Biochimica et Biophysica Acta, 553 (1979) 495—500

BBA Report

BBA 71389

SIDEDNESS OF THE ATP-Na^+-K^+ INTERACTIONS WITH THE Na^+ PUMP IN SQUID AXONS

L.A. BEAUGÉ and R. DiPOLO*

Department of Biophysics, University of Maryland School of Medicine, Baltimore, MD 21201 (U.S.A.)

(Received March 12th, 1979)

Key words: ($Na^+ + K^+$)-ATPase; Ion concentration regulation; Enzyme conformation; (Squid axon)

Summary

Using dialysed squid axons we have been able to control internal and external ionic compositions under conditions in which most of the Na^+ efflux goes through the Na^+ pump. We found that (i) internal K^+ had a strong inhibitory effect on Na^+ efflux; this effect was antagonized by ATP, with low affinity, and by internal Na^+, (ii) a reduction in ATP levels from 3 mM to 50 μM greatly increased the apparent affinity for external K^+, but reduced its effectiveness compared with other monovalent cations, as an activator of Na^+ efflux, and (iii) the relative effectiveness of different K^+ congeners as external activator of the Na^+ efflux, though affected by the ATP concentration, was not affected by the Na^+/K^+ ratio inside the cells. These results are consistent with the idea that the same conformation of the ($Na^+ + K^+$)-ATPase can be reached by interaction with external K^+ after phosphorylation and with internal K^+ before rephosphorylation. They also stress a nonphosphorylating regulatory role of ATP.

The ($Na^+ + K^+$)-ATPase can interact with K^+ in the absence of phosphorylation leading to an enzyme conformation (EK) with reduced ATP affinity [1]. This K^+-enzyme interaction can also be detected by changes in the patterns of *N*-ethylmaleimide inhibition [2] and trypsin inactivation [3] of the enzyme. The K^+ effects are antagonized by ATP, acting with low affinity, and also by Na^+. The apparent K^+ and Na^+ affinities suggest that both cations act at intracelllular sites [2,3]. These K^+ effects are presumably different from others taking place after phosphorylation, where K^+ acting at extracel-

*Permanent address: Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela.

lular sites produces an enzyme conformation in which K^+ is thought to be occluded. The release of K^+ from this occluded complex $E_2(K)$ is accelerated by ATP, acting also with low affinity, but seems to be insensitive to Na^+ [4]. In determining the sidedness of the interaction of ligands with the Na^+ pump in intact cells it is necessary to be able to control both the intra and extracellular medium at the same time; in this regard the dialysed squid axon becomes an ideal preparation.

The efflux of $^{22}Na^+$ was followed in axons whose internal cation composition and ATP content were controlled by the dialysis technique described in detail in Refs. 5 and 6. Excellent washout of ATP and other nucleotides was accomplished with glass porous capillaries without the use of metabolic inhibitors. In addition to being free of ADP and P_i the dialysis solutions contained 5 mM phosphoarginine to convert any endogenous ADP to ATP thus reducing to a minimum the Na^+-Na^+ exchange fluxes through the Na^+ pump. All dialysates were also free of Ca^{2+} and contained 1 mM EGTA in order to inhibit the Na^+-Ca^{2+} exchange mechanism [7,8]. The reasonable expectation was, then, that almost all Na^+ efflux in these axons would go through the Na^+ pump, either as Na^+-K^+ exchange or in the uncoupled mode. At high ATP concentrations, some Na^+-Mg^{2+} exchange component would still have been present [9,10].

