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Constitutive activation of gastric H^+,K^+-ATPase by a single mutation

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In the reaction cycle of P-type ATPases, an acid-stable phosphorylated intermediate is formed which is present in an intracellularly located domain of the membrane-bound enzymes. In some of these ATPases, such as Na^+,K^+-ATPase and gastric H^+,K^+-ATPase, extracellular K^+ ions stimulate the rate of dephosphorylation of this phosphorylated intermediate and so stimulate the ATPase activity. The mechanism by which extracellular K^+ ions stimulate the dephosphorylation process is unresolved. Here we show that three mutants of gastric H^+,K^+-ATPase lacking a negative charge on residue 820, located in transmembrane segment six of the α-subunit, have a high SCH 28080-sensitive, but K^+-insensitive ATPase activity. This high activity is caused by an increased 'spontaneous' rate of dephosphorylation of the phosphorylated intermediate. A mutant with an aspartic acid instead of a glutamic acid residue in position 820 showed hardly any ATPase activity in the absence of K^+, but K^+ ions stimulated ATPase activity and the dephosphorylation process. These findings indicate that the negative charge normally present on residue 820 inhibits the dephosphorylation process. K^+ ions do not stimulate dephosphorylation of the phosphorylated intermediate directly, but act by neutralizing the inhibitory effect of a negative charge in the membrane.

Keywords: ATPase/baculovirus expression system/cation-binding domain/H^+,K^+-ATPase/phosphorylation

Introduction

P-type ATPases (Pedersen and Carafoli, 1987) are responsible for active ion transport across membranes. During their catalytic cycle, they become phosphorylated by ATP on a conserved aspartyl residue present in a large intracellular loop of the catalytic subunit. Some of these ion pumps, such as Na^+,K^+-ATPase and gastric H^+,K^+-ATPase, need extracellular K^+ for their ATPase activity and transmembrane ion transport (Klaassen and De Pont, 1994; Møller *et al.*, 1996). Extracellular K^+ ions act on the ATPase activity by stimulating the rate of hydrolysis of the phosphorylated intermediate. During the catalytic cycle, K^+ ions become temporarily occluded within the transmembrane segments (Beaugé and Glynn, 1979; Capasso *et al.*, 1992; Rabon *et al.*, 1993). However. the mechanism by which occluded K^+ ions stimulate dephosphorylation of the phosphorylated intermediate within the large intracellular loop is still not clear. There are strong indications that polar and in particular negatively charged residues, present in transmembrane domains, are involved in cation binding in several P-type ATPases (Clarke *et al.*, 1989; Adebayo *et al.*, 1995; Kuntzweiler *et al.*, 1996; Rice and MacLennan, 1996; Pedersen *et al.*, 1997).

Because of the possibility that similar negatively charged residues play such a role in gastric H^+,K^+-ATPase, a number of mutants of the rat enzyme were prepared with the aid of the baculovirus expression system (Swarts *et al.*, 1996). In these mutants, several carboxyl residues in and around transmembrane segments five and six of the catalytic subunit were replaced by their corresponding acid amide residues. One of these mutants, in which Glu820 had been replaced by a Gln (E820Q), could normally be phosphorylated by ATP, but showed no K^+-stimulated ATPase activity. K^+ ions also had no effect on the rate of dephosphorylation of the phosphorylated intermediate, indicating that this mutant had lost its K^+ sensitivity. In order to obtain a better insight into the function of this glutamic acid residue, some other mutants of Glu820 were investigated (Hermsen *et al.*, 1998). A negative charge at position 820 was shown to be sufficient for K^+-stimulated ATPase activity, but mutants with a neutral or a positive residue at this site had lost the K^+ sensitivity of both the ATPase and the dephosphorylation reaction. Mutant E820A was an exception to this rule in the sense that it did show some K^+-stimulated ATPase activity, but only with a very low affinity for K^+, as was also found by Asano *et al.* (1997), after expression of pig H^+,K^+-ATPase in HEK-293 cells. In agreement with this, we found that the rate of dephosphorylation of the phosphorylated intermediate could be stimulated slightly by 100 mM K^+. Lower K^+ concentrations (1 and 10 mM K^+) had no effect. In this study (Hermsen *et al.*, 1998), we also showed that the stimulatory effect of K^+ on the ATPase activity of both the wild-type enzyme and the E820D mutant was completely inhibited by the specific gastric H^+,K^+-ATPase inhibitor SCH 28080 [2-methyl,8-methyl-(phenylmethoxy)imidazo(1,2-a) pyridine 3-acetonitrile] (Wallmark *et al.*, 1987; Keeling *et al.*, 1989). The inhibitor was not used with the other mutants since we assumed that the measured ATPase activity in these mutants was an endogenous property of the Sf9 cell membranes.

