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Conformation-Dependent Inhibition of Gastric H^+,K^+-ATPase by SCH 28080 Demonstrated by Mutagenesis of Glutamic Acid 820

HERMAN G.P. SWARTS, HARM P.H. HERMSEN, JAN B. KOENDERINK, PETER H.G.M. WILLEMS, and JAN JOEP H.H.M. DE PONT

Department of Biochemistry, Institute of Cellular Signalling, University of Nijmegen, Nijmegen, the Netherlands

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ABSTRACT

Gastric H^+,K^+-ATPase can be inhibited by imidazo pyridines like 2-methyl-8-[phenylmethoxy] imidazo-(*1,2a*) pyridine 3-acetonitrile (SCH 28080). The drug shows a high affinity for inhibition of K^+-activated ATPase and for prevention of ATP phosphorylation. The inhibition by SCH 28080 can be explained by assuming that SCH 28080 binds to both the E_2 and the phosphorylated intermediate (E_2-P) forms of the enzyme. We observed recently that some mutants, in which glutamic acid 820 present in transmembrane domain six of the catalytic subunit had been replaced (E820Q, E820N, E820A), lost their K^+-sensitivity and showed constitutive ATPase activity. This ATPase activity could be inhibited by similar SCH 28080 concentrations as the K^+-activated ATPase of the wild-type enzyme. SCH 28080 also inhibited ATP phosphorylation at 21°C of the mutants E820D, E820N, and E820A, although with varying efficacy and affinity. ATP-phosphorylation of mutant E820Q was not inhibited by SCH 28080; in contrast, the phosphorylation level at 21°C was nearly doubled. These findings can be explained by assuming that mutation of Glu^{820} favors the E_1 conformation in the order E820Q >E820A >E820N >wild-type = E820D. The increase in the phosphorylation level of the E820Q mutant can be explained by assuming that during the catalytic cycle the E_2-P intermediate forms a complex with SCH 28080. This intermediate hydrolyzes considerably slower than E_2-P and thus accumulates. The high tendency of the E820Q mutant for the E_1 form is further supported by experiments showing that ATP phosphorylation of this mutant is rather insensitive towards vanadate, inorganic phosphate, and K^+.

Gastric H^+,K^+-ATPase, a P-type ATPase, is responsible for gastric acid secretion. The enzyme is located in the tubulovesicular system of the parietal cell and is translocated to the apical plasma membrane after hormonal stimulation. It catalyzes an electroneutral transport of K^+ versus H^+ energized by ATP hydrolysis. Gastric H^+,K^+-ATPase can be reversibly inhibited by imidazo pyridines like 2-methyl-8-[phenylmethoxy] imidazo-(*1,2α*) pyridine 3-acetonitrile (SCH 28080), apparently by binding to a high-affinity site for K^+ (Wallmark et al., 1987, Keeling et al., 1989; Mendlein and Sachs, 1990). Munson et al. (1991) determined the binding site of a photoaffinity analog of SCH 28080 to be the domain including the first two transmembrane segments of the α-subunit as well as the extracellular loop between these segments. Lyu and Farley (1997) recently reported that Na^+,K^+-ATPase, in which twelve amino acids of the first transmembrane segment of the α-subunit had been replaced by the homologous amino acids from H^+,K^+-ATPase, showed a rather high sensitivity toward this drug. This suggests that the N terminal part of the α-subunit of H^+,K^+-ATPase is the main participant in SCH 28080 binding, as it is in the binding of ouabain to Na^+,K^+-ATPase (Lingrel and Kuntzweiler, 1994). On the other hand, Asano et al. (1997) suggested recently that glutamic acid 820 might also be important for the binding of SCH 28080.

Recently, we prepared in Sf9 insect cells with aid of the baculovirus expression system a series of mutants of rat gastric H^+,K^+-ATPase in which glutamic acid 820, located in the 6th transmembrane domain of the α-subunit, had been replaced by various amino acids (Hermsen et al., 1998). All mutants (E820D, E820A, E820Q, and E820N) could, like the wild-type enzyme, be phosphorylated by ATP. The hydrolysis of the phosphorylated intermediate (E-P) of mutant E820D could, like that of the wild-type enzyme, be enhanced by 1 mM K^+. This mutant showed a normal K^+-stimulated ATPase activity with a maximal activity at 1 mM K^+. The dephosphorylation of the E–P of the E820Q and E820N mutants could not be stimulated by K^+ and that of the E820A mutant only at 100 mM K^+. Similarly, the ATPase activity of the E820Q and E820N mutants was not stimulated by K^+,

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ABBREVIATIONS: SCH 28080, 2-methyl-8-[phenylmethoxy] imidazo-(*1,2a*) pyridine 3-acetonitrile; E-P, phosphorylated intermediate.

whereas, that of the E820A mutant was only slightly increased at 100 mM K^+. For clarity, these three mutants are coined in the present paper as "K^+-insensitive mutants".

