line near the bottom. Concentrations of ions in the labeling mixture were as described in Fig. 1. Ions present were as follows: 1 and 2, Mg; 3 and 4, Mg + Ca; 5 and 6, Mg + Na; 7 and 8, Mg + Na + Ca: 9 and 10, Mg + Na + K; 11, Mg + K + Ca. Note that the slower moving, Ca-stimulated band (P-1) actually consists of two components, the smaller of which migrates slightly faster than the other. Note also that Ca prevents the dephosphorylation of the faster-moving peak (P-2) which normally occurs in the presence of K.

To obtain information regarding the accessibility of the Ca-phosphoprotein from the outside intact red cells were exposed to Pronase. When ghosts of Pronase-treated cells were electrophoresed the results in the middle graph of Fig. 3 were obtained. Comparison with control cells (upper graph) reveals no

TABLE VI

EFFECT OF DIFFERENT CA CONCENTRATIONS ON P-1 AND P-2 PHOSPHORYLATION

Each value represents the average of two determinations. Phosphorylation conditions were as described in Table I, except that 0.01 mM Ca was used where indicated. Phosphorylation in peaks 1 or 2 is expressed either as pmol P/mg ghost protein or as percent of the appropriate control with Mg alone. Values are uncorrected for recovery. Total recovery in peaks P-1 and P-2 (±SEM) was 33.3 ± 0.9% (N = 6) with 0.01 mM Ca and 40.1 ± 1.0% (N = 6) with 0.5 mM Ca.

Condition	P-1				P-2			
	0.01 mM Ca		0.5 mM Ca		0.01 mM Ca		0.5 mM Ca	
	pmol/mg	%	*pmol/mg*	%	*pmol/mg*	%	*pmol/mg*	%
Mg	0.204	100	1.051	100	0.270	100	0.290	100
Mg+Na	0.168	82	1.092	104	0.477	177	0.643	222
Mg+Na+K	0.159	78	0.897	85	0.374	139	0.609	210

decrease in the molecular weight (increase in migration distance relative to Pyronin-Y) of either P-1 or P-2. This is most clearly demonstrated by the bottom trace which was obtained by electrophoresing a 50:50 mixture of solubilized protein from control and Pronase-treated cells. Here possible artifacts due to nonuniformity of the gels can be excluded yet only single peaks are seen for both P-1 and P-2 and there is no detectable peak broadening. The maximum change in molecular weight which might have occurred is less than 10%. Although the height of the peaks appears different in the upper and middle traces Pronase has no effect on the total number of counts in each peak as measured by counting sliced gels nor on the total binding of phosphate to the TCA precipitate. In this experiment, after Pronase treatment 3.17 pmol of phosphate were bound per milligram ghost protein in the presence of Mg, Na, K, and Ca as compared with 3.08 pmol/mg ghost protein in the control.

DISCUSSION

At least two different ^{32}P-labeled bands can be clearly distinguished in acrylamide gels of phosphorylated red cell ghosts. The first of these (P-1) is stimulated by Ca and has a molecular weight of about 150,000. It seems to consist of two components, a slower moving major band (mol wt = 150,000) which accounts for most of the phosphate and a very slightly faster moving minor component (mol wt = 142,000). The second major peak (P-2), described in the preceding paper (14), is stimulated by Mg and Na and has a molecular weight of about 103,000.

The Na-stimulated phosphoprotein (P-2) has characteristics which suggest that it may be involved in the Na,K-ATPase (2, 3, 4). By an analogous argument it is probable that the Ca-phosphoprotein characterized in this paper (Table I) represents an intermediate in the Ca-ATPase (Table II). Nevertheless more evidence is needed to relate either the Ca-ATPase or the Ca-