Recently, the incubation conditions for culturing *Spodoptera frugiperda* (Sf9) insect cells in our laboratory were slightly modified, leading to significantly higher

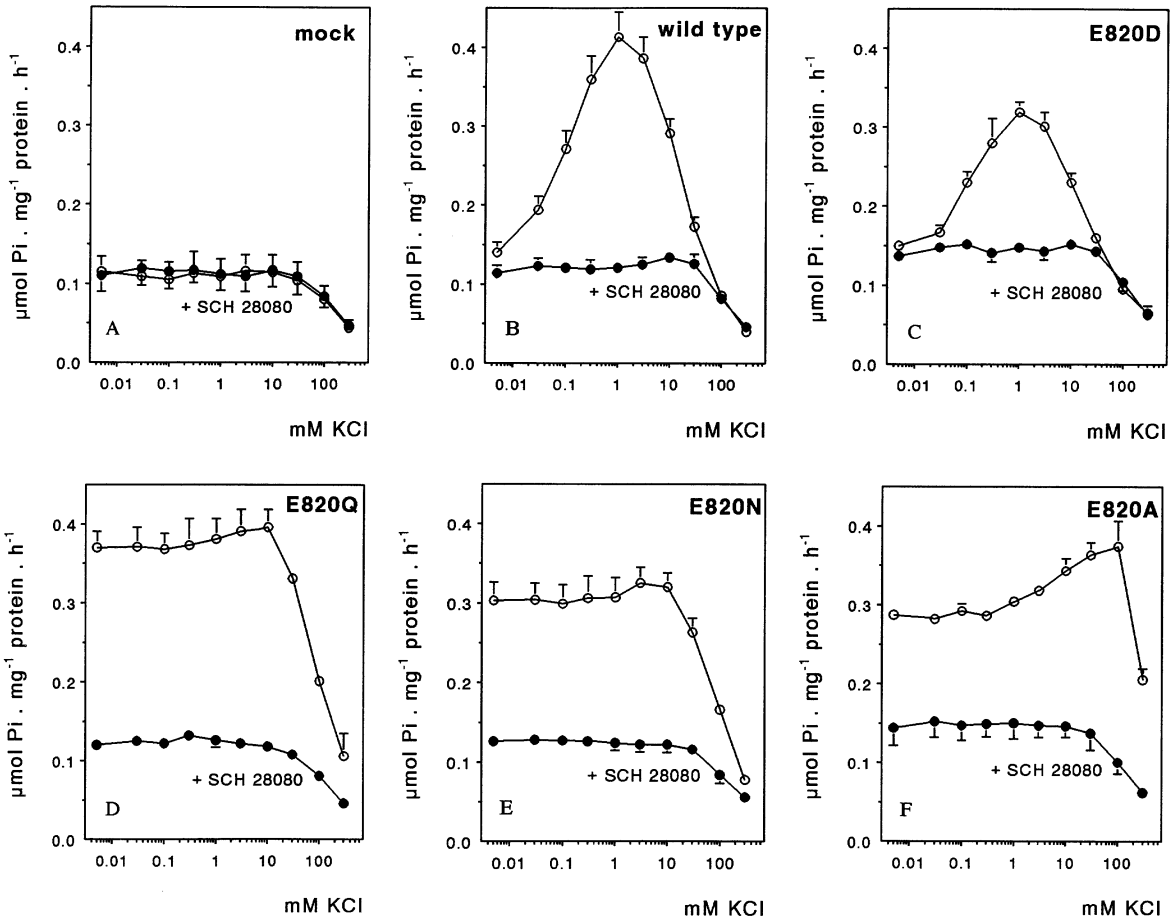

Fig. 1. Effect of K^+ and SCH 28080 on the ATPase activity of membranes from mock-infected cells (**A**), wild-type enzyme (**B**) and the E820D (**C**), E820Q (**D**), E820N (**E**) and E820A (**F**) mutants. Membranes were prepared and the ATPase activity in the presence of the indicated K^+ concentration was determined as described in Materials and methods. ATPase activity in the absence (○) and presence (●) of 100 µM SCH 28080. Mean values ± SE are given for three independent membrane preparations.