The experiments described in the legends to Figs. 1 and 3 and those summarized in Table I show that, in the presence of 70 mM internal Na^+, the removal of potassium from the dialysis solution produced almost no change in Na^+ efflux when the ATP concentration was 3 mM, but greatly increased the efflux of Na^+ if the ATP concentration was 50 μM. The ratio (total Na^+ efflux at 0 K_i^+)/(total Na^+ efflux at 310 mM K_i^+) averaged 1.17 ± 0.06 with 3 mM ATP, and 2.40 ± 0.23 with 50 μM ATP. The pattern of results was similar if one considers only the ATP-dependent Na^+ efflux. It is conceivable that the stimulation of Na^+ efflux by removing internal K^+ is not directly related to K_i^+ but to the accompanying membrane depolarization. As the Na^+ pump has been shown insensitive to membrane potential in axons with normal ATP con-

TABLE I

EFFECTS OF INTERNAL K^+ ON THE Na^+ EFFLUX IN AXONS DIALYSED WITH LOW AND HIGH ATP CONCENTRATIONS

The general experimental procedure, including ATP depletion and repletion, is described in detail in the legend to Fig. 1. To obtain the ATP-dependent Na^+ efflux the average values of efflux in the absence of ATP in the dialysate were subtracted from the individual values in the presence of 50 μM or 3 mM ATP. The entries represent the mean plus or minus the standard error of the mean of each group.

Dialysate (mM)		Na^+ efflux			Number of axons
ATP	K^+	Total (pmol · cm^{-2} · s^{-1})	ATP dependent pmol · cm^{-2} · s^{-1}	0 K^+/310 K^+	
0	310	0.78 ± 0.07	—	—	13
0	0	3.30 ± 0.32	—	—	4
0.05	310	4.87 ± 0.60	4.09 ± 0.62*	2.02 ± 0.26***	6
0.05	0	11.67 ± 1.78	8.37 ± 1.82*		
3	310	29.0 ± 3.6	28.2 ± 3.6**	1.08 ± 0.06***	3
3	0	33.7 ± 3.9	30.4 ± 3.9**		3

*$P < 0.05$.
**Non significant.
***$P < 0.01$.