Preincubation of the wild-type enzyme at 0°C with SCH 28080, a specific inhibitor of gastric H^+,K^+-ATPase, resulted in a decrease of the ATP phosphorylation level with an IC_{50} value of 20 nM (see Table 1). Similar IC_{50} values were obtained for the mutants E820D and E820N. The E820A mutant, however, was less sensitive to SCH 28080 and phosphorylation of the E820Q mutant could hardly be reduced by this compound. Thus, the three K^+-insensitive mutants have a different apparent affinity for SCH 28080 when ATP phosphorylation is used as the assay method. Moreover, these findings are difficult to match with the suggestion of Asano et al. (1997) that Glu^{820} is directly involved in SCH 28080 binding, and they make it likely that a more complex mechanism must exist for SCH 28080 inhibition.

In addition, we recently demonstrated (Swarts et al., 1998) that all three K^+-insensitive mutants (E820Q, E820N, and E820A) had an ATPase activity in the absence of K^+. This constitutive ATPase activity of these mutants could be inhibited by SCH 28080 with similar IC_{50} values (0.2–0.7 μM) as that of the wild-type enzyme (0.4 μM) and the E820D mutant (0.3 μM; see Table 1). In that study, we also measured phosphorylation and dephosphorylation at 21°C and found that the K^+-insensitive mutants had a high spontaneous dephosphorylation rate that could not be further stimulated by K^+.

The purpose of the present study is to provide a solution for these apparently contradictory findings. Why does SCH 28080 inhibit the ATPase activity (measured at 37°C) of all mutants with a rather similar affinity, whereas the IC_{50} values for reduction of phosphorylation at 0°C vary so much? To give a framework for the experiments to be described, a simplified version of the Post-Albers model for P-type ATPases is given in Fig. 1. This model takes into account that both the E_2 form and the E_2-P form of gastric H^+,K^+-ATPase are able to react with SCH 28080 (Keeling et al., 1989; Mendlein and Sachs, 1990; Van der Hijden et al., 1991).

The present study shows that the K^+-insensitive mutants have a high tendency for the E_1 form, in particular at 21°C, and therefore do not bind SCH 28080 very well under non-phosphorylating conditions. Importantly, under ATP-hydrolyzing conditions, they are temporarily in the E_2-P form, which does bind SCH 28080. During each catalytic cycle, part of the enzyme is thus trapped by SCH 28080 and therefore inhibited. The lack of reduction of the ATP phosphorylation level by SCH 28080 of mutant E820Q does not mean that residue Glu^{820} is directly involved in SCH 28080 binding, but only indicates the very low tendency of this mutant for the E_2 conformational state of the enzyme.

TABLE 1

IC_{50} values for SCH 28080 on the ATP phosphorylation level and the ATPase activity of mutants of glutamic acid 820

	ATP phosphorylation level[a]	ATPase activity[b]
	nM	*μM*
wild-type	20 ± 1	0.43 ± 0.06
E820D	8 ± 1	0.30 ± 0.04
E820A	126 ± 5	0.16 ± 0.01
E820N	23 ± 1	0.21 ± 0.04
E820Q	600 ± 100	0.73 ± 0.00

[a] Values from Hermsen et al. (1998). ATP phosphorylation level was measured for 10 s at 0°C (pH 6.0) after a preincubation for 60 min with 1 mM Mg^{2+}, 20 mM Tris-acetate (pH 6.0), and various concentrations of SCH 28080. IC_{50} values were determined after correction for the ATP phosphorylation level of mock-infected cells.

[b] Values from Swarts et al. (1998). ATPase activity was measured in the presence of 1 mM K^+ for 30 min at 37°C in the presence of various SCH 28080 concentrations. IC_{50} values were determined after correction for the ATPase activity of mock-infected cells.

Materials and Methods

Preparation of Mutants. Rat gastric H^+,K^+-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-HK$\beta\alpha$-wt and pAcUW51-HK$\beta\alpha$-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 instructions of the supplier. The viruses obtained by this method were further purified 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 analysis.

Production of Recombinant H^+,K^+-ATPase. 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 × 10^6 cells/ml were infected at a multiplicity of infection of 1 to 3 in the presence of 1% ethanol (Klaassen et al., 1995) and incubated for 3 days using Xpress medium (BioWhittaker, 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 to 3 times higher than previously found (Swarts et al., 1996).

Preparation of Sf9 Membranes. 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 3 × 15 s at 60 W (Branson Power Company, Denbury, CT). After centrifugation for 30 min at 10,000*g* the supernatant was recentrifuged 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.