ATPase activities in membrane preparations of these cells. We noticed that in the absence of K^+ the ATPase activity of the E820A, E820Q and E820N mutants was at least double that of the wild-type enzyme and the E820D mutant. The latter's ATPase activities were similar to that of mock-infected cells. To test the possibility that the high basal ATPase activity of these three mutants was caused by the expressed H^+,K^+-ATPase and not by variations in the expression of endogenous ATPases, the effect of SCH 28080 was measured. We found that this compound reduced the basal ATPase activity of these three mutants back to the level of mock-infected cells, suggesting that these three mutants showed constitutive ATPase activity. This completely unexpected finding has important consequences for our understanding of the mechanism of action of K^+-sensitive P-type ATPases.

Results

ATPase activity

When gastric H^+,K^+-ATPase is expressed by recombinant baculoviruses in Sf9 cells, the ATPase activity present in isolated membranes of these cells originates both from the expressed H^+,K^+-ATPase and from endogenous ATPases present in these membranes (Swarts *et al.*, 1996). In order to measure K^+-stimulated ATPase activity, we used a suboptimal (10 µM) ATP concentration since at higher ATP concentrations the endogenous activity increased relatively more than the H^+,K^+-ATPase activity. In mock-infected cells, the ATPase activity was ~0.12 ± 0.02 (n = 3) µmol/mg protein/h and was not affected by K^+ up to 30 mM (Figure 1A). With higher K^+ concentrations, the activity slightly decreased [0.08 ± 0.02 (n = 3) µmol/mg protein/h at 100 mM K^+], which is probably due to a non-specific ionic strength effect. Addition of 100 µM of the specific H^+,K^+-ATPase inhibitor SCH 28080 had no effect on the ATPase activity at any of the K^+ concentrations used. The ATPase activity of the wild-type enzyme in the absence of K^+ (Figure 1B) was similar to that of mock-infected cells. This ATPase activity could be stimulated by K^+, reaching a maximal activity of 0.41 ± 0.03 (n = 3) µmol/mg protein/h at 1 mM K^+. At higher K^+ concentrations, the ATPase activity decreased again, which is probably due to a conversion of the enzyme to the E_2-K^+ form, a process which could not be reversed by the low (10 µM) ATP concentration used in these experiments (Swarts *et al.*, 1995). The increase in activity due to addition of K^+ could be completely inhibited by 100 µM SCH 28080. Similar results were obtained with the charge-conserving E820D mutant (Figure 1C).

The E820Q (Figure 1D), E820N (Figure 1E) and E820A

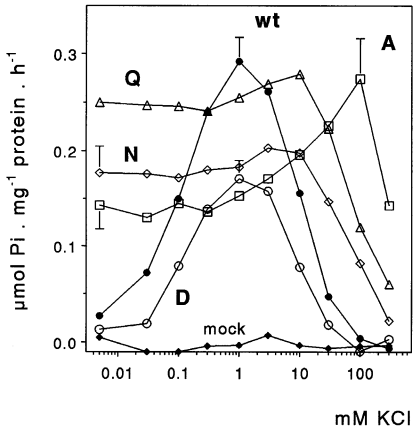

Fig. 2. K^+ dependence of SCH 28080-sensitive ATPase activity of (mutant) gastric H^+,K^+-ATPase. The difference in the ATPase activity measured in the experiments of Figure 1 with and without 100 µM SCH 28080 is given as function of the K^+ concentration. The following symbols are used; wild-type enzyme (wt; ●); E820D mutant (D; ○); E820Q mutant (Q; △); E820N mutant (N; ◇); E820A mutant (A; □). Mean values are given for three independent membrane preparations. Bars representing SE are for clarity only given in the absence of added K^+ and at the K^+ concentrations giving a maximal response.

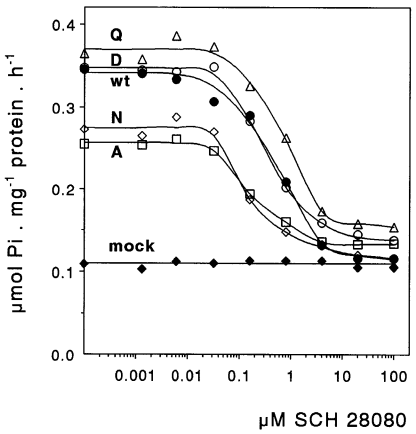

Fig. 3. Dose–inhibition curves for SCH 28080 on the ATPase activity of (mutant) gastric H^+,K^+-ATPase. Symbols are as in Figure 2. ATPase activity was measured with 1 mM K^+ in the presence of SCH 28080 at the indicated concentrations. ATPase activity was measured for 30 min as described in Materials and methods. The figure shows a representative experiment from two preparations.