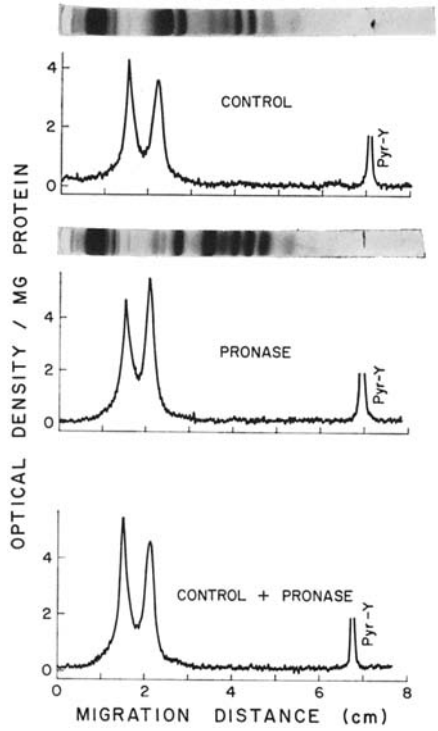

FIGURE 3. Effects of Pronase treatment of intact cells on the electrophoretic mobility of phosphorylated ghost proteins. Intact cells were exposed to 0.2 mg/ml Pronase for 30 min at 37°C and then washed six times with 5 vol of ice-cold isotonic saline. Control cells were treated in an identical manner except that Pronase was absent. Ghosts were prepared and were phosphorylated at 0°C for 15 s in a medium containing 2 μM [γ-^{32}P]-ATP, 12 μM $MgCl_2$, 50 mM NaCl, 10 mM KCl, 0.5 mM $CaCl_2$, 10 mM Tris (pH 7.5). Solubilization and electrophoresis were carried out as described in reference (14). The lower photograph shows a Coomassie Blue stained gel containing ghost protein from Pronase-treated cells. Comparison with the upper picture from control cells reveals that virtually all of the protein running in the P-2 region of the control is shifted to lower molecular weight, indicating that Pronase treatment was effective. The graphs represent densitometric scans of autoradiograms of longitudinally sliced disk gels, prepared as described in reference (14). The top and middle traces represent control and Pronase-treated cells, while the bottom graph is taken from a gel to which a 50:50 mixture of solubilized Pronase and control ghosts had been added. Differences in peak heights in the three graphs reflect different peak widths. The total amount of phosphoprotein in each peak, as determined by counting transversely sliced gels, was not significantly affected by Pronase treatment.

phosphoprotein as presented here to the (Mg + Ca)-ATPase activity as previously described (7, 8, 10, 24, 25, 26), since the conditions of study of the present and previous Ca-ATPase activities are different (e.g. ATP concentration, temperature) and also since there is the possibility that they may be more than one type of (Ca + Mg)-ATPase present in the membrane. If the

phosphoproteins, P-1 and P-2, are indeed components of Ca-ATPase and Na,K-ATPase, respectively, the fact that the phosphorylated proteins are different implies that the Ca-ATPase activity under these conditions is carried out by a different enzyme or enzyme complex from that which is responsible for Na,K-ATPase activity.

Although the results of hydroxylamine treatment (Table III) provide evidence that the Ca-phosphoprotein, like the Mg-phosphoprotein and Na-phosphoprotein, is an acyl phosphate, it seems to undergo spontaneous hydrolysis less readily (Tables III and IV). This suggests that the phosphates are situated in different environments which confer slightly different chemical properties. The Ca-phosphoprotein of sarcoplasmic reticulum is also resistant to spontaneous hydrolysis (12).

Like the Mg- and Na-phosphoproteins (14), the Ca-phosphoprotein is not affected by externally added Pronase. This result suggests that the Ca-phosphoprotein is inaccessible to the enzyme and therefore is not located near the outer surface of the membrane.

The amount of Ca-phosphoprotein is approximately equal to the sum of the Mg- and Na-phosphoproteins. Assuming one phosphate atom per molecule the Ca-phosphoprotein thus represents only about 0.02% of the total ghost protein. From the stained gels (Figs. 1 and 3) it is clear that very little protein runs in the region of the gel near P-1, so SDS-acrylamide gel electrophoresis provides at least a 100-fold purification of the Ca-phosphoprotein as well as separation of it from the Mg- and Na-phosphoproteins.

Separation of the Ca- from the Mg- and Na-phosphoproteins has made it possible to distinguish the effects of Ca on each of these components, revealing that 0.5 mM Ca (Fig. 1, Table V) and even much lower Ca concentrations (Table VI) interfere with the K-stimulated dephosphorylation of the Na-phosphoprotein (P-2). Ca also seems to slightly inhibit Mg-dependent phosphorylation and to slightly stimulate Mg + Na-dependent phosphorylation. The apparent additional stimulation of Ca-phosphorylation by K (Table I) actually has nothing at all to do with the Ca-phosphoprotein (P-1). In fact phosphorylation of P-1 seems to be slightly inhibited by K (Table V). The entire increase is accounted for by the effect of Ca in reducing the K-stimulated dephosphorylation of the Na-phosphoprotein (P-2). The presence of such multiple Ca effects suggests the need for extreme care in interpreting the effects of Ca on ATPase activity or on membrane phosphorylation when the various components which might be affected by Ca have not been studied individually.

Epstein and Whittam (9) have presented evidence that the inhibition by Ca of the Na,K-ATPase is due to competition of Ca-ATP with Mg-ATP. One might therefore expect Ca to reduce Na-stimulated phosphorylation. Although the rate of incorporation was not directly measured, the data on phos-

phorylation after 15 s indicate that if anything Ca stimulates Na-dependent incorporation of ^{32}P into P-2. The observed effect of Ca in decreasing the K-stimulated dephosphorylation (perhaps by competition of Ca with K) would imply that Ca (or Ca-ATP) acts later in the pump sequence. These disparities may be due to the different conditions under which the experiments were performed (0°C and 2 μM ATP vs. 37°C and 2 mM ATP). If not, they may mean either that Ca acts at the dephosphorylation step as well as the Mg-ATP utilizing step, or else that the phosphoprotein is not an intermediate in the main sequence of the ATPase reaction.

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