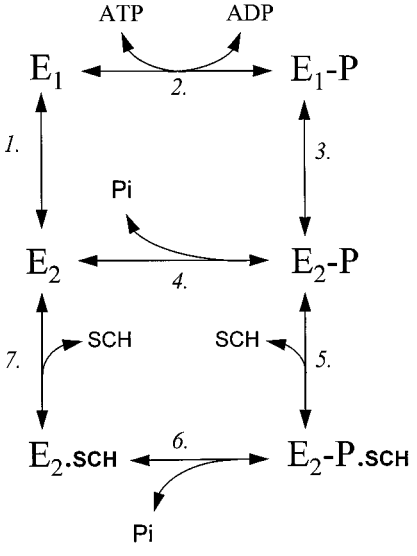

Fig. 1. Post-Albers reaction scheme for H^+,K^+-ATPase including the SCH 28080 inhibition mechanism. The clockwise reactions are indicated in the text with a positive (+) sign and the counter-clockwise reactions with a minus (−) sign. Reaction + 4 is the conversion of E_2-P to E_2.

Protein Determination. Protein was determined with the modified Lowry method described by Peterson (1983) using bovine serum albumin as a standard.

ATP Phosphorylation Capacity. ATP phosphorylation was determined as described before (Swarts et al., 1998). Sf9 cell membranes (amounts indicated in the legends) were incubated at 0° or 21°C in 50 mM Tris-acetic acid (pH 6.0), 1.2 mM $MgCl_2$, and 0.2 mM EDTA with and without 0.1 mM SCH 28080 in a volume of 50 μl. After 60-min preincubation, 10 μl of 0.6 μM [γ-^{32}P]ATP was added and incubated for the indicated time at either 0° or 21°C. The reaction was stopped by adding 5% trichloroacetic acid in 0.1 M phosphoric acid. The phosphorylated protein was collected by filtration over a 0.8 μm membrane filter (Schleicher and Schull, Dassel, Germany). After repeated washing, the filters were analyzed by liquid scintillation analysis.

Dephosphorylation Studies. For dephosphorylation studies, phosphorylation was carried out and the dephosphorylation mixture was diluted 8.3 times with nonradioactive ATP (final concentration 10 μM) to prevent rephosphorylation with radioactive ATP and was further incubated for 3 to 30 s at 21°C (Helmich-de Jong et al., 1985). The reaction was stopped at the time points indicated and the amount of E–P was determined as described above.

Analysis of Data. The IC_{50} values for SCH 28080 were iteratively determined 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 nonlinear regression computer program InPlot (GraphPAD Software for Science, San Diego, CA). All data are presented as mean values with S.E.M. Differences of average were tested for significance by means of Student's *t* test.

Chemicals. [γ-^{32}P]ATP (3000 $C_i mmol^{-1}$, Amersham, Buckinghamshire, UK) was diluted with nonradioactive Tris-ATP (pH 6.0) to a specific radioactivity of 20 to 100 $C_i mmol^{-1}$. SCH 28080, kindly provided by Dr. A. Barnett (Schering-Plough, Kenilworth, NJ), was dissolved in ethanol and diluted to its final concentration of 0.1 mM in 0.2% ethanol.

Results and Discussion

The mechanism of the SCH 28080-induced decrease of the steady-state ATP phosphorylation level and the ATPase activity of the pig gastric H^+,K^+-ATPase has previously been explained with the aid of the adapted form of the Post-Albers scheme given in Fig. 1 (Keeling et al., 1989; Mendlein and Sachs, 1990; Van der Hijden et al., 1991). This model will be used as a framework to explain the behavior of both the wild-type enzyme and the Glu^{820} mutants.

SCH 28080 Reduction of the ATP Phosphorylation Capacity. The effect of SCH 28080 on the phosphorylation level had previously been measured by preincubation with the drug for 1 h at 0°C followed by a 10-s phosphorylation at 0°C using 0.1 μM ATP (pH 6.0) (Hermsen et al., 1998). Because we recently observed with some mutants (E820D, E820Q) that a higher phosphorylation level could be reached at a (pre)incubation temperature of 21°C (Swarts et al., 1998), dose-inhibition curves for SCH 28080 were carried out at both temperatures. Fig. 2A shows that at 0°C the wild-type enzyme showed a sigmoid dose-inhibition curve with an IC_{50} value of 30 nM. At 21°C the dose-inhibition curve for the wild-type enzyme shifted to the right (IC_{50} = 0.2 μM), indicating a temperature-sensitive decrease in apparent affinity for SCH 28080. These findings can be explained by assuming that the wild-type enzyme is at least partly present in the E_2 conformation, which forms an E_2-SCH complex during the preincubation period (reaction −7). As a result, a new equilibrium between $E_1 \leftrightarrow E_2 \leftrightarrow E_2$-SCH is achieved, which depends on the SCH 28080 concentration used. Upon addition of ATP, the residual E_1 reacts and E_1-P is formed (reaction +2); this is rapidly converted into the E_2-P conformation (reaction +3). The decrease in apparent affinity for SCH 28080 upon increasing the temperature from 0° to 21°C might be due to a shift in the $E_1 \leftrightarrow E_2$ equilibrium to the left, resulting in formation of less E_2-SCH complex at suboptimal SCH 28080 concentrations. Alternatively, the increase in temperature might also shift the $E_2 \leftrightarrow E_2$-SCH equilibrium to the left, so indirectly resulting in more E_1 and, thus, a higher phosphorylation level.

