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STUDIES ON $(Na^+ + K^+)$-ACTIVATED ATPase

XXXVII. STABILIZATION BY CATIONS OF THE ENZYME-OUABAIN COMPLEX FORMED WITH Mg^{2+} AND INORGANIC PHOSPHATE

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SUMMARY

Dissociation of the $(Na^+ + K^+)$-ATPase ouabain complex, formed in the presence of Mg^{2+} and inorganic phosphate (Complex II), is inhibited by Mg^{2+} (21–45 %) and the alkali cations Na^+ (25–59 %) and K^+ (27–75 %) when kidney cortex tissue (bovine, rabbit, guinea pig) is the enzyme source. Choline chloride at 200 mM, equivalent to the highest concentration of NaCl tested, does not inhibit. Dissociation of Complex II from brain cortex (bovine, rat, rabbit) or heart muscle (rabbit) is much less inhibited: 0–11 % by Na^+ and 11–19 % by K^+. The degree of inhibition is not directly related to the size of the dissociation rate constant (k^-) of the various complexes, but rather to the extent of interaction between the cation and ouabain binding sites for these tissues.

Inhibition curves for Na^+ and K^+ are sigmoidal. Half-maximal inhibition for rabbit brain and kidney cortex is at 30–40 mM Na^+ and 6–10 mM K^+, and the maximally inhibitory concentrations are 50–150 and 15–20 mM, respectively. Maximal inhibition by Na^+ or K^+ for these tissues is the same. For guinea pig kidney cortex Na^+ and K^+ are almost equally effective, but 150 mM K^+ or 200 mM Na^+ are still not saturating, and inhibition curves indicate high- and low-affinity binding sites for the alkali cations.

The inhibition curve for Mg^{2+} is not sigmoidal. In the kidney preparations Mg^{2+} inhibits half-maximally at 0.4–0.5 mM, maximally at 1–3 mM. Maximal inhibition by Mg^{2+} is higher than by Na^+ or K^+ for rabbit kidney cortex and lower for guinea pig kidney cortex.

There is no competition or additivity among the cations, indicating the existence of different binding sites for Mg^{2+} and the alkali cations.

Complex II differs in stability, in the extent of inhibition, in the dependence of inhibition on the cation concentration and in the absence of antagonism between Na^+ and K^+, from the ouabain complex formed via phosphorylation by ATP (Complex I). This indicates that the phosphorylation states for the complexes are clearly different.

INTRODUCTION

Ouabain is a cardiac glycoside, which specifically inhibits the ($Na^+ + K^+$)-stimulated ATPase and has been used to demonstrate occurrence and function of this enzyme in a great many tissues [1]. In the reaction mechanism of the enzyme two types of phosphorylated intermediates are distinguished: a high-energy phosphorylated intermediate, designated $E \sim P$, and a low-energy phosphorylated intermediate E-*P* [2]. Two types of interpretation of the terms high- and low-energy phosphorylated intermediate are currently used. One is referring to a high- and low-energy conformation of the enzyme, the phosphoryl bond in both forms being identical [3, 4]. In this concept ouabain binds only to the low-energy phosphorylated intermediate, whether it is formed by phosphorylation with ATP in the presence of Na^+ and Mg^{2+} or by phosphorylation with inorganic phosphate in the presence of Mg^{2+} [5]. The other concept is that the terms high- and low-energy intermediate refer to chemically different types of phosphoryl bonds in the enzyme. [6, 7].

We tend to the latter interpretation in view of the differences displayed by the ouabain-enzyme complexes formed by phosphorylation with ATP (E-Ouabain $\sim$ P) or with inorganic phosphate (E-Ouabain-*P*). Following Allen et al. [8–10], these complexes will be designated Complex I and II, respectively, and their systems of formation accordingly System I and II.

Complex II is 3–10 times more stable than Complex I [8, 9, 11–14], although their ouabain binding capacities are the same [15–17]. Dissociation of Complex I is inhibited by both Na^+ and K^+ [8, 9, 11, 12]. On the other hand, the stability of Complex II is not affected by K^+, whether in the presence [18, 19] or the absence [8, 9, 11, 12] of Mg^{2+}. At variance with the latter results, Schönfeld et al. [16] have found that both Na^+ and K^+ stabilize Complex II in the presence of Mg^{2+}, although to a lesser extent than is found for K^+ in its effect on Complex I.

We have extended these studies of the effects of cations on the dissociation of ouabain from Complex II for a variety of tissues. We shall present further evidence for differences between Complex I and II, which are suggestive of a chemically different state of phosphorylation in the two complexes.

