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Phosphatase activity of $(Na^+ + K^+)$-ATPase. Ligand interactions and related enzyme forms

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The prevailing conformations of partially purified pig kidney $(Na^+ + K^+)$-ATPase interacting with ligands related to its phosphatase activity were determined following time-dependent trypsin digestion and inactivation as well as the amounts of Rb^+ or Ca^{2+} bound to the enzyme after passage through cation-exchange resin columns. In the presence of 150 mM choline chloride, alone or with 3 mM $MgCl_2$, 3 mM $MnCl_2$ or 1 mM $CaCl_2$, the major enzyme conformation was E_1. Similar forms were seen with 5 mM *p*-nitrophenyl phosphate with and without 3 mM $MgCl_2$. KCl, at 0.5 mM or 150 mM, produced an E_2 enzyme state; the effects of 0.5 mM KCl were completely counteracted by 5 mM *p*-nitrophenyl phosphate. Under optimal conditions for phosphatase activity (3 mM $MgCl_2$/5 mM *p*-nitrophenyl phosphate/10 mM KCl) the $(Na^+ + K^+)$-ATPase was in the E_2 state. At low ionic strength and 20°C and under 85% of maximal RbCl-stimulated phosphatase turnover (1 mM RbCl/3 mM $MgCl_2$/5 mM *p*-nitrophenyl phosphate) no Rb^+ occlusion could be detected. Ca^{2+}, at low ionic strength and in the presence of 3 mM $MgCl_2$, stimulated an ouabain-sensitive phosphatase activity. The rates of hydrolysis obtained with 1 mM $CaCl_2$ were similar to those seen with 0.5 mM KCl; under both conditions, similar patterns of trypsin digestion and inactivation of the enzyme were obtained. On the other hand, Ca^{2+} could not mimic Rb^+ in its ability to induce an E_2-occluding state. These results suggest that during phosphatase activity of $(Na^+ + K^+)$-ATPase, the most abundant form is a non-occluding E_2 and that at least one of the mechanisms of potassium stimulation of that activity it to take the enzyme into the E_2 state.

Introduction

All preparations showing $(Na^+ + K^+)$-ATPase activity display a K^+-dependent phosphatase activity as well. This activity requires Mg^{2+} as an essential activator, is inhibited by ouabain and vanadate and is modulated by Na^+ and ATP (see Refs. 1–3 for references). The potassium phosphatase could be linked to the K^+-stimulated dephosphorylation step of the $(Na^+ + K^+)$-ATPase activity [1–3] or result from a reaction cycle completely independent of the hydrolysis of ATP. The choice between these two alternatives is complicated by the fact that the enzyme forms related to phosphatase activity have not yet been unambiguously determined, although an E_2 type conformation is likely to be involved [4,5]. The phosphatase substrate site is matter of controversy; a low-affinity ATP site has been earlier proposed [2], but this idea has been recently challenged in view of the antagonism found between K^+ and *p*-nitrophenyl phosphate [6]. Magnesium activation takes place at cytoplasmic sites [7]. In red cells, increasing intracellular Mg^{2+} concentration pro-

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duces inhibition of $(Na^+ + K^+)$-ATPase with simultaneous stimulation in phosphatase activity [5]; this has been interpreted as an stabilization by Mg^{2+} of the E_2 enzyme form [5]. However, there is disagreement on the enzyme conformation that predominates in the presence of Mg^{2+}: trypsin inactivation of $(Na^+ + K^+)$-ATPase [8] and fluorescence studies [9,10] suggest that the predominant conformation is E_1, whereas trypsin inactivation of *p*-nitrophenyl phosphate hydrolysis [11] and the analysis of tryptic fragments in polyacrilamide gels [12] have been considered as the expression of an E_2 state. At high ionic strength, Ca^{2+} in the presence of Mg^{2+}, inhibits $(Na^+ + K^+)$-ATPase and potassium phosphatase activities; in Mg^{2+}-free solutions, Ca^{2+} can still stimulate some ATPase, but is completely unable to promote any phosphatase activity [13–15]. On the other hand, in K^+-free solutions of low ionic strength and pH around 7.0, Ca^{2+}, in the presence of Mg^{2+}, becomes a moderate activator of *p*-nitrophenyl phosphate hydrolysis [16]. The mechanism of this Ca^{2+} effect is not known, but it might be related to the acceleration of dephosphorylation of $(Na^+ + K^+)$-ATPase phosphorylated from inorganic phosphate (at 4°C and pH 7.1) when, besides Mg^{2+} either K^+ or Ca^{2+} is included in the media [17]. In the work described in this paper we looked into the prevailing conformations of $(Na^+ + K^+)$-ATPase in the presence of several ligands related to its phosphatase activity. This was done by following simultaneously the patterns of trypsin digestion and inactivation and also determining the amounts of Rb^+ and Ca^{2+} trapped by the enzyme after passing through cation-exchange resin columns.

Methods

The experiments were performed on pig kidney $(Na^+ + K^+)$-ATPase partially purified by the method of Jorgensen [18]. The specific activity was between 15 and 18 units · mg^{-1} and remained stable for several months when the preparation was stored at −85°C in a solution containing 25 mM imidazole (pH (20°C) 7.5)/2 mM EDTA · Tris/10% sucrose. Prior to use the enzyme was washed twice (1 : 10, v/v) and resuspended usually in the same solution without sucrose; in Rb^+ or Ca^{2+} trapping experiments, washing and resuspension solutions were free of EDTA.

ATPase activity was determined as in Ref. 6 following the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$. Phosphatase activity was measured as in Ref. 14 using *p*-nitrophenyl phosphate as a substrate.

Trypsin digestion was performed as described by Jorgensen [19]. Samples of enzyme suspension corresponding to different digestion times were centrifuged at $100\,000 \times g$ for 10 min in 250-μl tubes and the pellets were resuspended in 60 μl of 62.5 mM Tris-HCl (pH (20°C) 6.8); the final protein concentration was about 80 μg/100 μl. Aliquots of this suspension were used to determine (i) protein concentration, (ii) K^+-dependent *p*-nitrophenyl phosphatase activity, (iii) ouabain-sensitive $(Na^+ + K^+)$-ATPase activity, and (iv) polypeptide fragments released from the α-subunit of the enzyme.

SDS-polyacrylamide gel electrophoresis slabs were run as in Ref. 20 taking 40 μg samples and applying them on 3% stacking gel. The running gel was 7.5% and the slab was 1.5 mm thick. Bromophenol was used as tracking dye and the total length of the run was 10 cm. The molecular weight standards were phospholipase (M_r 94 000), bovine serum albumin (68 000) and carbonic anhydrase (29 000). Relative mobilities of the protein bands, stained with Coomassie blue, were determined with a Beckman spectrophotometer equipped with a gel-scanning device. When required, the areas under the scans were measured with an area-reading attachment adapted to an Apple II computer. In all inactivation graphs, the lines though the experimental points were drawn by eye. The ATPase and phosphatase activities as a function of digestion time for the enzyme in the E_1 state were considered to follow the equation $E = Ae^{-\alpha t} + Be^{-\beta t}$ [8]; the values of the rate constants, calculated manually, are given in the figure legends.