(Figure 1F) mutants showed an ATPase activity in the absence of K^+ between 0.29 and 0.37 µmol/mg protein/h. Addition of K^+ had no stimulatory effect on the ATPase activity of the two mutants with an acid amide residue on position 820 (E820Q and E820N), whereas the ATPase activity of the E820A mutant at K^+ concentrations between 10 and 100 mM was stimulated. At higher K^+ concentrations, the ATPase activities of all preparations decreased again. In the presence of 100 µM SCH 28080, the ATPase activity of these mutants was also reduced to the level of the mock-infected cells. The E820K and E820L mutants showed no ATPase activity above that of mock-infected cells and there was no inhibition by SCH 28080. These mutants were not studied further.

In Figure 2, the SCH 28080-sensitive ATPase activity is given as a function of the K^+ concentration for the wild-type enzyme and for the four mutants. The wild-type enzyme as well as the E820D mutant show a bell-shaped K^+-dependence, with a maximal stimulation at 1 mM. The maximal activity of the E820D mutant is 60% of that of the wild-type enzyme. The figure shows that at 10 µM ATP, the constitutive ATPase activity of the three mutants without a negative charge is 50–90% of the maximal activity of the wild-type enzyme. The maximal activity obtained with the E820A mutant is about twice its constitutive activity.

To exclude the possibility that the inhibitory effect of SCH 28080 is due to an non-specific 'drug' effect, dose–inhibition curves for SCH 28080 were determined for the wild-type enzyme and the mutants at 1 mM K^+ (Figure 3). The ATPase activity of the wild-type enzyme and the K^+-sensitive E820D mutant is maximal at this K^+ concentration and that of the other three mutants is similar to that in the absence of K^+ (Figure 2). All mutants as well as the wild-type enzyme had an IC_{50} value for SCH 28080 between 0.2 and 0.7 µM, which is rather similar to that of pig gastric H^+,K^+-ATPase (0.8 µM; Van der Hijden *et al.*, 1991).

Phosphorylation and dephosphorylation

Because of the high constitutive ATPase activity of the three mutants without a negative charge on residue 820, one would expect that they should show a high spontaneous dephosphorylation rate. We therefore phosphorylated the five preparations with 0.1 µM ATP at pH 6.0 and 0°C as described previously (Klaassen *et al.*, 1993; Swarts *et al.*, 1996). These conditions had been chosen since the endogenous phosphorylation of the membranes of the Sf9 cells is relatively low under these conditions. Table I shows that at 0°C all mutants yielded an acid-stable phosphorylated intermediate, but the phosphorylation level of the various preparations, although higher than that of the mock-infected cells, varied considerably. The dephosphorylation rate of these preparations was measured as the reduction in the level of phosphorylated intermediate over 3 s (after correction for phosphorylation levels in mock-infected cells) and was found to be rather similar for all preparations (Figure 4A). K^+ (1 mM) stimulated dephosphorylation of the wild-type enzyme and the E820D mutant, but had no effect on the phosphorylation level of the other mutants after 3 s (Figure 4B).

Since these results were difficult to match with those of the ATPase activity (Figure 2), we compared the conditions for ATPase activity and dephosphorylation measurements. One of the differences is that the ATPase activity was measured at 37°C and the (de)phosphorylation process at 0°C. We therefore increased the temperature for measuring phosphorylation and dephosphorylation to 21°C. At an even higher temperature, the dephosphorylation process would be too fast to be measured manually.

Table I shows that at this temperature the steady-state phosphorylation level was higher than at 0°C, in particular for those preparations (E820D, E820Q) which had a relatively low phosphorylation level at 0°C. This suggests that at 0°C the steady-state phosphorylation level has not been reached for all mutants. At 21°C, the dephosphorylation rate of the mutants with a high constitutive ATPase activity was indeed markedly increased, whereas the dephosphorylation rate for the preparations with a negative charge at position 820 was still relatively low (Figure

Table I. Phosphorylation level of recombinant gastric H^+,K^+-ATPase and its mutants at 0 and 21°C

	0°C		21°C	
		Minus mock-infected level		Minus mock-infected level
Mock-infected	0.58 ± 0.10	–	1.30 ± 0.22	–
Wild-type	4.19 ± 0.16	3.61 ± 0.19	5.73 ± 0.18	4.43 ± 0.28[a]
E820D	1.63 ± 0.03	1.05 ± 0.11	4.83 ± 0.14	3.53 ± 0.26[b]
E820Q	2.81 ± 0.03	2.23 ± 0.11	4.63 ± 0.03	3.33 ± 0.22[b]
E820N	4.19 ± 0.66	3.61 ± 0.67	6.12 ± 0.92	4.82 ± 0.95
E820A	6.33 ± 1.27	5.75 ± 1.27	6.81 ± 1.33	5.51 ± 1.35

Phosphorylation was carried out as described in Materials and methods both at 0 and 21°C. The phosphorylation level is expressed as pmol/mg protein and is given as the mean ± SE for three independent preparations.
[a]$P < 0.05$; [b]$P < 0.01$ comparison with 0°C.