MATERIALS AND METHODS

Preparation of microsomes treated with NaI or urea

Microsomes have been prepared from three different tissues (brain cortex, heart ventricular muscle and kidney cortex) from four different species (cattle, rabbit, guinea pig and rat).

Microsomes from brain cortex are prepared according to Uesugi et al. [20] in a buffer containing 0.25 M sucrose, 30 mM imidazole/HCl and 1 mM EDTA, pH 7.4. The tissue is homogenized in a Waring blender for 2 min. The centrifugation technique of Jørgensen and Skou [21] has been followed with a final centrifugation for 2 h at $23\,000 \times g$.

Microsomes from heart muscle are prepared according to Akera et al. [22] after Waring blender homogenization of the tissue in the above sucrose buffer. The LiBr treatment has been replaced by a NaI treatment (see below). Microsomal pellets from brain cortex and heart muscle after the final centrifugation are suspended in

sucrose buffer to a protein concentration of 20 mg protein/ml and stored at −70 °C. These microsomes are treated with 2 M NaI, essentially according to Uesugi et al. [20], in order to remove ouabain-insensitive Mg^{2+}-ATPase [23, 24]. The resulting preparation is stored at −70 °C in sucrose buffer at a protein concentration of 20 mg/ml.

Microsomes from kidney cortex are prepared according to the method of Post and Sen [25], omitting heparin from the second homogenization medium. Depletion of ouabain-insensitive Mg^{2+}-ATPase is achieved by treatment with 2 M urea. Storage of this preparation is the same as that for brain cortex and heart muscle preparations.

Protein determination and $(Na^+ + K^+)$-ATPase assay

The protein content of the microsomal preparations is determined according to Hess and Lewin's modification [26] (method C) of the procedure of Lowry et al.

$(Na^+ + K^+)$-ATPase activity in the different preparations is determined according to Bonting (ref. 1, p. 261) as the difference in phosphate liberation during 10 min at 37 °C in media A and E in a slightly modified composition. Medium A contains: 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 5 mM ATP (disodium salt) and 30 mM imidazole/HCl, pH 7.0. Medium E has the same composition and pH as medium A, except that KCl is omitted and 10^{-4} M ouabain is added. Average specific $(Na^+ + K^+)$-ATPase activities of the different NaI- or urea-treated microsome preparations are: bovine brain cortex 0.50 (97 ± 1 %; $n = 2$), rabbit brain cortex 0.37 (94 ± 2 %; $n = 2$), rat brain cortex 1.38 (88 ± 2 %; $n = 2$), rabbit heart muscle 0.04 (45 %; $n = 1$), bovine kidney cortex 0.11 (56 ± 6 %; $n = 2$), rabbit kidney cortex 0.54 (78 ± 4 %; $n = 5$) and guinea pig kidney cortex 0.54 (87 ± 4 %; $n = 6$) μmol $P_i \cdot mg^{-1}$ protein $\cdot$ min^{-1}. The values between parentheses indicate the percentage of total ATPase activity, which is ouabain sensitive and the number of preparations (n).

Formation of the ouabain complex

The formation of the ouabain complex is essentially according to Tobin and Sen [27]. The association medium (pH 7.4) contains 0.25 M sucrose, 30 mM imidazole/HCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM Tris/phosphate, 2 mM $MgCl_2$, [^{3}H]ouabain (10^{-6} M, unless otherwise specified; specific radioactivity 13.3 Ci/mmol) and 1 mg protein/ml of the NaI- or urea-treated microsomes. Concentrations above 10^{-6} M ouabain are obtained by adding non-radioactive ouabain to the [^{3}H]ouabain preparation. After 10 min at 37 or 25 °C (specified under Results), the process is stopped by rapid cooling in acetone-solid CO_2, followed by centrifugation for 30 min at $27\,000 \times g$ and a two-fold washing of the sediment by resuspension and centrifugation, all at 0–4 °C. Washing is carried out with the same medium, except that phosphate, $MgCl_2$ and ouabain have been omitted. The final pellet is suspended in the washing medium to a protein concentration of 20 mg/ml.