Rb^+ occlusion in $(Na^+ + K^+)$-ATPase was estimated under equilibrium and/or steady state turnover conditions. The procedure was essentially the same as that described previously [6,21]. The enzyme suspension at 20°C was forced through a Dowex 50-X8-400 resin column in a Tris form and the effluent was collected in scintillation vials containing 3 ml distilled water. Once counted in a

Beckman scintillation counter (Cherenkov radiation), the amount of proteins in each vial was determined as described in Ref. 6 using the Ponceau-S staining [22]. The experiments on Ca^{2+} trapping by $(Na^+ + K^+)$-ATPase were performed in a similar way except that column effluents were collected in vials containing 3 ml of scintillation solution. In this case, protein contents were determined in parallel columns with no radioactive Ca^{2+}, as described for Rb^+ experiments.

In all other cases protein was determined by the method of Lowry et al. [23] with modifications [24], using bovine serum albumin as standard.

All solutions were made with bidistilled deionized water. NaCl, KCl and RbCl were Baker Ultrex; all other chemicals were reagent grade. ADP, ATP (Na^+ salts), *p*-nitrophenyl phosphate (Tris salt), ouabain, trypsin and soybean trypsin inhibitor were obtained from Sigma, U.S.A. Nucleotides were transformed into Tris salts after passing through Amberlite IR-120-P columns. Rubidium-86 and calcium-45, as chloride salts, were purchased from New England Nuclear. Phosphorus-32, as inorganic phosphate, was provided by the Argentine Atomic Energy Commission. The composition of solutions varied in different experiments and is given in the corresponding figure and table legends. Unless otherwise stated, all experimental points are the means of triplicate determinations, and each experiment was repeated at least once.

The estimation of free ligands concentrations was based in the following dissociation constants: (a) 37°C and 150 mM ionic strength: Mg-*p*-nitrophenyl phosphate, 10 mM (6); (b) 20°C and 50 mM ionic strength: MgADP, 0.22 mM; CaADP, 0.85 mM [25]; in addition, those of Mg- and Ca-*p*-nitrophenyl phosphate were arbitrarily taken as 3 mM.

Results

Trypsin digestion and inactivation of $(Na^+ + K^+)$-ATPase

(a) Effects of Mg^{2+} and Mn^{2+}

Time-dependent trypsin inactivation of $(Na^+ + K^+)$-ATPase and *p*-nitrophenyl phosphatase activities and tryptic fragments released were followed in choline chloride, and in some cases Tris/Tris-HCl. They were used instead of NaCl because in their presence, although the predominant enzyme form is E_1 the $(Na^+ + K^+)$-ATPase is more sensitive to ligands (like K^+ and its congeners) favouring the E_2 conformation. The results of typical experiments are described in Fig. 1. In the absence and presence of 3 mM $MgCl_2$ the tryptic cleavage (A and B right-hand side) is qualitatively the same and corresponds to that described for the E_1 state: a gradual disappearance of the 94 kDa band and appearance of 77 kDa fragment [12]. (In this and the rest of the figures, only the region between 94 and 58 kDa is shown, for the changes seen there are the most conspicuous to differentiate between E_1 and E_2 states of the enzyme.) The time-dependent inactivation of both $(Na^+ + K^+)$-ATPase and *p*-nitrophenyl phosphatase activities is also consistent with E_1 being the predominant enzyme form in the presence of Mg^{2+}. Similar results were obtained with 3 mM $MnCl_2$ (Fig. 1C). On the other hand, it has been reported [8] that increasing concentration of $MgCl_2$ gradually decreases the sensitivity of the enzyme to trypsin attack. The observation was confirmed and extended also for Mn^{2+}; this can be seen in Fig. 1 and more clearly in Fig. 2, where the time-dependent release of 77 kDa fragments plotted as percentage of the α sub-unit is much higher in the absence than in the presence of either 3 mM $MgCl_2$ or 3 mM $MnCl_2$. The small difference between the two divalent cations is not significant. Similar behaviour was observed when Tris-HCl instead of choline chloride was used in the digestion mixtures (not shown). Regarding the effects on the estimated rate constants for inactivation (legend to Fig. 1), $MgCl_2$ reduced the slow (β) more markedly than the fast (α) (see also Ref. 8); on the other hand, $MnCl_2$ produced a more noticeable reduction in the value of α.

(b) Effects of K^+ in the absence and presence of Mg^{2+} and p-nitrophenyl phosphate

In the absence of any other ligand and at constant ionic strength (about 170 mM), 0.5 mM KCl and 150 mM KCl produced digestion and inactivation patterns characteristic of the E_2 state [12,22]: (i) progressive reduction of the 94 kDa band and growing of the 58 kDa band without