The phosphorylation level of the E820Q mutant (Fig. 2C) was not reduced by SCH 28080 at 0°C. At 21°C, the phosphorylation level increased, resulting in a near doubling of the phosphorylation level at SCH 28080 concentrations between 0.1 and 10 μM (50% of maximal activation at 0.4 μM).

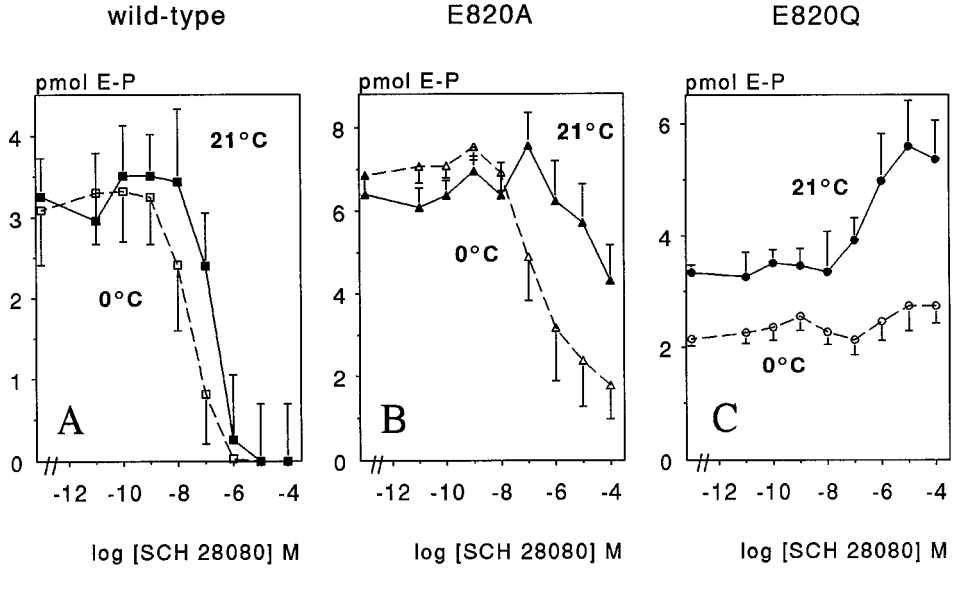

Fig. 2. Effect of SCH 28080 on ATP phosphorylation capacity of the H^+,K^+-ATPase mutants at 0° and 21°C. Membranes (4–8 μg) obtained from Sf9 cells infected with either wild-type virus (A), mutant E820A (B), or mutant E820Q (C) were preincubated for 60 min at either 0° or 21°C in the presence of 1.2 mM $MgCl_2$, 0.2 mM EDTA, 50 mM Tris-acetic acid (pH 6.0) and the indicated SCH 28080 concentrations. After phosphorylation for 10 s at 0° or 21°C with 0.1 μM [γ-^{32}P]ATP the phosphorylation level (E–P; pmol · mg^{-1} protein) was determined and corrected for that of mock-infected cells. Mean values ± S.E.M. of three enzyme preparations.

The latter increase can be explained by assuming that the E820Q mutant, in the absence of SCH 28080, is nearly completely present in the E_1 form. Upon addition of ATP, phosphorylation occurs resulting in the formation of E_2-P (reactions +2 and +3). When SCH 28080 is present, E_2-P will at least in part be converted into the E_2-P-SCH form (reaction +5). The residual part of E_2-P will be rapidly hydrolyzed into E_2 (reaction +4), because of the high spontaneous hydrolysis rate of this mutant (Swarts et al., 1998). Because the $E_1 \leftrightarrow E_2$ equilibrium for this mutant is directed to the left, E_1 is subsequently formed (reaction +1) and phosphorylation starts again (reactions +2 and +3). It is likely that after a number of cycles all the enzyme accumulates in the E_2-P-SCH form; this explains the increase in the phosphorylation level obtained with this mutant.

We suppose that at 0°C the $E_1 \leftrightarrow E_2$ equilibrium for the E820Q mutant is, as for the wild type and all other mutants, slightly more directed toward the E_2 form. We postulate that the lack of effect of SCH 28080 on the phosphorylation level might be a combination of a reducing effect (resulting in E_2-SCH) and a stimulatory effect (resulting in the E_2-P-SCH form). In our previous experiments (Swarts et al., 1996; Hermsen et al., 1998), we found maximally 40% reduction at very high SCH 28080 concentrations, suggesting that in the preparation used in those studies the inhibitory effect was slightly higher than the stimulatory effect.