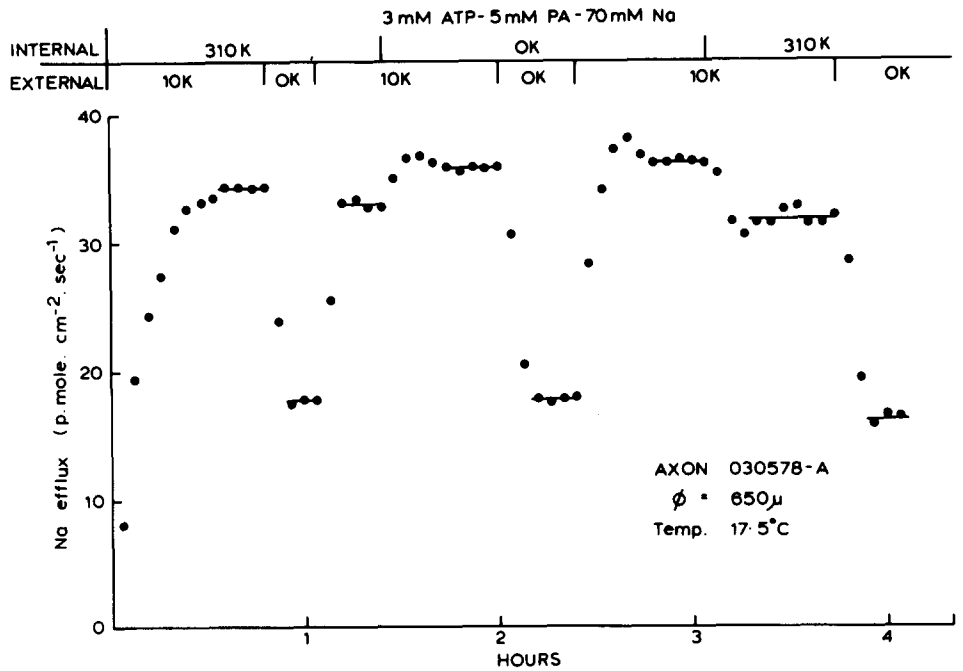

Fig. 1. The effects of removal of internal K^+ on the Na^+ efflux in an axon dialysed with 3 mM ATP. The composition of the dialysis solutions was (mM): Na^+ 70, K^+ 0 or 310, Mg^{2+} 4 in excess of the ATP concentration; Tris 5 (in K^+-containing solutions) or 315 (in K^+-free solutions); Cl^-, 83; aspartate$^-$, 310; EGTA, 1; glycine 330. The osmolarity was 980 mosM. and the pH (20° C) 7.1. ATP was obtained from Boehringer and phosphoarginine (PA) from Calbiochem. Both were neutralized to pH 7.1 with Tris-OH (ATP) or -HCl (PA) and stored at −80° C as 250 mM solutions. The ATP solution also contained 250 mM $MgCl_2$. The composition of the standard artificial sea water was as follows (mM): Na^+ 440; K^+ 10; Mg^{2+} 50; Ca^{2+} 10; Tris 10; Cl^- 580; EDTA 0.1. The osmolarity was 1050 and the pH (20° C) 7.6. The removal of external K^+ was made without change of the other constituents. At zero time the dialysis began with the indicated solutions plus radioactive Na^+. The initial apparent rise in Na^+ efflux does not represent a real flux but the time taken to reach steady-state distribution of the isotope. The flow of dialysis solution was 1 μl/min and the flow of external solutions about 1 ml/min. More details about the dialysis technique can be found in Refs. 5 and 12.

tent [6], this would mean that the rate of pumping becomes sensitive to membrane polarization when the ATP concentration is reduced. However, other experiments (not shown) indicated that reducing internal Na^+ from 70 to 10 mM (Na^+ being replaced with choline) greatly increased the sensitivity of Na^+ efflux to internal K^+, and with low Na_i^+ even axons dialysed with 3 mM ATP showed a noticeable increment in Na^+ efflux when K_i^+ was removed. This suggests that the basis for that effect is more likely to be a Na^+-K^+ antagonism (which is increased at low ATP) than a change in the membrane potential.

When the dialysates were free of ATP the removal of K_i^+ also resulted in a large increase in the efflux of Na^+ (Table I). The efflux of Na^+ in axons dialysed with no ATP might be entirely passive 'leak' [5]; as such, a reduction in K_i^+ with the resulting membrane depolarization would be expected to increase it. On the other hand, it could be that under these conditions there is enough ATP in the vicinity of the membrane to drive some Na^+ efflux through the Na^+ pump. If this were the case, from the above results the flux would be expected to be highly sensitive to inhibition by K_i^+. Unfortunately

cardiac glycosides cannot be used to test whether the Na^+ efflux goes through the pump because they are known to promote Na^+ efflux in axons nominally free of ATP [11].

Fig. 2 shows the increase in the apparent affinity for external K^+ as an activator of the Na^+ efflux when the ATP concentration is decreased. With 5 mM ATP complete saturation was not attained even at 50 mM K^+, whereas with 50 μM ATP 10 mM K^+ showed almost full activation. Taking the Na^+ efflux at 50 mM K^+ as 100% (insert in Fig. 2) the $K_{1/2}$ for potassium becomes about 8 mM at 5 mM ATP and about 1 mM at 50 μM ATP.

It has been shown previously in squid axons [12] that when ATP is reduced there is an increased effectiveness of external NH_4^+ over K^+ and Rb^+, and of K^+ over Rb^+ in activating Na^+ efflux. These effects were similar to those described for the levels of phosphoenzyme [4] and are consistent with the hypothesis that an ATP-sensitive occluded conformation of the Na^+ pump is formed after dephosphorylation. The experiment of Fig. 3 indicates that internal K^+ (or the Na^+/K^+ ratio for that matter) does not influence the relative effectiveness of external cations in activating the Na^+ pump. With 50 μM ATP, and in agreement with previous work, at 10 mM concentration external NH_4^+ was twice as effective as K^+ in promoting Na^+ efflux above the K^+-free levels, whereas the addition of Rb^+ did not produce any detectable stimulation. The removal of K_i^+ produced a general increase in the levels of Na^+ efflux but the relative effectiveness of the three forementioned cations did not change at all.