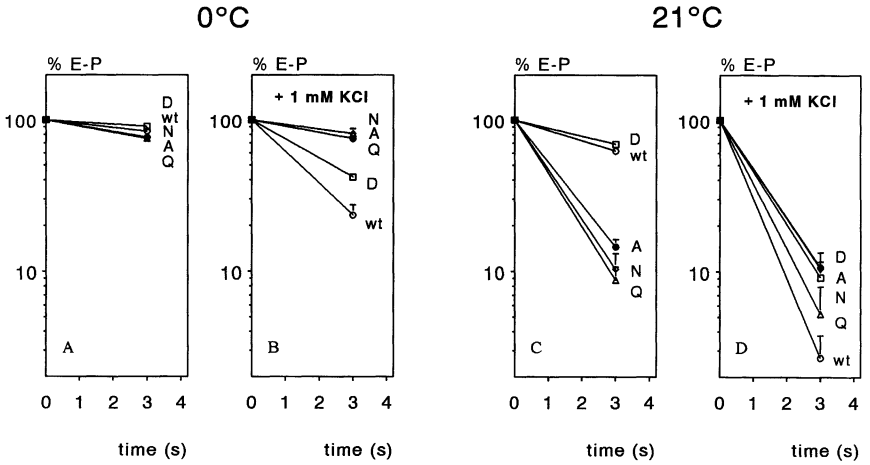

Fig. 4. Dephosphorylation of phosphorylated intermediate of the wild-type enzyme and the E820 mutants. Symbols are as in Figure 3. The membrane preparations were phosphorylated for 10 s with 0.1 μM radioactive ATP at either 0°C (**A** and **B**) or 21°C (**C** and **D**) as described in Materials and methods. Dephosphorylation was started by addition of excess non-radioactive ATP with (B and D) or without (A and C) 1 mM K^+. Samples were taken immediately before dephosphorylation and 3 s after the start of the dephosphorylation step. In parallel experiments, membranes of mock-infected Sf9 cells were phosphorylated and dephosphorylated, and the results obtained with the wild-type enzyme and the mutants were corrected for those of mock-infected cells. The residual phosphorylation level was expressed as a percentage of the phosphorylation level before the start of the dephosphorylation step (values are given in Table I) and is given as mean ± SE for three preparations.

4C). In the latter two preparations, 1 mM K^+ stimulated the dephosphorylation rate up to the level of the basal dephosphorylation rate of the three mutants with a neutral charge on residue 820 (Figure 4D).

In the experiment described in Figure 4, SCH 28080 could not be used since this drug does not completely inhibit ATP phosphorylation of some of the mutants (Swarts *et al.*, 1996). To be sure that the results given above are due to phosphorylation of H^+,K^+-ATPase, the phosphorylation state of the 100 kDa band was measured after separation on SDS–PAGE. The autoradiogram of Figure 5 shows that the wild-type enzyme and all the mutants indeed gave a 100 kDa phosphorylated band. Dephosphorylation for 3 s at 21°C in the absence of K^+ preserved the phosphorylated intermediate of 100 kDa for the wild-type enzyme and the E820D mutant, whereas the phosphorylated band disappeared completely when K^+ was present during the dephosphorylation period. Incubation of the mutants without a negative charge at position 820 resulted, both in the absence and presence of K^+, in an almost complete disappearance of the phosphorylated intermediate in the 3 s period.

It is theoretically possible that the mutants with an apparent intrinsic activity in the absence of added K^+

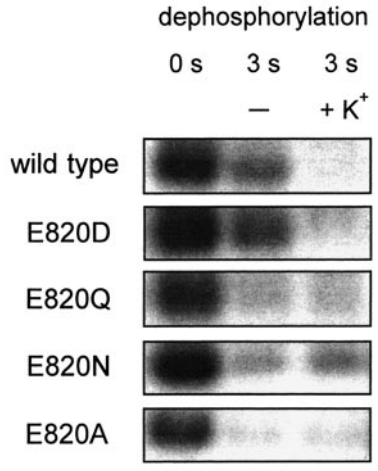

Fig. 5. Phosphorylation status of the 100 kDa band of gastric H^+,K^+-ATPase and its mutants after 0 (left lane) and 3 s dephosphorylation (middle and right lanes) both with (right lane) and without (middle lane) 1 mM K^+ at 21°C. ATP phosphorylation and dephosphorylation, followed by SDS–PAGE, was carried out as described in Materials and methods.

have an extremely high affinity for K^+, since the K^+ concentration in the incubation media is always of the order of 5 μM (Swarts *et al.*, 1996). We therefore measured the dephosphorylation reaction at 21°C in the presence of 50 mM triallylamine which decreases the affinity of H^+,K^+-ATPase for K^+ by a factor of 100 (Swarts *et al.*, 1994). Whereas the effect of K^+ on the dephosphorylation process of the wild-type enzyme was reduced, similarly to the effect observed by lowering the temperature to 0°C, no effect of K^+ on the dephosphorylation process of the E820Q mutant was observed (not shown), excluding the possibility that this mutant had a K^+ affinity in the micromolar range.