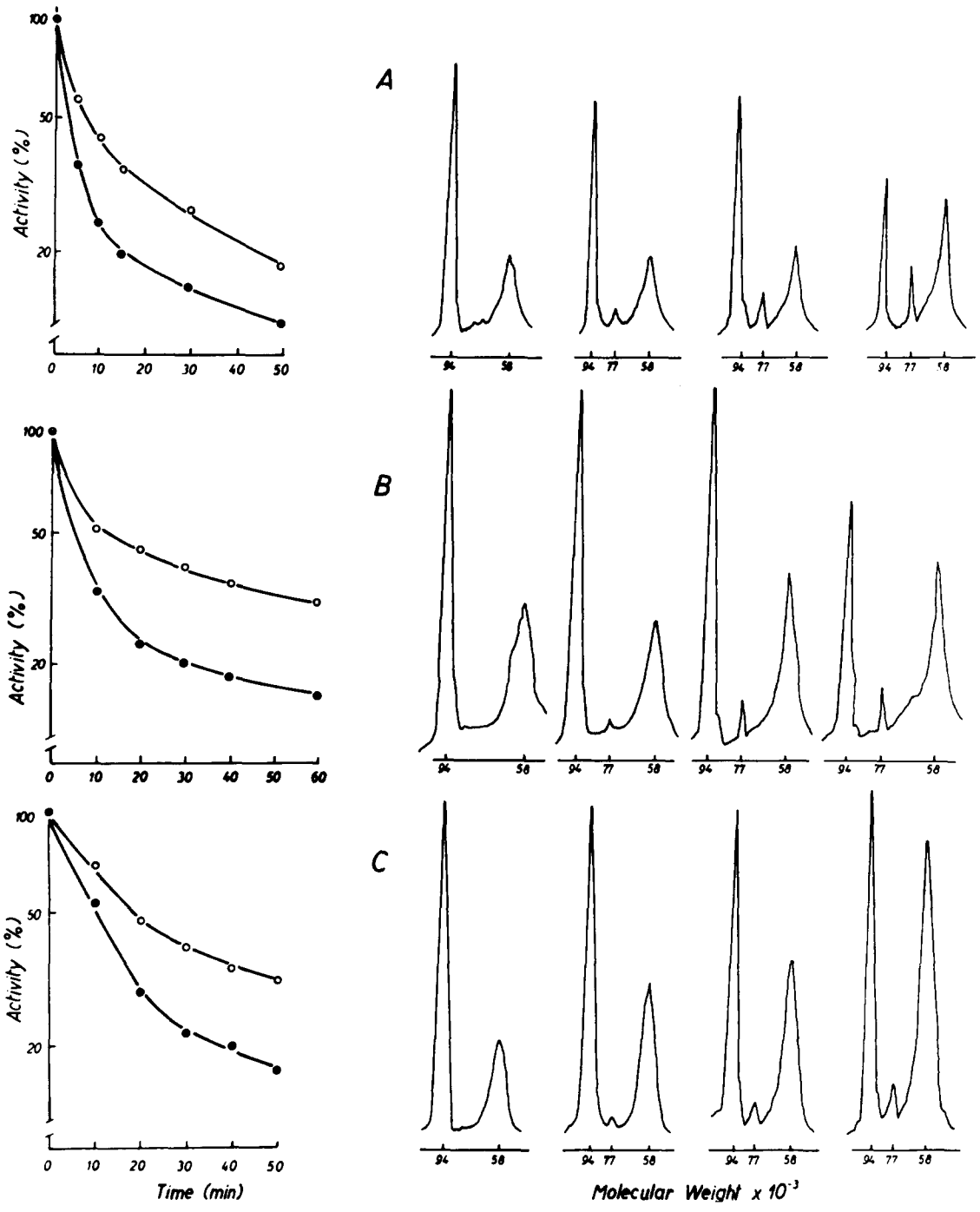

Fig. 1. Tryptic digestion of $(Na^+ + K^+)$-ATPase in the presence of 150 mM choline chloride/25 mM imidazole (pH 20°C 7.4) alone (A) and with the addition of 3 mM $MgCl_2$ (B) or 3 mM $MnCl_2$ (C). Left panel: time-course of inactivation of $(Na^+ + K^+)$-ATPase (○) and *p*-nitrophenyl phosphatase (●) activities. Right panel: densitometric scans of tryptic cleavage followed in 7.5% SDS-polyacrylamide gels. The scans correspond, from left to right, to samples taken at the following digestion times: (A), 0, 5, 15 and 50 min; (B) and (C), 0, 10, 30 and 50 min. Digestion was started by mixing 6 μg of trypsin with 300 μg of enzyme protein in a total volume of 1 ml and stopped at the indicated times with trypsin inhibitor; the weight ratio inhibitor to trypsin was 4:1. Temperature was 37°C. Enzymatic activities and fragments released were determined as described in Methods. Absorbance in the scans is given in arbitrary units. On the basis that enzymatic activities as a function of time are given by the equation $E = Ae^{-\alpha t} + Be^{-\beta t}$ [8], the following values for the rate constants were calculated (min^{-1}): (A) $(Na^+ + K^+)$-ATPase: $\alpha = 0.254$, $\beta = 0.018$; potassium phosphatase: $\alpha = 0.333$; $\beta = 0.014$. (B) $(Na^+ + K^+)$-ATPase: $\alpha = 0.205$, $\beta = 0.008$; potassium phosphatase: $\alpha = 0.224$, $\beta = 0.008$. (C) $(Na^+ + K^+)$-ATPase: $\alpha = 0.112$, $\beta = 0.011$; potassium phosphatase: $\alpha = 0.135$, $\beta = 0.013$.

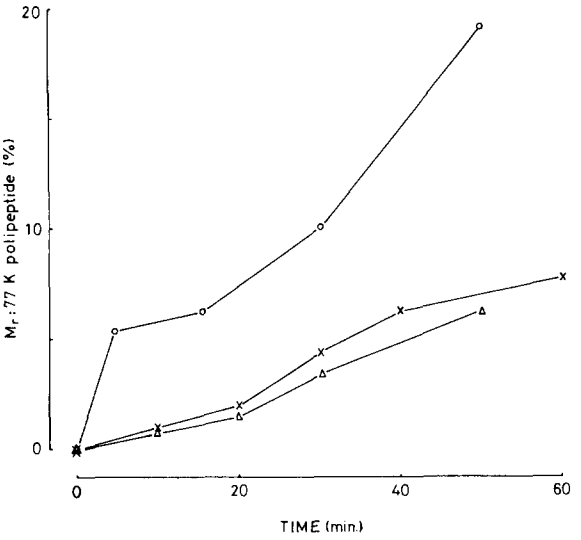

Fig. 2. Time-course of appearance of the 77 kDa polypeptide in the presence of 150 mM choline chloride alone (○) and with the addition of 3 mM $MgCl_2$ (×) or 3 mM $MnCl_2$ (△). The amounts of 77 kDa polypeptide are given as percentage of the α-subunit at zero time and were determined from the area under the scans using the graphic tablet of an Apple II computer. The data were taken from the experiments shown in Fig. 1.

appearance of the 77 kDa fragment; (ii) single exponential time-dependent inactivation of $(Na^+ + K^+)$-ATPase and (iii) an initially slow followed by a faster single exponential decay in the K^+-dependent hydrolysis of *p*-nitrophenyl phosphate (Fig. 3A and B). The rates of inactivation and fragmentation of the 94 kDa band were distinctively lower with 150 mM K^+ than with 0.5 mM K^+ (se also Ref. 19); i.e., besides bringing the enzyme into the E_2 state, K^+ seems to protect against trypsin attack.

In the absence of $MgCl_2$, addition of 5 mM *p*-nitrophenyl phosphate to 0.5 mM KCl in the digestion mixture induces inactivation and digestion patterns typical of the E_1 conformation. Quantitatively, the values of the rate constants α and β in KCl-*p*-nitrophenyl phosphate were lower than in *p*-nitrophenyl phosphate alone or in choline (or Tris) without any of the aforementioned ligands (see legend to Fig. 3). This concurs with previous results where *p*-nitrophenyl phosphate was able to release Rb^+ from the $E_2(Rb)$ occluded complex [6]. On the other hand, in the presence of 3 mM $MgCl_2$, 5 mM *p*-nitrophenyl phosphate and 10 mM KCl (Fig. 4B), the unconventional inactivation pattern of $(Na^+ + K^+)$-ATPase already reported [6] was reproduced; this was associated with inactivation of *p*-nitrophenyl phosphate hydrolysis which was also complex: an initial delay followed by a straight line that bent upwards after 40 min. However, the tryptic fragments released under these conditions unambiguously corresponded to those of an enzyme in the E_2 conformation [12].

(c) Effects of Ca^{2+} and K^+ in the absence and presence of Mg^{2+} at low ionic strength

Fig. 5 shows that a Ca^{2+}-stimulated *p*-nitrophenyl phosphatase activity in the presence of Mg^{2+} can be observed when the ionic strength is lowered, even if the pH is kept at the usual 7.4 units. This effect takes place with no delay (within the resolution of the method), for there was no difference in Ca^{2+} stimulation with and without preincubation at 37°C and at 20°C (not shown). Regardless of the actual K^+ and Ca^{2+} phosphatase activation sites, if what matters is to attain a given enzyme conformation, one would expect that the conformations stabilized by K^+ and Ca^{2+} in the presence of Mg^{2+} at low ionic strength should be the same, or very similar, for similar levels of phosphatase activity. This point was checked in experiments like those described in Figs. 6 and 7. Both inactivation and digestion patterns (in this case the actual picture of the gels is given) indicate that in the presence of $MgCl_2$ or $CaCl_2$ alone (Fig. 6A and B) the enzyme is predominantly in the E_1 form (as at high ionic strength). When Mg and Ca are present together (Fig. 6C) the picture is ill-defined. On the one hand, the appearance of the 77 kDa fragment can still be observed, although it is much less marked than with either cation alone. On the other hand, the inactivation curve of $(Na^+ + K^+)$-ATPase activity follows a single exponential up to 60 min and then bends upwards, whereas that of the phosphatase activity increases its slope after 10 min and then decreases it, resulting in a curved line bent upwards. This behaviour was observed in all experiments performed. At low ionic strength, 0.5 mM KCl and 1 mM $CaCl_2$ produced similar stimulation of a ouabain-sensitive *p*-nitrophenyl phosphate hydrolysis (not