The results for the E820A mutant (Fig. 2B) were intermediate between those for the wild-type enzyme and the E820Q mutant. The ATP phosphorylation level measured at 0°C was less sensitive toward SCH 28080 than that of the wild-type enzyme and could not be fully reduced by 100 μM SCH 28080. The SCH 28080 concentration range over which inhibition occurred was rather broad. At 21°C, ATP phosphorylation became even more insensitive toward SCH 28080. At 100 μM SCH 28080 the residual phosphorylation level was still more than 50% of the control level. These findings can at least qualitatively be explained by assuming for the E820A mutant that at both temperatures the $E_1 \leftrightarrow E_2$ equilibrium lies more in the direction of E_2 than for the E820Q mutant but less as compared with that of the wild-type enzyme. The part that is in the E_2 form cannot be phosphorylated by ATP, whereas the part that is in the E_1 form can be phosphorylated, but might result in the more stable E_2-P-SCH through reaction 5. The final result is probably a concentration-dependent combination of a decrease of E_2-P and an increase in E_2-P-SCH.

Time Course of SCH 28080 Effect. We investigated the difference between the effect of high SCH 28080 concentrations on the phosphorylation level at 21°C for the wild-type enzyme (decrease) and the E820Q mutant (increase) in more detail by adding 100 μM SCH 28080 15 s after the start of the phosphorylation. Figure 3 shows that addition of SCH 28080 resulted in an immediate decrease in the phosphorylation level of the wild-type enzyme and in an immediate increase in that of the E820Q mutant. In both cases a new steady-state level was reached within 20 to 30 s; this level was similar to that obtained when the enzyme was preincubated for 60 min with SCH 28080 prior to the addition of ATP (dotted line for E820Q). The rate of equilibrium change is in the same order of magnitude as described for the binding of SCH 28080 at this temperature (Keeling et al., 1989), suggesting that the binding rate primarily determined the rate of this process.

If the wild-type enzyme is first phosphorylated resulting in formation of E_2-P, addition of SCH 28080 will, according to the model of Fig. 1, lead to formation of E_2-P-SCH (reaction +5), which dephosphorylates to E_2-SCH (reaction +6). Alternatively, E_2-P dephosphorylates to E_2 (reaction +4), which form can also react with SCH 28080 (reaction −7) to E_2-SCH. Independent of the route, this explains the SCH 28080-induced decrease of the phosphorylation level.

With the E820Q mutant, the E_2-P intermediate will in part be converted into E_2-P-SCH and in part be dephosphorylated into E_2. Because of the direction of the $E_1 \leftrightarrow E_2$ equilibrium E_1 will be formed (reaction +1) and phosphorylation starts again. This will finally result in an accumulation of E_2-P-SCH and thus to an increased phosphorylation level.

Dephosphorylation Kinetics of the Phospho-Intermediates. If the above reasoning is correct, the type of E-P obtained with the E820Q mutant will be different in the absence of SCH 28080 (E_2-P) from that obtained in the presence of SCH 28080 (E_2-P-SCH). We therefore studied the dephosphorylation kinetics of the E–Ps obtained under various conditions in the absence of K^+. Figure 4A shows the