Dissociation studies

The suspended complex is diluted 20-fold with the washing medium, to which $2.5 \cdot 10^{-4}$ M non-radioactive ouabain has been added. NaCl, KCl or $MgCl_2$ are added, separately or in combination, in the concentrations indicated under Results. The suspension is incubated at 37 °C in 1 ml aliquots, which are withdrawn after certain time intervals, are immersed in acetone-solid CO_2 and are centrifuged for

30 min at 27 000 × *g* at 0–4 C. After removal of the supernatant the pellet is suspended in 1 ml distilled water. An aliquot from each tube is assayed for protein to correct for losses during the procedure. The remainder is transferred to counting vials, taken to dryness and dissolved overnight at 50 °C in 1 ml Hyamine hydroxide 10-X (Packard Instrument Co., Inc.), followed by addition of 10 ml Aquasol (New England Nuclear). The radioactivity is counted in a liquid scintillation analyzer (Philips 12 channel L.S.A.). A 2-μl aliquot (0.15 nmol) of the original [^{3}H]ouabain preparation serves as a standard for the conversion of cpm to pmol of protein-bound [^{3}H]ouabain. A 50 μl aliquot (approx. 1 mg protein) from the washed stock suspension of the complex is used to determine the experimental point at zero time. The first order dissociation rate constant (k_d) is determined from the slope of the linear graph of the logarithm of protein-bound [^{3}H]ouabain concentration vs time [27].

Materials

All chemicals are analytical grade reagents. Ouabain is from Merck, Darmstadt, Germany; randomly labeled [^{3}H]ouabain, specific radioactivity 13.3 Ci/mmol, is obtained from New England Chemicals GmbH, Frankfurt/Main, Germany, as a solution of 1 mCi/ml benzene/ethanol (1 : 9, v/v) and is stored at −20 °C.

RESULTS

Effect of Na^+ on ouabain binding levels

Fig. 1 shows the effect of 200 mM NaCl on the levels of ouabain binding by bovine brain cortex microsomes during association (10 min at 25 °C) in the presence of Mg^{2+} and inorganic phosphate. Na^+ decreases the binding in this system over the total range of ouabain concentrations. This binding is the resultant of association and dissociation kinetics. As dissociation for this tissue is not influenced by Na^+ (see below), this means that Na^+ decreases the association rate constant for this complex, since the cation does not affect the total number of ouabain binding sites [17]. A similar result has been reported for K^+ [18].

Effect of alkali cations on the dissociation rate constant

Table I shows the effect of Na^+ or K^+ on the dissociation rate constant (k_d) of Complex II from various tissues, representing a 40- to 50-fold variation in dissociation rate constant without added metal ions. Na^+ at 200 mM has no effect on the dissociation rate constant of Complex II from bovine brain cortex whereas 20 mM K^+ exerts only a slightly inhibitory effect. The same is more or less true for the other brain cortex preparations and for rabbit heart muscle, where k_d is lowered by Na^+ or K^+ 0–11 % and 11–19 %, respectively. A more pronounced decrease of k_d by Na^+ (42–59 %) or K^+ (34–75 %) is found for the kidney preparations.

It is evident from these results that no relation exists between the size and the lowering of k_d for the various tissues. For example, for rabbit tissues the increase of the dissociation rate constant in the order brain → kidney cortex → heart muscle is not accompanied by a stepwise increase in inhibition by Na^+ or K^+. Even for a single tissue the inhibition does not always follow variations in k_d values. This is illustrated in the first and second lines for guinea pig kidney cortex and rabbit heart muscle in Table I. Despite a 20 % variation in k_d (column a) the variation in the ratio b/a is

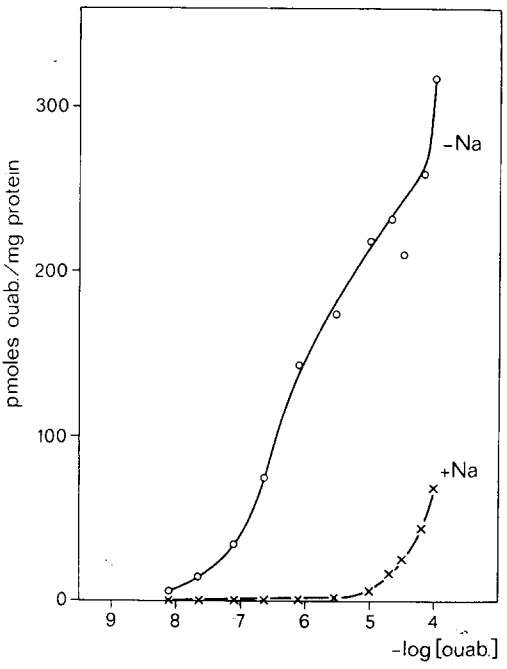

Fig. 1. Ouabain binding to NaI-treated bovine brain cortex microsomes as a function of the ouabain concentration. Formation of the enzyme-ouabain complex in the absence (○) or presence (×) of 200 mM NaCl and subsequent washing as described under Materials and Methods, except that association took place at 25 °C in 1-ml samples at [^{3}H]ouabain concentrations indicated on the abscissa. Pellets after the final wash are suspended in 1 ml washing medium, an aliquot is taken for protein determination and the remainder is counted against [^{3}H]ouabain as reference.

only 2–10 %. Neither do the results suggest that there is a transition in k_d values above which only an inhibitory cation effect might become apparent. The most striking proof against this hypothesis is presented by the kidney cortex group in which bovine and guinea pig kidney cortex represent both extremes of the k_d scale, but undergo about equal inhibition by Na^+ or K^+ (Table I and Fig. 6).