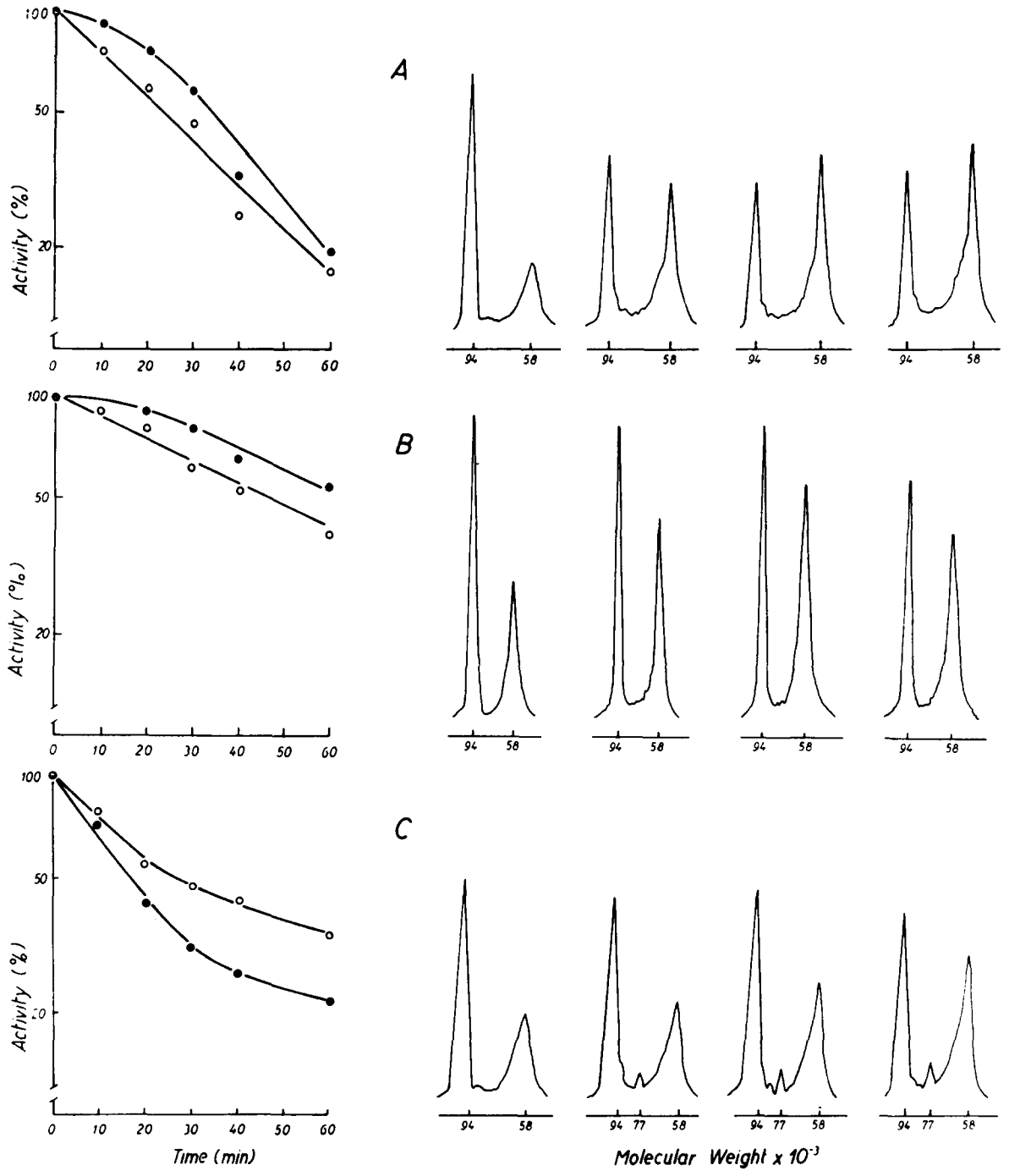

Fig. 3. Trypsin digestion of purified $(Na^+ + K^+)$-ATPase in the presence of 0.5 mM KCl (A), 150 mM KCl (B) or 0.5 mM KCl plus 5 mM *p*-nitrophenyl phosphate (C). Experimental procedures were as indicated in Fig. 1. Left panel: time-course of inactivation of $(Na^+ + K^+)$-ATPase (○) and *p*-nitrophenyl phosphatase (●) activities. Right panel: densitometric scans of tryptic cleavage followed in SDS-polyacrylamide gels. High ionic strength (150 mM monovalent salt plus 25 mM imidazole-HCl) was kept constant with choline chloride. The scans correspond, from left to right, to samples taken at the following digestion times: (A): 0, 20, 30 and 60 min; (B) and (C): 0, 30, 40 and 60 min. The estimated rate constants for the time-dependent trypsin inactivation in C were (min^{-1}): $(Na^+ + K^+)$-ATPase: $\alpha = 0.095$; $\beta = 0.011$; potassium phosphatase: $\alpha = 0.090$, $\beta = 0.013$. See text and legend to Fig. 1 for details.