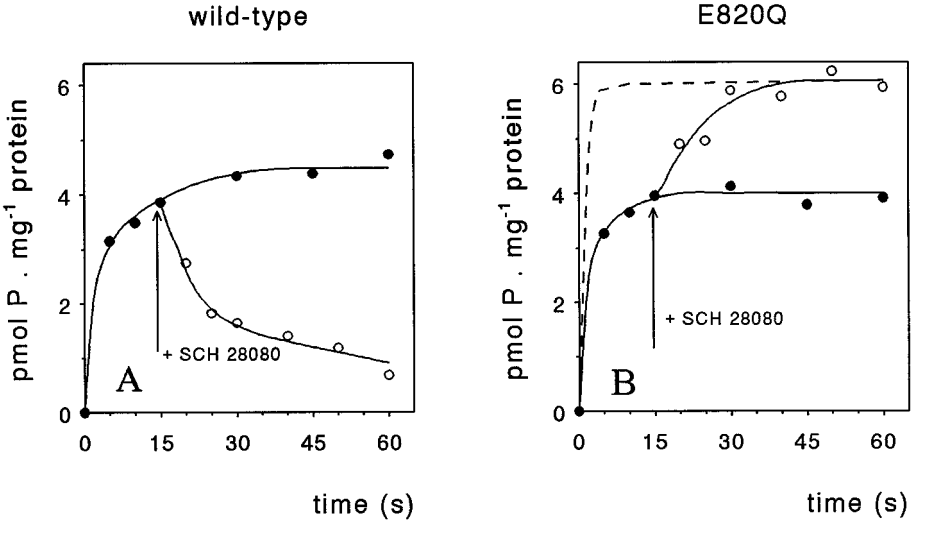

Fig. 3. Time course of the effect of SCH 28080 on the ATP-phosphorylation capacity of the wild-type enzyme and the E820Q mutant. Membranes (4 μg) obtained from Sf9 cells infected with either wild-type virus (A) or mutant E820Q (B) were preincubated for 60 min at 21°C in the presence of 1.2 mM $MgCl_2$, 0.2 mM EDTA, and 50 mM Tris-acetic acid (pH 6.0). At t = 0 s, 0.1 μM [γ-^{32}P]ATP (final concentration) was added and the level of the phosphorylated intermediate was followed in time. At t = 15 s, 100 μM SCH 28080 (final concentration) was added. Data were corrected for those of mock-infected cells. Representative for 2 to 3 experiments.

dephosphorylation process of the phospho-intermediate of the wild-type enzyme (reaction 4). The measured rate constant at 21°C was 0.08 ± 0.011 s^{-1} (n = 4). In the presence of 0.1 mM SCH 28080, no E-P was formed.

In the absence of SCH 28080, both the E820A and the E820Q mutants formed an E–P that dephosphorylated rapidly. After 3 s only 19 ± 4.2% (n = 3; E820A) and 12 ± 3.6% (n = 3; E820Q) of the E–P was left, indicating that the dephosphorylation rates were at least 0.5 s^{-1}. The dephosphorylation rate constants of the phospho-intermediate generated in the presence of SCH 28080 of the mutants E820A (0.05 ± 0.004 s^{-1}; n = 3) and E820Q (0.05 ± 0.002 s^{-1}; n = 3) were very low, indicating that a different type of E–P (E_2-P-SCH instead of E_2-P) was formed. With the E820A mutant the initial phosphorylation level was decreased due to partial inhibition of the phosphorylation process (see Fig. 2B), whereas with the mutant E820Q the initial phosphorylation level was increased by 100 μM SCH 28080 (see Fig. 2C).

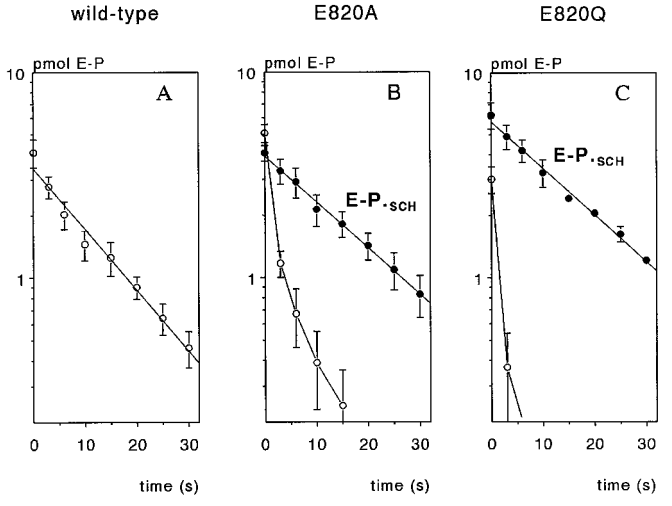

Fig. 4. Dephosphorylation rate of the phospho-intermediates of the H^+,K^+-ATPase (mutants) generated in the absence and presence of SCH 28080. Membranes (13–19 μg) obtained from Sf9 cells infected with either wild-type virus (A), mutant E820A (B), or mutant E820Q (C) were preincubated with (●) and without 0.1 mM SCH 28080 (○) in the presence of 1.2 mM $MgCl_2$, 0.2 mM EDTA and 50 mM Tris-acetic acid (pH 6.0) for 30–60 min at 21°C and phosphorylated with 0.1 μM [γ-^{32}P]ATP. After 10 s (t = 0), the incubation medium was diluted from 60 to 500 μl with non-radioactive ATP (final concentration 10 μM) in the presence of 50 mM Tris-acetic acid (pH 6.0), 1 mM Mg^{2+}, with (●) and without (○) SCH 28080 to prevent rephosphorylation with radioactive ATP and incubated for 3–30 s. The residual phosphorylation level (E–P; pmol.mg^{-1} protein), corrected for the mock infected levels, is plotted as a function of time. Mean values ± S.E.M. of three enzyme preparations.