Choline chloride at 200 mM has no effect on the k_d of Complex II from rabbit or guinea pig kidney cortex (data not shown). This proves that the effects of Na^+ or K^+ on k_d are specific cation effects, and are not due to the increase in ionic strength caused by addition of their chloride salts.

The decrease of k_d caused by Na^+ and K^+ is not equivalent for the two cations for several tissues (Table I). However, it is not sure that 200 mM Na^+ and 20 mM K^+ are saturating in these cases, except for rabbit brain cortex Complex II, where dissociation is maximally inhibited by 15–20 mM K^+ and 150–200 mM Na^+ and half-maximally with 10 mM K^+ and 40 mM Na^+. Therefore, the effects of increasing cation concentrations have been investigated with two other tissues, rabbit kidney cortex (Fig. 2) and guinea pig kidney cortex (Fig. 6). The results are reported in the following sections, including experiments with Mg^{2+}. It should be recalled here that the large excess of non-radioactive ouabain in the dissociation medium makes reassociation of liberated [^{3}H]ouabain negligible, even in the presence of Mg^{2+} [28, 29].

TABLE I

EFFECT OF Na^+ AND K^+ ON THE DISSOCIATION OF THE ($Na^+ + K^+$)-ATPase-OUABAIN COMPLEX FROM DIFFERENT SOURCES

The dissociation rate constant (k_d) is determined at 37 °C and pH 7.4, in the absence or presence of 200 mM NaCl or 20 mM KCl. Each line represents results obtained for one preparation of the relevant tissue.

Source	Dissociation rate constant (k_d) ($min^{-1} \times 10$)			Ratio	
	no addition (a)	+200 mM Na^+ (b)	+20 mM K^+ (c)	b/a	c/a
Bovine kidney cortex	0.092	0.038	0.038	0.41	0.41
	0.092	0.046	0.023	0.50	0.25
Bovine brain cortex	0.115	0.122		1.06	—
	0.123	0.123	0.107	1.00	0.87
Rat brain cortex	0.165	0.146	—	0.89	—
	0.107	—	0.088	—	0.82
Rabbit brain cortex	0.179	0.160		0.89	
	0.170	—	0.152	—	0.89
Rabbit kidney cortex	2.19	1.21	—	0.55	
	2.18	—	1.26	—	0.58
Rabbit heart muscle	2.37	2.16	—	0.91	—
	2.82	2.82	2.27	1.00	0.81
Guinea pig kidney cortex	4.09	2.42	—	0.59	—
	4.84	2.80	—	0.58	—
	4.28	—	2.85	—	0.66

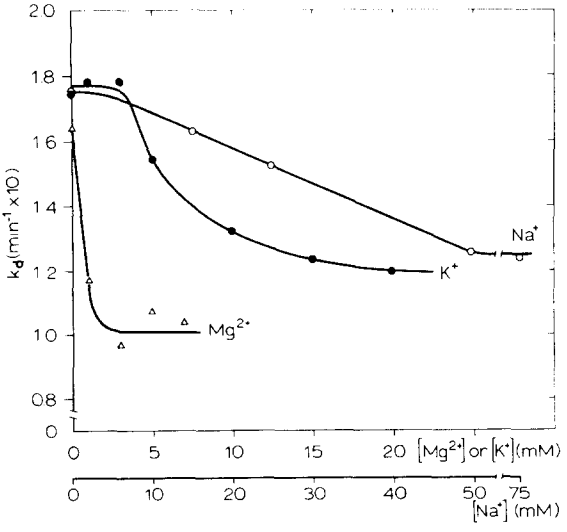

Fig. 2. Inhibition by Mg^{2+} (△), K^+ (●) and Na^+ (○) of the dissociation of rabbit kidney cortex Complex II. Inhibition is expressed as decrease of the dissociation rate constant (k_d). Mg^{2+} concentrations have been reduced by 1 mM for binding by EDTA.

Concentration dependence of cation effects in rabbit kidney cortex

Fig. 2 shows that k_d for rabbit kidney cortex is inhibited maximally (30 %) by 50 mM Na^+ or 20 mM K^+, and half-maximally at about 30 mM Na^+ or 6 mM K^+. The curves are sigmoidal, indicating cooperative interaction [30] of at least two Na^+- or K^+-binding ligands. Na^+ and K^+ give the same maximal inhibition to a consistent minimum value of k_d in the presence of these cations: 0.122 (S.E.: 0.003) min^{-1} for Na^+ and 0.120 (S.E.: 0.003) min^{-1} for K^+ in 6 experiments with three different preparations. Apparently the same absolute minima are attained for the preparations of Table I. However, relative inhibitions in Fig. 2 and Table I vary due to the difference in k_d in the absence of alkali metal ions.