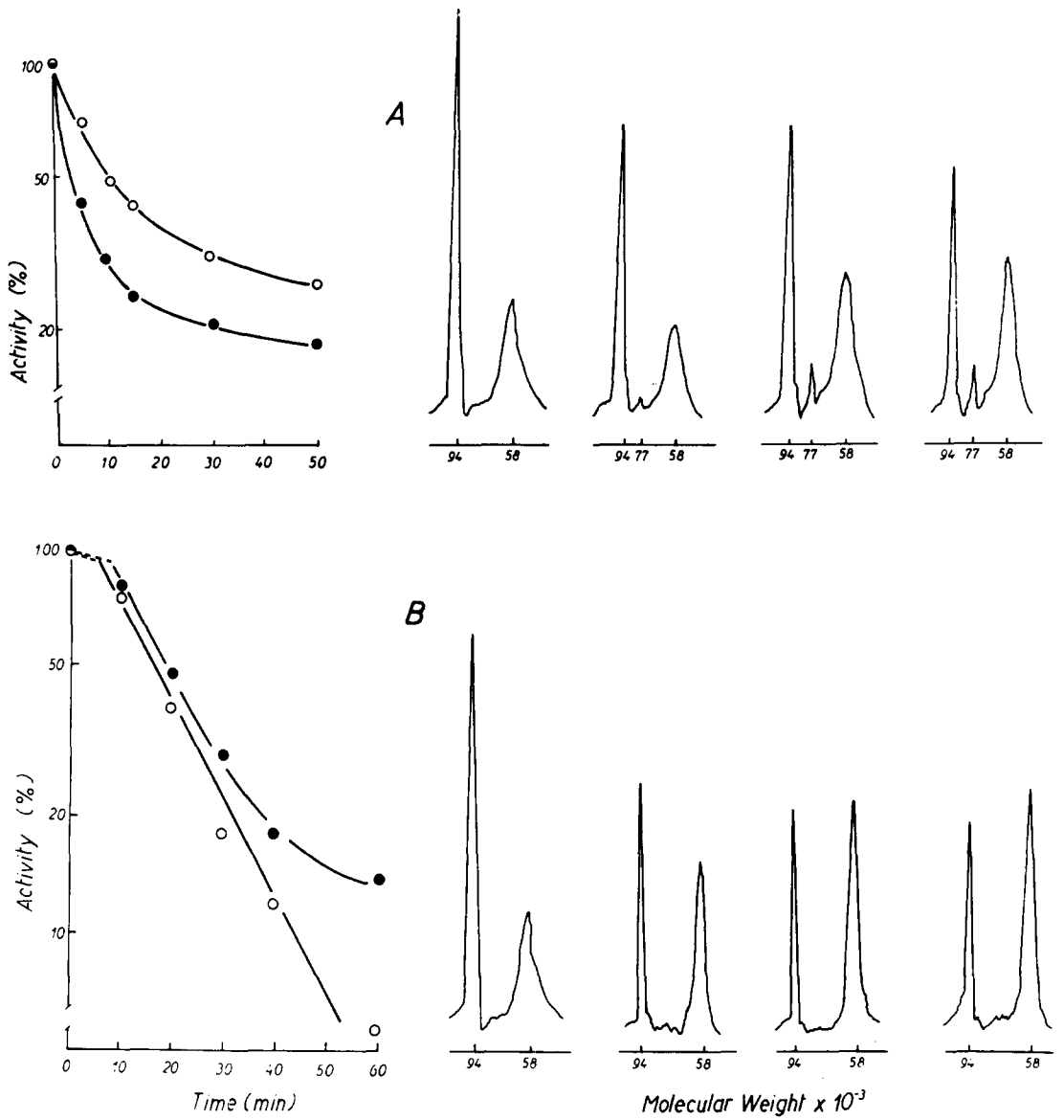

Fig. 4. Effects of 5 mM *p*-nitrophenyl phosphate (A) and 5 mM *p*-nitrophenyl phosphate plus 10 mM KCl (B) on trypsin digestion of $(Na^+ + K^+)$-ATPase in the presence of 3 mM $MgCl_2$ at high ionic strength. Ionic strength (150 mM monovalent salt/25 mM imidazole·HCl) was kept constant with choline chloride. Experimental procedures were as indicated in Fig. 1. Left panel: time-course of inactivation of $(Na^+ + K^+)$-ATPase (○) and *p*-nitrophenyl phosphatase (●) activities. Right panel: densitometric scans of tryptic cleavage followed in SDS-polyacrylamide gels. The scans correspond, from left to right, to samples taken at the following digestion times: (A): 0, 15, 30 and 60 min; (B): 0, 20, 40 and 60 min. The estimated rate constants for the time-dependent trypsin inactivation in A were (min^{-1}): $(Na^+ + K^+)$-ATPase: $\alpha = 0.150$; $\beta = 0.010$; potassium phosphatase: $\alpha = 0.284$, $\beta = 0.009$. For details see text and legend to Fig. 1.

shown). Fig. 7 shows that tryptic digestion and inactivation, with 0.5 mM KCl/3 mM $MgCl_2$ are identical to those seen with 1 mM $CaCl_2$/3 mM $MgCl_2$. This is reflected by the similar values observed for the α and β inactivation rate constants under both conditions (see legends for Figs. 6 and 7). On the other hand, with 1 mM KCl/3 mM $MgCl_2$, the inactivation patterns correspond

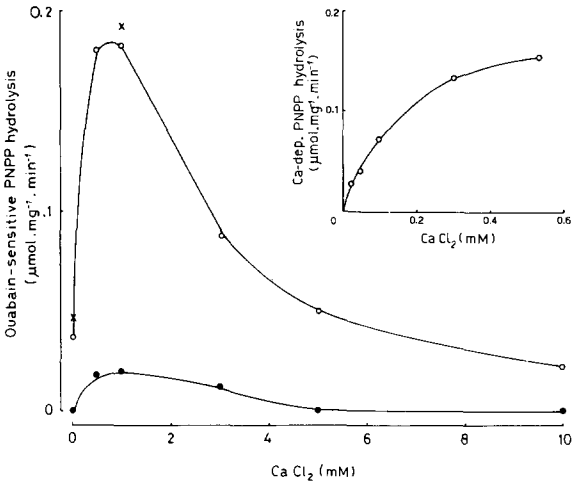

Fig. 5. Ouabain-sensitive hydrolysis of *p*-nitrophenyl phosphate by purified $(Na^+ + K^+)$-ATPase as a function of $CaCl_2$ concentration at fixed concentration of $MgCl_2$ in solutions of low (50 mM) and high (170 mM) ionic strength. The composition of solutions was the following: *p*-nitrophenyl phosphate, 5 mM; $MgCl_2$ 3 mM; $CaCl_2$ 0–10 mM; Tris-HCl enough to keep ionic strength equal to 50 mM (○) or to 170 mM (●). The assays were run with (○, ●) or without (×) 1 h preincubation of the enzyme at 37°C in the whole reaction mixture without the substrate. Insert: Ca^{2+}-dependent *p*-nitrophenyl phosphatase activity at low ionic strength.

to an enzyme predominantly in the E_2 form, though small amounts of 77 kDa fragments can still be detected in the gels. The possibility that the low ionic strength is responsible for the presence of these fragments, independently of the cation composition, is ruled out by the experiment with 0.5 mM KCl and no $MgCl_2$, where this band is not detected (Fig. 7A).

Trapping of Rb^+ and Ca^{2+} by $(Na^+ + K^+)$-ATPase after passage through cation-exchange columns

(a) Unphosphorylated enzyme under equilibrium conditions

One way to explain the data presented in the previous sections would be if, in the presence of Mg^{2+} and low ionic strength, Ca^{2+} has a K^+-like effect on $(Na^+ + K^+)$-ATPase. If this is the case, and if the mechanism involves the binding of Ca^{2+} to the K^+ sites taking the enzyme into $E_2(Ca)$, it should be possible to detect a certain amount of Ca^{2+} strongly bound to the unphosphorylated enzyme after passing it through a cation-exchange column; in addition this 'Ca^{2+}-occluding state' should be sensitive to ADP and Na^+, which are known to shift the equilibrium to E_1 [21,26], and to ouabain which, in the presence of Mg^{2+}, takes the enzyme into a E_2 state where K^+ is not occluded [26]. These possibilities were explored in the experiments summarized in Tables I and II.

The first column of Table I, used as control, reproduces and extends results already reported in the literature: in the presence of Mg^{2+}, Rb^+ brings the enzyme into an occluding form which is disoccluded by ADP [21,26], Na^+ [6,26] and ouabain [26]. Actually, the difference between the Rb^+ remaining in the effluent with and without ADP or Na^+ in the reaction mixture is taken as a measure of the occluded cation. On the other hand, although there is certain amount of Ca^{2+} strongly bound to the unphosphorylated enzyme in the presence of Mg^{2+} (second column in Table I), its sensitivity to the disoccluding agents in-

TABLE I

EFFECTS OF ADP, NaCl AND OUABAIN IN THE PRESENCE OF $MgCl_2$ ON THE TRAPPING OF Rb^+ OR Ca^{2+} BY UNPHOSPHORYLATED $(Na^+ + K^+)$-ATPase INCUBATED AT LOW IONIC STRENGTH UNDER EQUILIBRIUM CONDITIONS

Aliquots of 0.25 mg of $(Na^+ + K^+)$-ATPase (spec. act. 17 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$) were incubated at 20°C in 0.5 ml of media containing 3 mM $MgCl_2$, enough Tris-HCl (pH (20°C) 7.4) to keep total ionic strength at 50 mM and 0.5 mM $^{86}RbCl$ or 1 mM $^{45}CaCl_2$. ADP, NaCl, ouabain and RbCl were added at the indicated concentrations. After 2 min (10 min in the case of ouabain-containing solutions) the mixture was forced through 0.5 ml Dowex 50-X8-400 resin columns (in Tris form) at such a flow rate that the enzyme spent 0.9 s in contact with the resin. The effluent from the columns (the volume of which was determined by weight) was used for estimation of protein and radioactivity content. Each entry is the mean ± S.E. of triplicate determinations. For details see text.