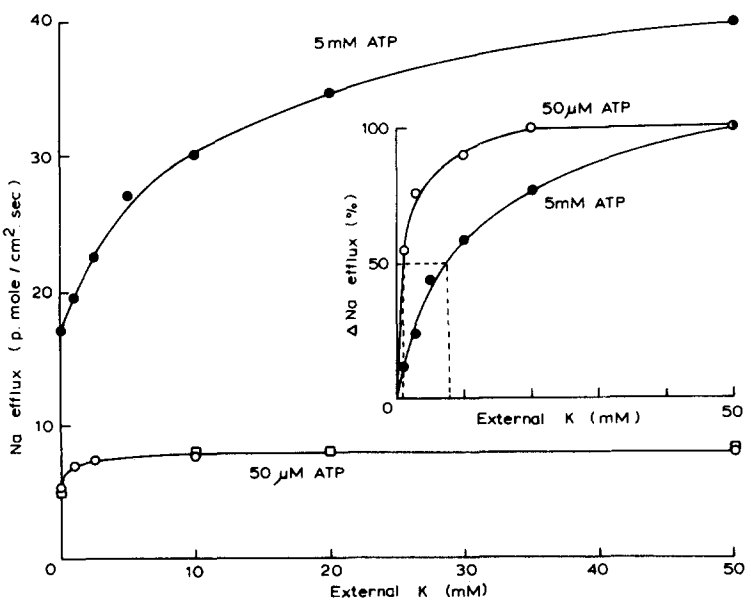

Fig. 2. Activation curve of Na^+ efflux by external K^+ in axons dialysed with 5 mM or 50 μM ATP, together in both cases with 5 mM phosphoarginine. The typical experimental protocol was to wash out the ATP by previous dialysis with a solution free of ATP. Upon the addition of ATP at the specified concentrations, and once a steady Na^+ efflux was obtained, the effect of different K^+ concentrations in the sea water was assayed. The filled circles represent an axon dialysed with 5 mM ATP. The open circles and squares are two axons where the internal ATP was 50 μM. In the insert the effects of K^+ at 5 mM ATP, and the averaged effects at 50 μM ATP are expressed as percentage increments in Na^+ efflux over the levels of flux in solutions nominally free of K^+. Temperature was 17.5° C.