Inhibition by Mg^{2+} is maximal at about 3 mM, and half-maximal at 0.4 mM Mg^{2+} (Fig. 2). The effect is significantly greater than for Na^+ or K^+. The lower limit of k_d in the presence of Mg^{2+} is 0.097 (S.E.: 0.003) min^{-1} (5 experiments with two different preparations). In contrast to the inhibition curves for Na^+ and K^+, inhibition by Mg^{2+} does not give a sigmoidal curve (Fig. 2).

Non-additivity of cation effects in rabbit kidney cortex

The inhibitory effects of Na^+ and K^+ appear to be non-additive in the sense that the decrease of k_d caused by a combination of these cations does not exceed the maximal decrease set by either of them. Varying the K^+ concentration at a maximally inhibitory concentration of Na^+ (Fig. 3) or of Na^+ at a maximally inhibitory concentration of K^+ (Fig. 4) results in straight lines, representing k_d values, which are virtually the same as the lower limits reached by K^+ or Na^+ alone.

Varying the Mg^{2+} concentration at maximally inhibitory concentrations of Na^+ or K^+ (Fig. 5) leads to analogous results. In the experiments of Fig. 5, although performed with two different preparations of urea-treated rabbit kidney cortex membranes, virtually the same lower limit of k_d in the presence of Mg^{2+} is obtained, whether Na^+ or K^+ is present or not. Moreover, Na^+ or K^+ does not affect the course of inhibition, which is maximal in all cases at 3 mM Mg^{2+}. Raising K^+ to

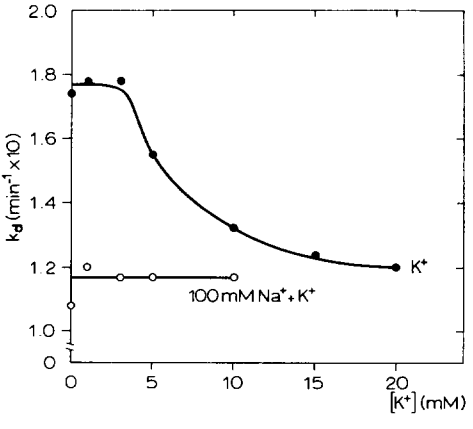

Fig. 3. Inhibition of dissociation of rabbit kidney cortex Complex II at increasing K^+ concentrations in the presence of a maximally inhibitory concentration (100 mM) of Na^+ (○). For comparison inhibition by K^+ alone (●) is included. Inhibition is expressed as in Fig. 2.

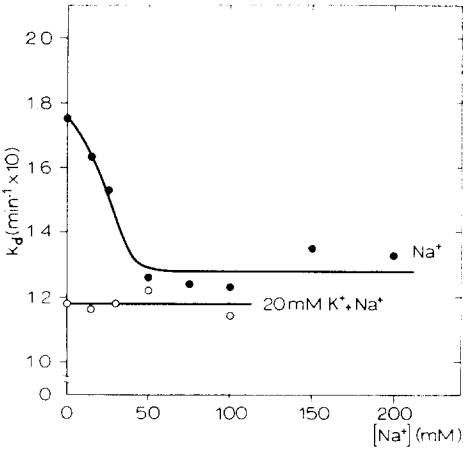

Fig. 4. Inhibition of dissociation of rabbit kidney cortex Complex II at increasing Na^+ concentrations in the presence of a maximally inhibitory concentration (20 mM)of K^+ (○). For comparison inhibition by Na^+ alone (●) is included. Inhibition is expressed as in Fig. 2.

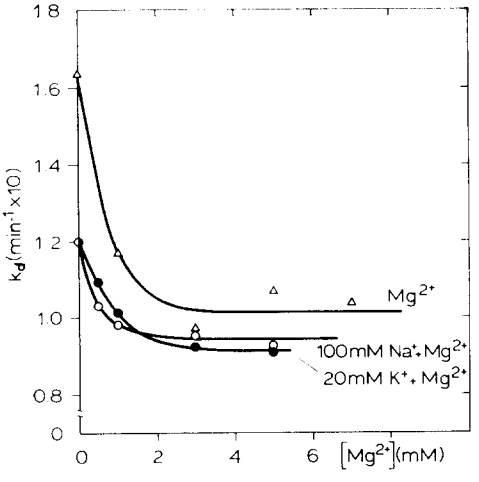

Fig. 5. Inhibition of dissociation of rabbit kidney cortex Complex II at increasing Mg^{2+} concentrations in the presence of a maximally inhibitory concentration of Na^+ (100 mM; ○) or K^+ (20 mM; ●). For comparison inhibition by Mg^{2+} alone (△) is included. Inhibition is expressed as in Fig. 2. The values of k_d in min^{-1} without added cations are: 0.160 (●) and 0.169 (○). The Mg^{2+} concentrations have been reduced by 1 mM for binding by EDTA.