Added ligand (mM)	Ion detected in effluent (nmol/mg protein)	
	Rb^+	Ca^{2+}
–	3.12 ± 0.16	4.20 ± 0.18
1 ADP	0.28 ± 0.12	4.12 ± 0.28
20 NaCl	0.18 ± 0.10	3.33 ± 0.09
1 ouabain	0.73 ± 0.20	6.34 ± 0.21
1 RbCl	–	4.14 ± 0.11
10 RbCl	–	3.38 ± 0.08

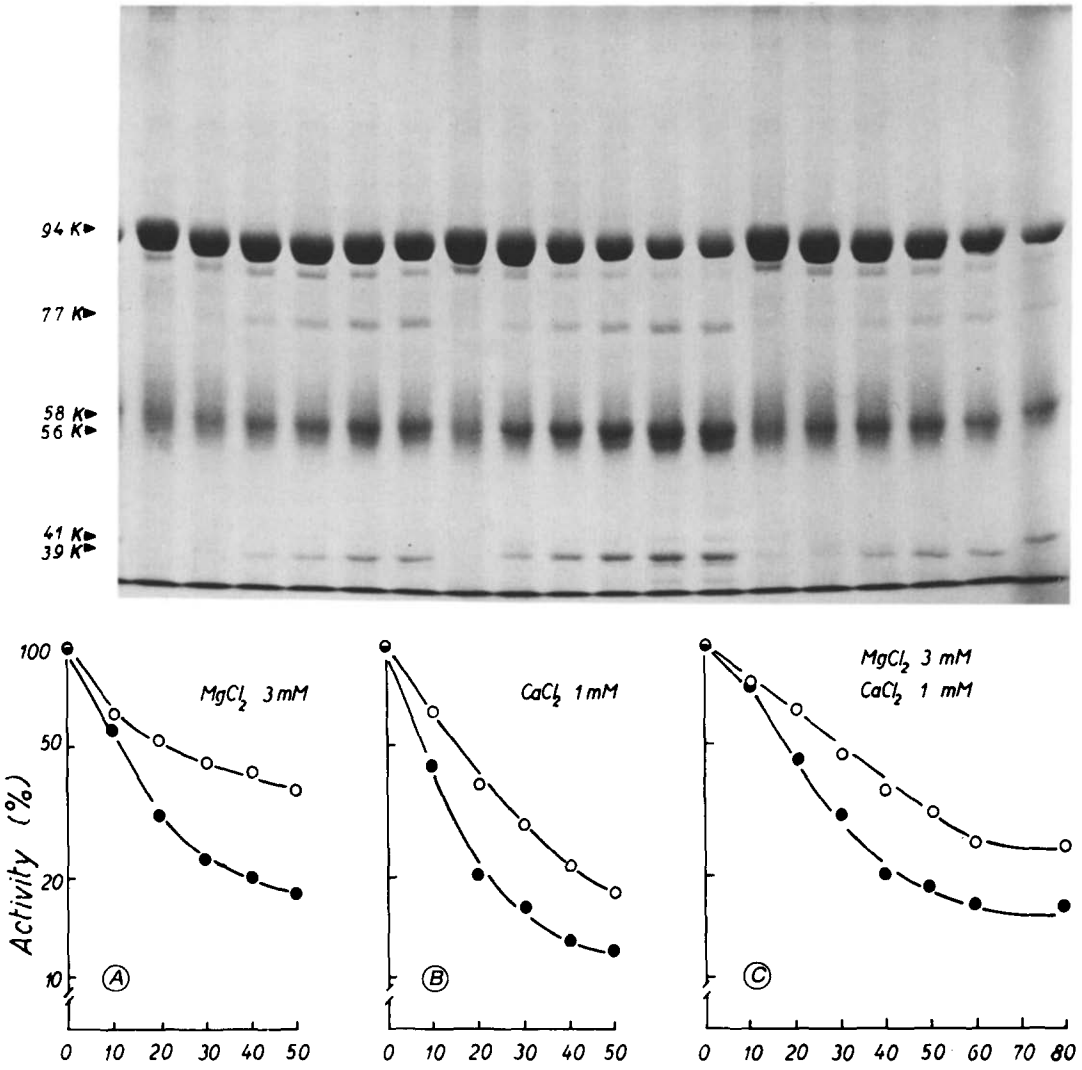

Fig. 6. Trypsin digestion of $(Na^+ + K^+)$-ATPase at low ionic strength in the presence of 3 mM $MgCl_2$ (A), 1 mM $CaCl_2$ (B) or 3 mM $MgCl_2$ plus 1 mM $CaCl_2$ (C). Top: pictures of the SDS-polyacrylamide gels showing the α-subunit and tryptic fragments released at different times (from left to right: 0, 10, 20, 30, 40 and 60 min). Bottom: time-course of inactivation of $(Na^+ + K^+)$-ATPase (○) and *p*-nitrophenyl phosphatase (●) activities. Digestion was started by mixing 0.24 μg of trypsin with 300 μg of enzyme in a total volume of 1 ml; at the indicated times it was stopped adding soybean trypsin inhibitor (the weight ratio of inhibitor to trypsin was 4:1). Aliquots were then taken for (i) analysis of released polypeptides by SDS-polyacrylamide slab gels, and (ii) assays of remaining $(Na^+ + K^+)$-ATPase and *p*-nitrophenyl phosphatase activities. The ionic strength was kept constant at 50 mM with Tris-HCl; pH (37°C) was 7.4 and temperature was 37°C. The estimated rate constants for the time-dependent trypsin inactivation were (min^{-1}): (A) $(Na^+ + K^+)$-ATPase: $\alpha = 0.160$, $\beta = 0.011$; potassium phosphatase: $\alpha = 0.165$, $\beta = 0.013$. (B) $(Na^+ + K^+)$-ATPase: $\alpha = 0.130$, $\beta = 0.024$, potassium phosphatase: $\alpha = 0.175$, $\beta = 0.018$. (C) $(Na^+ + K^+)$-ATPase: $\alpha = 0.041$, $\beta = 0.001$. See Methods for details.

vestigated is completely different from that of Rb^+: ADP had no effect, 20 mM NaCl produced only about 20% Ca release (which was statistically significant), whereas ouabain, instead of reducing, increased by 50% the amount of Ca^{2+} trapped. In addition, 10 mM RbCl displaced as much Ca^{2+} as 20 mM NaCl, whereas 1 mM RbCl was ineffective. Table II indicates that (i) the amount of Ca^{2+}