Discussion

The results presented here indicate that elimination of the negative charge of Glu820, present in transmembrane segment six of the catalytic subunit of gastric H^+,K^+-ATPase, results in a constitutively active ATPase. The K^+-insensitive ATPase activity in these mutants is explained by the high basal dephosphorylation rate of the phosphorylated intermediate. The low ATPase activity in the absence of K^+ of the wild-type enzyme might be due to an inhibitory effect of the negatively charged Glu820 on the phosphorylated intermediate, so preventing its hydrolysis.

The hydrolysis of the phosphorylated intermediate of the native enzyme can be enhanced by extracellular K^+. The ion is assumed to bind in a specific binding pocket in the membrane, whereupon it is released at the cytosolic side. Binding of this cation to its binding pocket results in an enhanced hydrolysis of the phosphorylated intermediate. It probably does so by inducing a conformational change resulting in a change in the environment of the aspartyl–phosphate bond. There are several indications that binding of K^+ to its binding pocket in the membrane has an effect on the conformation of the large intracellular loop between the transmembrane segments four and five, in which K^+ binding and phosphorylation occur. A readily accessible tryptic digestion site at Lys668 is no longer available in the presence of K^+, whereas a cleavage site at Arg455 is exposed (Helmich-de Jong *et al.*, 1987; Munson *et al.*, 1991; Van Uem *et al.*, 1991). Fluorescence by fluorescein isothiocyanate (Jackson *et al.*, 1983) and eosin (Helmich-de Jong *et al.*, 1986), both of which agents interact with the intracellular loop, is affected by K^+. The binding of the monoclonal antibody 5B6 to this loop is also affected by K^+ (Van Uem *et al.*, 1990, 1991). The conformational change in the loop could have an effect on the accessibility of the aspartyl–phosphate bond for water. De Meis (1989) has shown for both Ca^{2+}-ATPase and Na^+,K^+-ATPase that the opposite reaction (formation of a phosphorylated intermediate from inorganic phosphate) is facilitated when a more hydrophobic environment is created.

The present study shows that the hydrolysis of the phosphorylated intermediate can also be enhanced when the negative charge of residue 820 is eliminated. When the negative charge is retained, no enhanced hydrolysis occurs. This suggest that by removal of the negative charge a similar conformational change occurs as by binding of K^+ to its binding pocket. Moreover, by removal of the negative charge from residue 820, the affinity for K^+ is no longer measurable (E820Q, E820N) or markedly reduced (E820A). This strongly suggests that Glu820 is involved in K^+ binding and that this residue plays a role in K^+-stimulated dephosphorylation (for a model, see Figure 6).

It thus seems that binding of K^+ to its binding pocket, in which Glu820 is involved, has a similar effect on both ATPase activity and dephosphorylation of the phosphorylated intermediate as does removal of the negative charge of this residue. There is, however, a difference: K^+ can enhance the dephosphorylation rate at 0°C, whereas the mutation alone does not result in an enhanced rate of dephosphorylation at this temperature. At 21°C, both binding of K^+ and elimination of the negative charge result in an enhanced dephosphorylation rate. The most likely explanation for the difference at 0°C is that the conformational change induced by binding of K^+ to its binding pocket is larger than by removal of the negative charge from residue 820. Binding of K^+ to its binding pocket also has an effect on amino acid residues other than Glu820. Possible candidates are Asp824, Glu795 and Glu343 (Asano *et al.*, 1996; Swarts *et al.*, 1996; Hermsen *et al.*, 1998), since these amino acids are conserved and seem to play a similar role in other P-type ATPases (Clarke *et al.*, 1989; Lingrel and Kuntzweiler, 1994; Adebayo *et al.*, 1995; Vilsen, 1995; Pedersen *et al.*, 1997). At 21°C, the conformational change induced by the mutation is apparently sufficient to increase the hydrolysis rate of the phosphorylated intermediate. With the E820A mutant, containing a relatively small methyl group at site 820, a further stimulation of the ATPase activity is possible by increasing the K^+ concentration above 10 mM. The latter effect can be explained by assuming that Glu820 is a crucial, but not the only residue in the binding pocket for K^+. Upon binding of K^+, more residues in this pocket are neutralized, whereas with the mutation only the inhibitory effect of a single coordinating group in the K^+-binding pocket is eliminated. Interestingly, Vilsen *et al.* (1995, 1997) reported a high Na^+-ATPase activity and a high basal dephosphorylation rate for mutant E781A of rat α_1 Na^+,K^+-ATPase, which also has a reduced K^+ affinity. Glu781 is equivalent to Glu795 in gastric H^+,K^+-ATPase.