15 mM in the presence of 5 mM Mg^{2+} for one of these preparations (not shown) gives a straight line ($k_d = 0.097\ min^{-1}$), which is intermediate between the lower limits shown in Fig. 5. These data indicate that Na^+ and K^+ are not competitive with respect to Mg^{2+} and that their inhibitory effects are non-additive. This suggests that Mg^{2+} binds to sites different from those binding Na^+ and K^+.

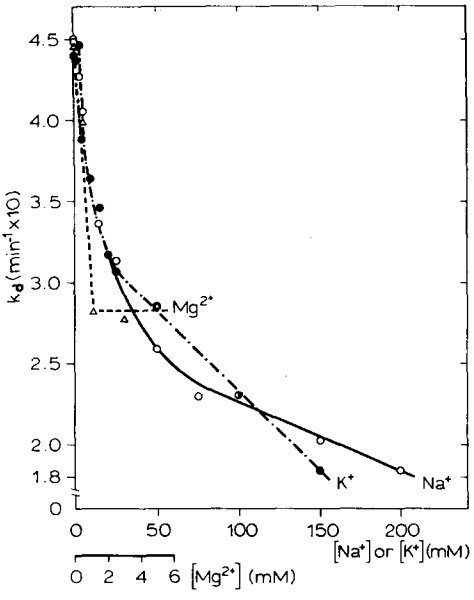

Fig. 6. Inhibition of dissociation of guinea pig kidney cortex Complex II by Mg^{2+} (△), K^+ (●) or Na^+ (○). Inhibition is expressed as in Fig. 2. The Mg^{2+} concentrations have been reduced by 1 mM for binding by EDTA.

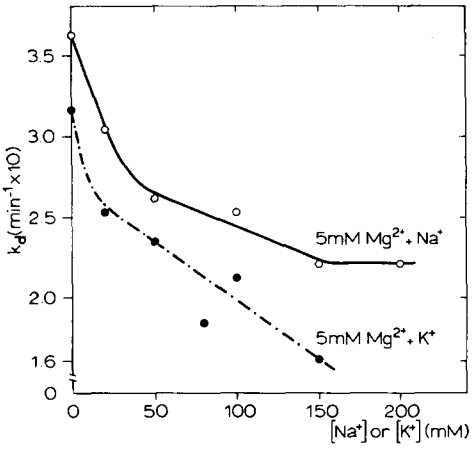

Fig. 7. Inhibition of dissociation of guinea pig kidney cortex Complex II at increasing Na^+ (○) or K^+ (●) concentrations in the presence of a maximally inhibitory concentration (5 mM) of Mg^{2+}. Inhibition is expressed as in Fig. 2. The values of k_d in min^{-1} without added cations are: 0.460 (○) and 0.446 (●). The Mg^{2+} concentration has been reduced by 1 mM for binding by EDTA.

Concentration dependence of cation effects in guinea pig kidney cortex

In contrast to the situation for rabbit kidney cortex no absolute lower limit for k_d in the presence of cations can be determined for guinea pig kidney cortex. This is obvious for Na^+ or K^+, which even at concentrations of 200 and 150 mM, respectively, do not lower k_d maximally (Fig. 6). Mg^{2+}, which is optimally effective between 1 and 5 mM (Fig. 6), causes a maximal (22–37 %) decrease of k_d to 0.28–0.36 min^{-1} (Figs 6 and 7) without any relation to the magnitude of k_d without added cations.

Another difference with rabbit kidney cortex is that the maximal inhibition by Mg^{2+} is less than for Na^+ and K^+. The latter two ions are virtually equally effective up to a concentration of 100 mM, above which K^+ begins to be slightly more effective (Fig. 6). Half-maximal inhibition by Mg^{2+} is at 0.5 mM, which is very close to the value of 0.4 mM determined for rabbit kidney Complex II (Fig. 2). Again the curve is not sigmoidal (Fig. 6). In view of the risk of ionic strength effects, cation concentrations above 200 mM have not been used. So no half-maximal effective Na^+ and K^+ concentrations are presented, and no attempts have been made to investigate fully the additivity of the K^+ and Na^+ effects.