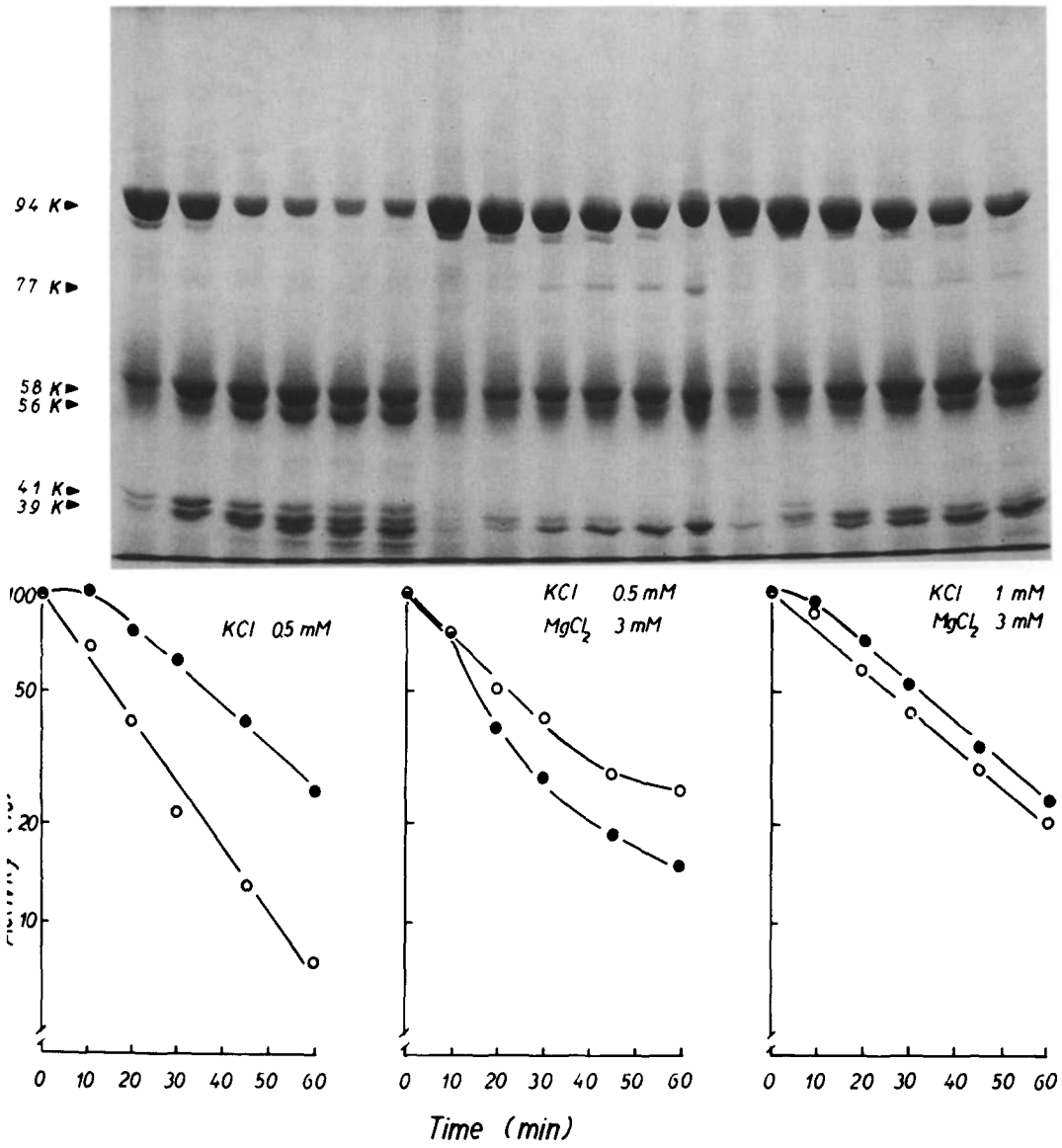

Fig. 7. Trypsin digestion of $(Na^+ + K^+)$-ATPase at low ionic strength in the presence of 0.5 mM KCl (A), 0.5 mM KCl plus 3 mM $MgCl_2$ (B) or 1 mM KCl plus 3 mM $MgCl_2$ (C). The general procedure was similar to that described in the legend to Fig. 6. Top: pictures of the SDS-polyacrylamide gels showing the α-subunits and tryptic fragments released at different times (from left to right: 0, 10, 20, 30, 40 and 60 min). Bottom: time-course of inactivation of $(Na^+ + K^+)$-ATPase (○) and *p*-nitrophenyl phosphatase (●) activities. The estimated rate constants for the time dependent trypsin inactivation of $(Na^+ + K^+)$-ATPase in B were (min^{-1}): $\alpha = 0.055$, $\beta = 0.007$. For details see text and legend to Fig. 6.

remaining in the effluent is directly proportional to the Ca^{2+}/Mg^{2+} ratio in the incubation mixture, and (ii) regardless of that ratio, ADP is consistently unable to release Ca^{2+} from the Ca-enzyme complex.

(b) Enzyme under phosphatase turnover

The present results indicate that the major conformation of $(Na^+ + K^+)$-ATPase under optimal conditions of *p*-nitrophenyl phosphate hydrolysis is E_2, but they do not tell whether in that form

TABLE II

LACK OF EFFECT OF ADP ON Ca^{2+} IONS TRAPPED BY UNPHOSPHORYLATED $(Na^+ + K^+)$-ATPase AT LOW IONIC STRENGTH EQUILIBRATED WITH VARIABLE Ca^{2+}/Mg^{2+} RATIOS

The general procedure was the same as that described in the legend to Table I. Between parentheses are the $CaCl_2/MgCl_2$ concentrations given in mM. Each entry is the mean ± S.E. of triplicate determinations. See text for details.

Ca^{2+}/Mg^{2+} ratio in incubation mixture	Ca^{2+} in effluent from columns (nmol/mg protein)		
	No ADP	1 mM ADP	difference
0.25 (0.5/2.0)	1.46 ± 0.07	1.32 ± 0.07	0.14 ± 0.10
0.30 (0.3/1.0)	1.71 ± 0.13	1.88 ± 0.10	−0.17 ± 0.17
0.33 (1.0/3.0)	4.20 ± 0.18	4.12 ± 0.28	0.08 ± 0.33
0.50 (0.5/1.0)	4.39 ± 0.17	4.40 ± 0.10	−0.01 ± 0.19

K^+ is occluded or not. The experiments described in this section were designed to answer that question. The enzyme was exposed to 1 mM $^{86}RbCl$/Tris · HCl at a concentration sufficient to give a total ionic strength of 50 mM and different ligand combinations at 20°C. The enzyme spent 30 s in the different media before being forced through the cation-exchange resin. The conditions investigated were: addition of no other ligand, 1.4 mM $MgCl_2$ alone, 5 mM *p*-nitrophenyl phosphate alone, combination of 3 mM $MgCl_2$ and 5 mM *p*-nitrophenyl phosphate (1.4 mM ionized Mg^{2+}) and 1.6 mM $MgCl_2$ plus 0.5 mM inorganic phosphate (1.4 mM Mg^{2+}). The latter mixture was included because it was found that during 30 s incubation about 10% of the *p*-nitrophenyl phosphate was hydrolyzed in the presence of $MgCl_2$ (not shown); adding 0.5 mM P_i was a way to deal with any possible effects of that P_i concentration on Rb^+ occlusion [27]. With 1 mM RbCl, 3 mM $MgCl_2$ and 5 mM *p*-nitrophenyl phosphate the rate of phosphatase activity was 85% of its maximal value at saturating Rb^+ concentrations (not shown). As usual, the total amount of occluded Rb^+ was taken as the difference between the Rb^+ present in the effluent in the absence and presence of 2 mM ADP. The results, summarized in Table III, show the following: (i) when $MgCl_2$ was the only added ligand 86% of Rb^+ remained occluded in the enzyme; (ii) in the absence of $MgCl_2$, Rb^+ occlusion was reduced to 37% by 5 mM *p*-nitrophenyl phosphate (it should be noted that, under these conditions, phosphatase activity is nonexistent; (iii) the amount of Rb^+ occluded in

TABLE III

EFFECTS OF *p*-NITROPHENYL PHOSPHATE AND INORGANIC PHOSPHATE ON RUBIDIUM OCCLUSION BY $(Na^+ + K^+)$-ATPase INCUBATED AT LOW IONIC STRENGTH IN MEDIA CONTAINING 1 mM $^{86}RbCl$ IN THE ABSENCE AND PRESENCE OF Mg^{2+}