Further evidence supporting the importance of residue 820 for the K^+ effects arises from the fact that in K^+-sensitive ATPases a negatively charged residue is present at this site [Asp in Na^+,K^+-ATPase (Shull *et al.*, 1985) and colon H^+,K^+-ATPase (Crowson and Shull, 1992), Glu in gastric H^+,K^+-ATPase (Shull and Lingrel, 1986)], while K^+-insensitive ATPases, such as Ca^{2+}-ATPases, both from the plasma membrane type (Verma *et al.*, 1988) and from the sarco- and endoplasmic reticulum type (MacLennan *et al.*, 1985), contain a neutral Asn at this position. It would be interesting to investigate whether a mutation of this Asn residue into an Asp or Glu residue would result in a K^+-sensitive Ca^{2+}-ATPase.

The ion transport caused by H^+,K^+-ATPase is electroneutral. However, both H^+ transport in the E_1-P $\rightleftarrows$ E_2-P step and K^+ transport in the $E_2 \rightleftarrows E_1$ step are electrogenic, although with an opposite sign (Van der Hijden *et al.*, 1990; Stengelin *et al.*, 1993). It will be very interesting to know whether the constitutive ATPase activity of the three mutants without a negative charge on residue 820

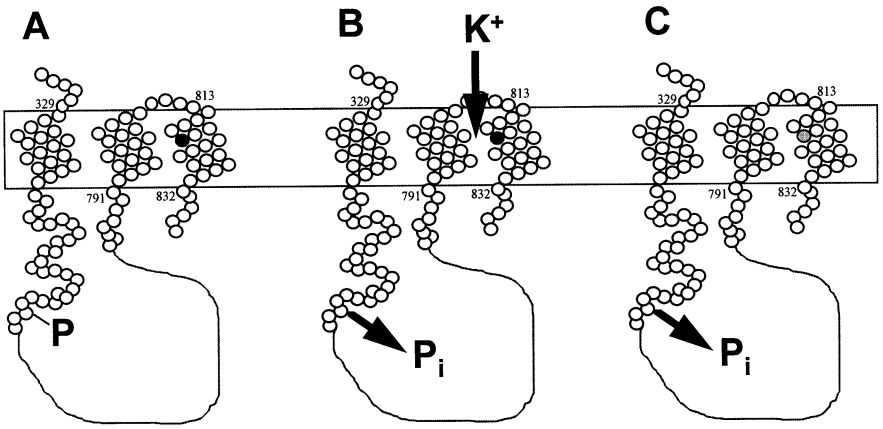

Fig. 6. Model for the effects of K^+ binding to the native H^+,K^+-ATPase or of removal of the negative charge on Glu820 on the dephosphorylation of the phosphorylated intermediate. The fourth, fifth and sixth transmembrane segments and part of the intracellular loop between transmembrane segments four and five are shown, including Asp386 which becomes phosphorylated during the catalytic cycle. Residue Glu820 is drawn in black. (**A**) The situation for the wild-type enzyme after phosphorylation in the absence of K^+. (**B**) The effect of extracellular K^+ resulting in dephosphorylation. (**C**) Spontaneous dephosphorylation of the phosphorylated intermediate after mutation of Glu820 to either Asn, Gln or Ala (indicated in grey).

is accompanied by ion transport and, if so, whether the transport would be electroneutral or electrogenic. The extremely low K^+ affinity of the mutants suggests that either H^+ is also transported in the opposite direction or the conversion of the E_2 into the E_1 form occurs without accompanying ion transport. It might also be that no ion transport occurs in either direction. The relatively low ATPase activity of the expressed enzyme, however, has precluded transport measurements until now.

The findings reported here lead to a fundamental new concept on the mechanism of action of K^+ on this ATPase and possibly other ATPases. The enhancing influence of K^+ on the rate of dephosphorylation is not caused by a direct positive effect on the dephosphorylation process, but by neutralization of an inhibitory effect of the binding pocket for K^+, in which pocket Glu820 plays a crucial role.