Nevertheless, the inhibition curves for Na^+ and K^+ (Fig. 6) easily explain the difference in inhibition by Na^+ (41–42 %) and K^+ (34 %) in Table I, as these values were determined at 200 mM Na^+ and 20 mM K^+, respectively.

The inhibition curves in Fig. 6 suggest that there are two different binding sites for the alkali cations: sites with a high affinity for K^+ or Na^+ being saturated below 50 mM, and low-affinity sites becoming saturated above 50 mM. When the K^+ concentration is gradually increased in the presence of 100 mM Na^+ (not shown), then a straight line with a slope intermediate between that evoked by Na^+ or K^+ alone is obtained. This indicates that for the high-affinity sites there is no additivity of Na^+ and K^+ and that partial occupation of the low affinity sites by Na^+ lowers the affinity for K^+. Evidence for such a two-site binding behaviour is absent from the inhibition curves for k_d of rabbit kidney cortex Complex II (Fig. 2).

Non-additivity of Mg^{2+} and alkali cation effects in guinea pig kidney cortex

The additivity of the Mg^{2+} and Na^+ or K^+ effects (Fig. 7) could be tested, since inhibition by Mg^{2+} is already maximal at low concentrations (Fig. 6). However, inhibition by 150–200 mM Na^+ in the presence of 5 mM Mg^{2+} reaches 52 % and that by 150 mM K^+ in the presence of 5 mM Mg^{2+} 64 %, which inhibition values are not much different from the 59 % inhibition by 200 mM Na^+ or 150 mM K^+ alone (Fig. 6). Therefore, the inhibitory effects of Mg^{2+} and Na^+ or K^+ on Complex II dissociation of guinea pig kidney cortex are also considered to be non-additive.

Fig. 7 shows that the inhibition curves for Na^+ and K^+ diverge as they do without Mg^{2+} in Fig. 6, possibly as a consequence of the higher affinity for K^+ than for Na^+ of the low-affinity binding sites. It is equally clear from this figure that Mg^{2+} does not abolish the high-affinity site effect in the curves for the alkali metal ions, as 100 mM Na^+ does in the curve for K^+ mentioned above. Therefore, the Mg^{2+} site is probably different from the high-affinity alkali cation site, like it is different from the low-affinity alkali cation site.

DISCUSSION

Species and tissue differences in response to cations

The results of this study definitely show that the activating cations of $(Na^+ + K^+)$-ATPase, Na^+, K^+ and Mg^{2+}, stabilize Complex II. This is particularly the case for kidney cortex, while brain and heart muscle respond much less. These results agree with findings by other authors for rat [12, 19], dog [11] and bovine brain [8, 18] and rabbit and bovine heart muscle [9]. They are at variance with results for dog and guinea pig kidney by Tobin et al. [11, 14], who report that Complex II dissociation rates are insensitive to alkali cations. However, careful examination of their results [14] for urea-treated guinea pig kidney microsomes shows that 16 mM K^+ or Na^+ exerts a 16 % decrease in the Complex II dissociation rate constant in the presence of 2 mM ATP, as compared to a 26 % decrease at the same cation concentration in Fig. 6. Furthermore, the presence of ATP may destabilize the complex [31] and thereby antagonize the stabilizing action of the alkali cations, so that they may appear to be ineffective.

At least two types of responses to the alkali cations emerge from our study. One response is that K^+ gives maximal inhibition at a lower concentration than Na^+ (rabbit brain and kidney cortex, and possibly bovine kidney cortex). For these tissues 50–150 mM Na^+ and 15–20 mM K^+ are saturating and yield the same maximal inhibition. Another response is that K^+ and Na^+ are almost equally effective, but at 150–200 mM they are still submaximally effective (guinea pig kidney cortex). There may even be a third type of response, in which 20 mM K^+ yields a higher inhibition than 200 mM Na^+, neither of the two being saturating (bovine brain cortex, rabbit heart muscle, and possibly rat brain cortex). Mg^{2+} inhibits the dissociation of Complex II of rabbit kidney cortex more than the alkali cations do (Fig. 2). The reverse is true for Complex II of guinea pig kidney cortex, which shows in addition a two-site alkali cation behaviour (Fig. 6). So the conclusion appears to be justified that differences in response to the cations depend on intrinsic species and tissue differences in the chemical composition or conformation of $(Na^+ + K^+)$-ATPase.