Aliquots of 0.25 mg of $(Na^+ + K^+)$-ATPase (spec. act. 17.5 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$) were mixed at 20°C in 0.5 ml of media containing 1 mM $^{86}RbCl$, 0.05 mM CDTA · Tris, enough Tris-HCl (pH (20°C) 7.4) to give a total ionic strength of 50 mM, with and without 1.4 mM Mg^{2+} in the absence and presence of *p*-nitrophenyl phosphate and inorganic phosphate (at the concentrations given in the table) and of 2 mM ADP. After 30 s the mixture was force through 0.5 ml Dowex 50-X8-400 resin columns equilibrated with Tris-HCl. The flow rate was such that the enzyme spent 0.9 s in contact with the resin. The effluents from the columns (the volume of which was determined by weight) were use for estimation of protein and radioactivity content. Total occluded Rb^+ was taken as the difference between Rb^+ present in the effluent when the enzyme was incubated without and with 2 mM ADP. The rate of *p*-nitrophenyl phosphate hydrolyzed in the presence of 1 mM RbCl, 5 mM *p*-nitrophenyl phosphate and 1.4 mM Mg^{2+} was 85% of the maximum rate of hydrolysis at saturating RbCl and resulted in an accumulation of about 0.5 mM inorganic phosphate after 30 s. Each entry is the mean ± S.E. of the differences of triplicate determinations. See text for details. In the presence of 2 mM ADP the Rb^+ in the effluent was (nmol/mg^{-1}) 0.87 ± 0.04 in the absence of Mg^{2+} and 0.95 ± 0.08 in the presence of Mg^{2+}.

Added ligand (mM)	Rb^+ remaining in effluent after 0.9 s (total minus ADP) (nmol/mg protein)	
	Mg^{2+}-free medium	1.4 mM Mg^{2+} medium
–	3.60 ± 0.10	3.11 ± 0.10
5 *p*-nitrophenyl phosphate	1.31 ± 0.13	0.21 ± 0.15
0.5 inorganic phosphate	3.72 ± 0.11	1.94 ± 0.12

the $(Na^+ + K^+)$-ATPase incubated in 1 mM RbCl, 3 mM $MgCl_2$ and 5 mM *p*-nitrophenyl phosphate was not different from zero; i.e., no Rb occlusion was detected; (iv) the results in (iii) cannot be ascribed to P_i accumulation for with 0.5 mM P_i and 1.6 mM $MgCl_2$ about 55% Rb^+ remained trapped in the enzyme.

Discussion

A very important point emerging from the present study is that the predominant enzyme form of the $(Na^+ + K^+)$-ATPase present during K^+-dependent phosphatase activity probably belongs to an E_2 type where K^+ is not occluded. This being the case, the questions that immediately arise are (i) what role do the different ligands play which are involved in the phosphatase reaction, and (ii) on what sites do they act? In the presence of Mg^{2+} as the sole ligand the enzyme is definitely in the E_1 state; this is also true for Mn^{2+}, a divalent cation with higher affinity for the enzyme and which can sustain phosphatase activity [11], and even for Ca^{2+}, a competitor of Mg^{2+} which is unable to activate the phosphatase reaction [13,16]. The E_1Mg (or E_1Mn) complexes are different from E_1 without the divalent cations, for they are less sensitive to trypsin attack (see also Ref. 8). Binding of *p*-nitrophenyl phosphate to the enzyme also favors the E_1 state; in doing so, *p*-nitrophenyl phosphate antagonizes K^+, accelerating its release from $E_2(K)$ (Ref. 6; this paper); this makes it very unlikely that the substrate site for phosphatase activity is the low-affinity ATP site [6]. The simultaneous presence of Mg^{2+} and *p*-nitrophenyl phosphate keeps the enzyme in the E_1 conformation; this indicates that the substrate does not behave like inorganic phosphate, which requires Mg^{2+} for binding and, as a consequence of phosphorylation, takes the enzyme into an E_2 form where K^+ is not occluded (Refs. 10, 27; Beaugé, L. and Pedemonte, C., unpublished data). On the other hand, inorganic phosphate is a competitive inhibitor of *p*-nitrophenyl phosphatase activity [28]; an interesting possibility then is that the phosphatase substrate site is the inorganic phosphate-binding site, which accepts *p*-nitrophenyl phosphate only when the $(Na^+ + K^+)$-ATPase is in the E_2 conformation. With Mg^{2+} and *p*-nitrophenyl phosphate, K^+ being excluded, ouabain-sensitive phosphatase activity of $(Na^+ + K^+)$-ATPase is either completely absent or exceedingly low; the only conditions under which a significant, although still small, fraction of that activity is obtained is in solutions with low ionic strength (Fig. 5) or when the $(Na^+ + K^+)$-ATPase has been treated with thimerosal [10]. A possible explanation is that these conditions change the reactivity of the enzyme; in agreement with this view is the fact that at low-ionic-strength, Ca^{2+}, in the presence of Mg^{2+}, becomes stimulatory to ouabain-sensitive *p*-nitrophenyl phosphate hydrolysis. This Ca^{2+} stimulation reaches its peak at 1 mM $CaCl_2$ (for 3 mM $MgCl_2$) and declines above that concentration; the decline very likely expresses displacement of Mg^{2+} from its essential activator site [14,16], suggesting that Ca^{2+} activation takes place at other sites. Moreover, the patterns of trypsin digestion and inactivation obtained with 1 mM $CaCl_2$ or 0.5 mM KCl, which give similar stimulation of phosphatase activity, are indistinguishable from each other and agree with a mixed populations of E_1 and E_2 forms. This not only concurs with the idea that E_2 is the enzyme state required for phosphatase activity, but suggests that the key role played by K^+ is actually to take the enzyme from an E_1 (catalytically inactive) into an E_2 (catalytically active) form. This may not be the only function of K^+ for, in the presence of Na^+ and ATP, K^+ is required at both intra- and extracellular sites [28,29]. Regarding the role played by Mg^{2+}, there are two possibilities: by analogy with what happens in phosphorylation from inorganic phosphate, the function of Mg^{2+} could be to allow the binding of the substrate; on the other hand, by analogy with what happens in the $(Na^+ + K^+)$-ATPase activity, it might be that the phosphatase substrate binds with or without Mg^{2+} and the role of the divalent cation is to allow hydrolysis to occur.

Ca^{2+}, in the presence of Mg^{2+}, can be strongly bound to $(Na^+ + K^+)$-ATPase. Per mg of protein the total amount of Ca^{2+} retained by the enzyme was a little higher than the occluded Rb^+. However, the reactivity of this Ca^{2+}-binding complex to different ligands (ADP, NaCl and ouabain) was completely different from that of the Rb^+-occluding $(Na^+ + K^+)$-ATPase. It being extremely un-

likely that Ca^{2+} leads to a Ca^{2+}-occluded conformation of the $E_2(K)$ form, it becomes difficult to guess at which site(s) Ca^{2+} exerts its stimulatory role of phosphatase activity. Perhaps both K^+ and Ca^{2+} act on the same site(s) [16], but it is not inconceivable that, under certain conditions (such as low ionic strength), different cations, acting on different sites, might produce similar enzyme conformations.

Finally, it is interesting to note that oligomycin, which stabilizes the E_1P conformation of (Na^+ + K^+)-ATPase [30], has no effect on phosphatase activity in the presence of Mg^{2+}, K^+ and *p*-nitrophenyl phosphate, but it becomes inhibitory if Na^+ and CTP (an hydrolyzable ATP analogue) are added to the reaction mixture [31]. This observation, together with the results given here, strongly suggests that an E_1P form of the enzyme is not involved in the phosphatase cycle. According to this view, E_1P appears when, in addition to phosphatase, the ATPase reaction is simultaneously turning over.

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