Materials and methods

Preparation of mutants

Rat gastric ATPase was expressed in Sf9 cells as described previously (Klaassen *et al.*, 1993; Swarts *et al.*, 1996). The BaculoGold transfer vector pAcUW51 (Pharmingen, San Diego, CA), containing the full-length cDNA of the rat H^+,K^+-ATPase α- and β-subunits was used for site-directed mutagenesis (Deng and Nickoloff, 1992). The obtained pAcUW51-HKβα-wt and pAcUW51-HKβα-mutants, with the DNA code of the α-subunit under control of the polyhedrin promoter and that of the β-subunit under control of the p10 promoter, were used to produce recombinant viruses. The (mutated) transfer vectors and linearized AcNPV DNA (BaculoGold™ DNA) were co-transfected in Sf9 cells according to the supplier's instructions. The viruses obtained by this method were purified further via a plaque assay, and expression of the α-subunit was screened by Western blotting. The presence of the desired mutation in the viral genome was checked by sequence and restriction analyses. Sf9 cells were grown at 27°C in 100 ml spinner flask cultures (Klaassen *et al.*, 1993). For production of H^+,K^+-ATPase, $1.0–1.5\times10^6$ cells/ml were infected at a multiplicity of infection of one to three in the presence of 1% ethanol (Klaassen *et al.*, 1995) and incubated for 3 days using Xpress medium (BioWittaker, Walkersville, MD) containing additionally 0.1% pluronic F-68 (Sigma Bornem, Belgium). By using the latter incubation conditions, both the phosphorylation capacity and the H^+,K^+-ATPase activity of the expressed enzyme were 2–3 times higher than previously found (Swarts *et al.*, 1996). The Sf9 cells were harvested by centrifugation at 2000 *g* for 5 min. After resuspension at 0°C in 0.25 M sucrose, 2 mM EDTA and 25 mM HEPES–Tris (pH 7.0), the membranes were sonicated twice for 30 s at 60 W (Branson Power Company, Denbury, USA). After centrifugation for 30 min at 10 000 *g*, the supernatant was recentrifugated for 60 min at 100 000 *g* at 4°C. The pelleted membranes were resuspended in the above-mentioned buffer and stored at –20°C.

ATPase assay

The ATPase activity was determined with a radiochemical method (Swarts *et al.*, 1995). For this purpose 0.5–2.0 μg of Sf9 membranes were added to 100 μl of medium, which contained 10 μM [γ-^{32}P]ATP (Amersham, Buckinghamshire, UK; sp. act. 100–500 mCi/mmol), 1.3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 0.1 mM ouabain, 1 mM NaN_3, 25 mM Tris–HCl (pH 7.0), with or without 100 μM SCH 28080 (provided by Dr A.Barnett, Schering-Plough, Bloomfield, NJ, dissolved in ethanol and diluted to its final concentration of 0.1 mM in 0.2% ethanol) and varying concentrations of KCl. In the absence of added K^+, the K^+ concentration in the incubation medium was ~5 μM. After incubation for 30 min at 37°C, the reaction was stopped and analysed further as described before (Swarts *et al.*, 1996). Protein was determined with the modified Lowry method according to Peterson (1983) using bovine serum albumin as a standard.

Phosphorylation and dephosphorylation assays

Phosphorylation and dephosphorylation were performed by incubating Sf9 membranes (10–50 μg) for 10 s at either 0 or 21°C in 50 mM Tris–acetic acid (pH 6.0), 1 mM $MgCl_2$ and 0.1 μM [γ-^{32}P]ATP in a volume of 60 μl. For dephosphorylation studies, part of the reaction mixture was diluted 8.3 times with non-radioactive ATP (final concentration 10 μM), in order to prevent rephosphorylation with radioactive ATP, with and without 1 mM K^+, and was incubated further for 3 s at either 0 or 21°C (Helmich-de Jong *et al.*, 1985). Thereafter, the reaction was stopped and samples were either solubilized and analysed by SDS–PAGE or the amount of phosphorylated intermediate was determined by a filtration method (Swarts *et al.*, 1996).

Analysis of data

The IC_{50} values for SCH 28080 were determined iteratively by fitting the concentration relationship to the logistic equation:

$$Y = A + (B–A)/1 + (10^C/10^X)^D$$

(A = bottom plateau; B = top plateau; C = IC_{50}; D = Hill coefficient; the values of X and C were entered as the logarithm of concentration) using the non-linear regression computer program InPlot (GraphPAD Software for Science, San Diego, CA). All data are presented as mean values with standard error of the mean. Differences were tested for significance by means of the Student's *t*-test.

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