Effects of urea

However, the question arises of whether urea treatment of the kidney preparations has induced the sensitivity to the cations tested. Whittam and Chipperfield [32] found a two- to three-fold increase in ouabain binding capacity following urea treatment of ox brain microsomes. In addition to increasing the accessibility for ouabain, urea might also increase the number of available cation sites. Against a specific effect of urea would speak the results of Schönfeld et al. [16] for Complex II of pig heart: 22, 32 and 49 % inhibition of k_d by 4 mM Mg^{2+}, 4 mM Mg^{2+} + 80 mM Na^+ and 4 mM Mg^{2+} + 80 mM K^+, respectively. The pig heart enzyme has been prepared by NaI treatment, not involving the use of urea. In other NaI-treated heart preparations (rabbit and beef) [9] the dissociation of Complex II is not inhibited by 10 mM K^+. In our hands 20 mM K^+ gives 19 % inhibition of k_d for rabbit heart muscle (Table I). It appears that these differences between authors are due to differences in concentration, and possibly also to partial additivity of the effects of Mg^{2+} and Na^+ or K^+ for this kind of tissue.

Site specificity of the cations

Mg^{2+} may exert its stabilizing effect at sites different from the alkali metal ion sites for the following reasons. For rabbit kidney cortex Complex II there is no competition between Mg^{2+} and Na^+ or K^+ (Fig. 5). For guinea pig kidney cortex Na^+ and K^+ cause more inhibition than Mg^{2+} (Fig. 6), without competition by the latter cation (Fig. 7). However, a difference in site specificity for Na^+ and K^+ is less likely, since there is no additivity of the Na^+ and K^+ effects for rabbit kidney cortex (Figs 3 and 4) and for the high-affinity sites in guinea pig kidney. The only indication for different sites for Na^+ and K^+ comes from experiments in the presence or absence of Mg^{2+} where K^+ inhibits more than Na^+ (Fig. 7; Table 1: bovine brain cortex and rabbit heart muscle). A similar result has been obtained by Schönfeld et al. [16] for pig heart Complex II (see previous paragraph).

Mechanism of the cation effects; comparison with Complex I

Half-maximally inhibitory concentrations of Na^+ and K^+ for Complex I dissociation in dog and rat brain are 400 mM and 0.2–0.75 mM, respectively [11, 12, 19]. For Complex II dissociation (rabbit brain and kidney cortex) these concentrations are 30–40 mM and 6–10 mM, respectively. Thus half-maximally effective Na^+ concentrations for Complex II are lower and the corresponding K^+ concentrations are higher than for Complex I. In addition, Yoda and Yoda [31] report an antagonism between Na^+ and K^+ for Complex I dissociation, whereas we find no such antagonism for Complex II dissociation. In addition to the difference in stability, the above findings provide two more arguments for the supposition that the complexes E-Ouabain $\sim$ *P* and E-Ouabain-*P* are chemically different. The higher half-maximal K^+ concentrations for Complex II are in agreement with the finding of Erdmann and Schoner [18] that the dissociation constant of Complex II is less sensitive to K^+ than that of Complex I.

Another difference exists in the relation between the magnitude of k_d for the ouabain complex and the relative inhibition of the dissociation by alkali cations for different tissues. For Complex II there is no clear relationship, while the dissociation of Complex I for a number of tissues (dog kidney, beef and dog heart, calf, dog and rat brain and rabbit heart) representing a 20-fold range in k_d values displays a remarkably constant K^+-induced inhibition (mean, 81 %; S.D., 9 %) [8, 9, 11, 12]. This suggests that steric hindrance of ouabain release by occupation of the alkali cation sites may be partly or totally prevented in Complex II of the less sensitive tissues (brain cortex and rabbit heart muscle), owing to a state of phosphorylation different from that in Complex I. An alternative explanation might be that interaction between alkali cation and ouabain binding sites is different for different tissues, but that phosphorylation by ATP increases this interaction to the same extent.

The difference in stability of Complex I and II may be caused by binding of Na^+ and ATP to the first complex rather than by a different phosphorylation state. Indeed, binding of different ligands can destabilize ($Na^+ + K^+$)-ATPase-ouabain complexes formed under different conditions [19], and so can Na^+ plus ATP destabilize Complex II, the latter effect being reversible upon dilution and being antagonized by K^+ [31]. Thus, the inhibitory effect of K^+ on Complex I dissociation may be caused by displacement of Na^+ and ATP [33, 34]. However, the complex regains its original dissociation characteristics after K^+ treatment and a wash-out procedure

[19], which should remove Na^+ and ATP and hence their destabilizing effect (see above). The washed Complex I is again sensitive to inhibition by K^+ [19]. Therefore, we conclude that the stabilizing effect of K^+ on Complex I is probably not due to removal of Na^+ and ATP and that there is an intrinsic stability difference between Complexes II and I, which is not due to binding of destabilizing ligands.

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