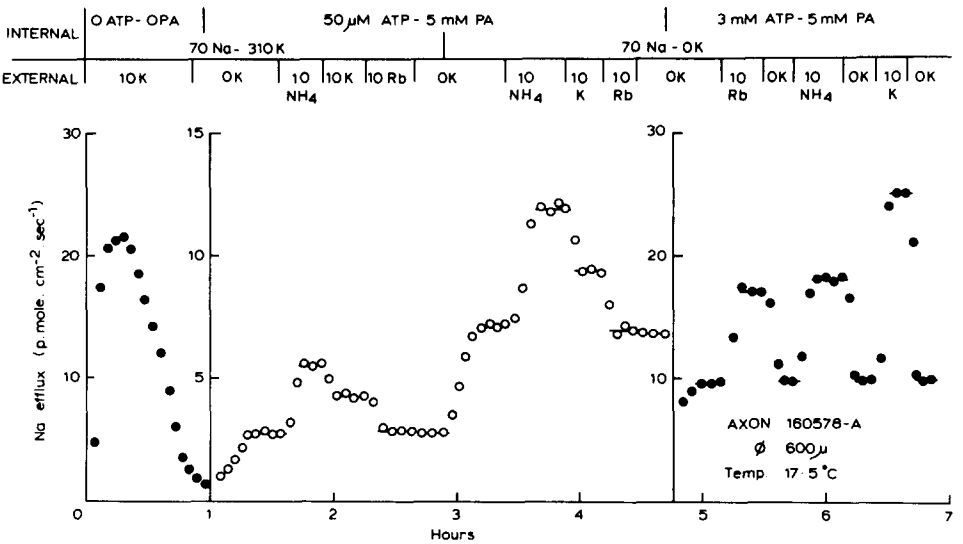

Fig. 3. The effects of internal K^+ at different internal ATP concentrations on the external cation activation of Na^+ efflux in a dialysed squid axon. The general dialysis technique is the same as that described in the legend to Fig. 1 and in Refs. 5 and 12.

When the ATP concentration was increased to 3 mM a pattern of response was obtained similar to that in axons dialysed with normal ATP and K^+ concentrations.

The interactions of external K^+ with the Na^+ pump resemble those with the $(Na^+ + K^+)$-ATPase leading to the occluded $E_2(K)$ conformation [4,12], whereas the interactions of internal K^+ with the Na^+ pump can be explained on the basis of the effects of K^+ on the $(Na^+ + K^+)$-ATPase in the absence of phosphorylation [1,2,3]. The present results agree with the sequence of events proposed by Karlish et al. [13], namely

$$K_o^+ + E_2P \rightleftharpoons E_2PK \rightleftharpoons E_2(K) \rightleftharpoons E_1K \rightleftharpoons E_1 + K_i^+$$

where $E_2(K)$ can be formed by the action of K^+ from inside or outside the cell. ATP, binding to $E_2(K)$ with low affinity, shifts the $E_2(K) \rightleftharpoons E_1K$ equilibrium to the right (with or without Na_i^+) and Na_i^+ competes with K^+ for E_1.

A one hundred-fold reduction in the ATP concentration increased the apparent affinity for external K^+ about 8-fold (Fig. 2). The increased K_o^+ affinity can be explained by the scheme described above, for upon reducing the ATP concentration the rate limiting step is shifted from the K_o^+-promoted dephosphorylation of the enzyme to the release of K^+ from the $E_2(K)$ complex. An increase in the apparent affinity for external K^+ at low ATP has also been reported by Skou [2] for the $(Na^+ + K^+)$-ATPase activity of ox brain enzyme. These results suggest that the invariance with changing ATP concentration of the apparent affinity for external K^+, which has been used as an argument against consecutive models for the Na^+ pump [14], does not hold under all conditions.

We wish to thank the Director and staff of the Marine Biological Laboratory, Woods Hole, for the facilities put at our disposal. This work was supported by US NSF Grant BNS 76-81050 to L.A.B.

References

1 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104—116
2 Skou, J.S. (1975) Q. Rev. Biophys. 7, 401—434
3 Jørgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399—415
4 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530—6540
5 Brinley, F.J. and Mullins, L.J. (1967) J. Gen. Physiol. 50, 2303—2331
6 Brinley, F.J. and Mullins, L.J. (1968) J. Gen. Physiol. 52, 181—211
7 Baker, P.F. (1970) in Calcium and Cellular Function (Cuthbert, A.W., ed.), pp. 96—107, Macmillan
8 DiPolo, R. (1978) J. Gen. Physiol. 73, 91—113
9 Baker, P.F. and Crawford, A.C. (1972) J. Physiol. (Lond.) 227, 855—874
10 De Weer, P. (1976) J. Gen. Physiol. 68, 159—178
11 Beaugé, L.A. and Mullins, L.J. (1976) Proc. R. Soc. B 194, 279—284
12 Beaugé, L.A. and DiPolo, R. (1978) Nature 271, 777—778
13 Karlish, S.J.D., Yates, D.W. and Glynn, I.M. (1978) Biochim. Biophys. Acta 525, 252—264
14 Garay, R. and Garrahan, P.J. (1975) J. Physiol. 249, 51—67