Effects of Inorganic Phosphate, Vanadate, and K^+ on the ATP Phosphorylation Capacity. Table 2 describes the results from some additional experiments at 21°C that confirm the model of Fig. 1. In this table, results with two other mutants (E820D and E820N) are included. In the absence of SCH 28080 the 10-s phosphorylation level at 21°C varied for these mutants between 2.9 and 5.4 pmol E–P per mg protein. The observed difference might either be because under these conditions the maximal phosphorylation level had not been reached for all mutants or to differences in the amount of correct folded enzyme. It is known, at least for P-type ATPases, that in the baculovirus system, only part of the expressed protein is active (De Tomaso et al., 1993; Xie et al., 1996; Swarts et al., 1996; Liu and Guidotti, 1997).

After preincubation with 100 μM SCH 28080, the phosphorylation level of the E820D mutant was like that of the wild-type enzyme, nearly completely reduced. Comparison of the phosphorylation level of the three other mutants shows that the E820N mutant was the only one of these K^+-insensitive mutants that was significantly, although only partly, reduced. The phosphorylation level of the E820A mutant was slightly decreased and that of the E820Q mutant was enhanced as described above. This suggests that the $E_1 \leftrightarrow E_2$ equilibrium for the E820N mutant lies farther in the E_2 direction than for the E820A and the E820Q mutants. The inhibition by SCH 28080 of the phosphorylation level of the E820N mutant at 21°C is again less than at 0°C (Hermsen et al., 1998), which is in agreement with a temperature-dependent shift of the $E_1 \leftrightarrow E_2 \leftrightarrow E_2$-SCH equilibrium towards E_1.

H^+,K^+-ATPase can be phosphorylated by inorganic phosphate on the same aspartyl residue that becomes phosphorylated by ATP (Van der Hijden et al., 1991). Once part of the enzyme molecules is phosphorylated by nonradioactive inorganic phosphate, only the unphosphorylated part of the enzyme molecules can react with radioactive ATP. Table 2 (column 3) shows that after preincubation with 1 mM inorganic phosphate, the ATP phosphorylation level of both the wild-type enzyme and the E820D mutant was some 60% lower than that in the absence of inorganic phosphate, whereas phosphorylation of the K^+-insensitive mutants (E820Q, E820A, and E820N) was not reduced. This indicates that maximally 40% of the wild-type enzyme and the E820D mutant is not phosphorylated by inorganic phosphate under these conditions. The fact that preincubation with inorganic phosphate has no effect on the ATP phosphorylation level of the three K^+-insensitive mutants suggests that either these mutants do not form an E–P with inorganic phosphate or

TABLE 2

The effect of SCH 28080, inorganic phosphate, vanadate, and K^+ on the steady-state ATP phosphorylation level

Sf9 membranes (4–8 μg) were preincubated in the presence of 50 mM Tris-acetic acid (pH 6.0), 1.2 mM $MgCl_2$, 0.2 mM EDTA, and in addition with either 0.1 mM SCH 28080, 1 mM inorganic phosphate pH 6.0 (Pi), 0.1 mM SCH 28080 + 1.0 mM Pi, 1 mM vanadate, or 10 mM KCl at 21°C. After 60 min 0.1 μM [γ-^{32}P]ATP (final concentration) was added and after 10 s the amount of E–P was determined. The data are corrected for the levels obtained with membranes of mock infected Sf9 cells. Averages with S.E.M. for three enzyme preparations are presented. Differences of the data compared to the control values are tested for significance by means of Student's t test (*$p < .05$; **$p < .01$).

	ATP phosphorylation capacity (pmol P · mg^{-1})					
	Control	SCH 28080	Pi	Pi SCH 28080	Vanadate	KCl
wild-type	4.06 ± 0.56	0.01 ± 0.25**	1.40 ± 0.28*	−0.09 ± 0.14**	−0.05 ± 0.08**	0.06 ± 0.28**
E820D	3.67 ± 0.04	0.33 ± 0.08**	1.39 ± 0.19**	0.16 ± 0.08**	0.07 ± 0.18**	0.23 ± 0.07**
E820Q	2.86 ± 0.22	6.27 ± 0.44**	2.65 ± 0.11	3.66 ± 0.43	1.49 ± 0.10**	1.60 ± 0.16**
E820N	3.94 ± 0.25	1.65 ± 0.64*	3.21 ± 0.25	0.11 ± 0.27**	0.10 ± 0.10**	2.65 ± 0.28*
E820A	5.38 ± 1.66	4.02 ± 1.61	4.80 ± 1.44	0.55 ± 0.36*	4.11 ± 1.02	4.20 ± 1.12

form a very labile intermediate which hydrolyzes during the phosphorylation period with ATP. Because phosphorylation with inorganic phosphate only occurs with the E_2 form of the enzyme, this supports the hypothesis that the K^+-insensitive mutants favor the E_1 conformation.

We previously observed with pig gastric ATPase that SCH 28080 increased the steady-state phosphorylation level by inorganic phosphate, which we attributed to formation of an E_2-P-SCH complex (Van der Hijden et al., 1991). We therefore preincubated the preparations with 1 mM inorganic phosphate and 0.1 mM SCH 28080 before phosphorylation with radioactive ATP. With the wild-type enzyme and the E820D mutant no radioactive E–P was found. Similar findings were observed with the E820N and the E820A mutants. Only the E820Q mutant formed a radioactive E–P under this condition, although the level was lower than with SCH 28080 alone. These findings confirm that SCH 28080 promotes phosphorylation with inorganic phosphate in such a way that for the wild-type enzyme and all mutants, except E820Q, no significant phosphorylation with ATP could be measured. The results with the E820Q mutant indicate that in the combined presence of SCH 28080 and inorganic phosphate relatively little E–P is formed from inorganic phosphate resulting in a relative large phosphorylation with ATP. This suggests that from the tested mutants the E820Q mutant has the highest preference for the E_1 form.

Vanadate is an inhibitor of P-type ATPases (Yamasaki and Yamamoto, 1991; Faller et al., 1983) and reacts like SCH 28080 with the E_2 form of these enzymes. Previously we observed that preincubation of the E820Q mutant with 1 mM vanadate only partly prevented ATP phosphorylation, whereas ATP phosphorylation of the wild-type enzyme was completely reduced (Swarts et al., 1996). These findings can again be explained by the preference of the E820Q mutant for the E_1 conformation. Table 2 shows that phosphorylation of the E820D mutant is like that of the wild-type enzyme reduced by preincubation with 1 mM vanadate. Phosphorylation of the E820N mutant was also sensitive to vanadate. Vanadate had surprisingly no significant effect on the E820A mutant. If the E820Q mutant would be more directed to the E_1-form than the E820A mutant as the experiments with SCH 28080 and inorganic phosphate suggest, one would expect relatively more inhibition by vanadate of the E820A mutant than of the E820Q mutant.

Preincubation with 10 mM K^+ also largely prevented the phosphorylation of both the wild-type enzyme and the E820D mutant but did not or only partly (22–44%) prevent phosphorylation of the three other mutants. Both the wild-type enzyme and the E820D mutant are poised in the direction of the E_2 conformation by K^+ and therefore do not phosphorylate. In experiments with intact gastric vesicles we previously showed that this effect of K^+ occurs from the cytosolic side through a low-affinity binding site (Swarts et al., 1995). Mutation of Glu^{820} into a Asn or Gln results in a complete abolishment of the high-affinity binding site, located at the extracellular side, as shown by dephosphorylation studies (Hermsen et al., 1998, Swarts et al., 1998). The fact that preincubation with 10 mM K^+ partly prevents phosphorylation of the mutants E820N (33%) and E820Q (44%) suggests that the low-affinity K^+ binding site is also modified in these mutants, although the effects on both sites are not completely similar.

SCH 28080 Inhibition in the ATPase Activity Measurements. Under ATP hydrolyzing conditions (37°C and long incubation periods), the wild-type enzyme as well as the Glu^{820} mutants come temporarily in the E_2 or E_2-P forms both of which bind SCH 28080. During each catalytic cycle, part of the enzyme is thus trapped by SCH 28080 and therefore inhibited. This explains why the IC_{50} values of SCH 28080 for the wild-type enzyme and the E820 mutants in the ATPase differ only slightly (Table 1).

Asano et al. (1997) came recently to the conclusion that Glu^{822} in rabbit H^+,K^+-ATPase (equivalent to Glu^{820} in the rat enzyme) is one of the important sites that binds SCH 28080. Their conclusion was based on a significantly lower sensitivity of the K^+-stimulated ATPase reaction to SCH 28080 for the E822D mutant (IC_{50} = 15 μM) than for the wild-type enzyme (2.1 μM). These findings contrast with our results, in which we found that the E820D mutant had a slightly higher sensitivity for SCH 28080 than the wild-type enzyme, both in the phosphorylation and the ATPase experiment (see Table 1). In addition, our studies indicate that a direct involvement of the Glu^{820} residue in SCH 28080 binding is unlikely and that the measured effects are due to conformational changes.

Concluding Remarks. The present study shows that differences in sensitivity for SCH 28080 on the ATP phosphorylation level between various Glu^{820} mutants of gastric H^+,K^+-ATPase are not due to a direct involvement of Glu^{820} for SCH 28080 binding, but to differences in K^+-sensitivity and the direction of the $E_2 \leftrightarrow E_1$ equilibrium. In general, if mutation of a certain amino acid has an effect on the degree of enzyme inhibition, it does not prove that the mutated residue is directly involved in binding of the inhibitor.

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Send reprint requests to: Dr. J.J.H.H.M. De Pont, Department of Biochemistry, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands. E-mail: J.dePont@bioch.kun.nl