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Involvement of Glutamic Acid 820 in K+ and SCH 28080 Binding to Gastric H+,K+-ATPase

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Negatively charged residues present in transmembrane segments of P-type ATPases may be involved in cation binding and transport. There are several glutamic and aspartic acid residues in or around the fifth and sixth transmembrane domain of the catalytic subunit of gastric H+,K+-ATPase that are conserved in other P-type ATPases. The role of six of these amino acid residues was investigated by site-directed mutagenesis, resulting in conversion of these acid groups into their corresponding acid amides. SF9 cells were used as an expression system using a single baculovirus with coding sequences for both the α and β subunits of H+,K+-ATPase. Both subunits of all mutants, like the wild-type enzyme, were expressed in intracellular membranes of SF9 cells as indicated by western blotting experiments, an enzyme-linked immunosorbent assay, and confocal laser scan microscopy studies. The amount of the specific H,K-ATPase phosphorylated intermediate was measured as the difference between the ATP-phosphorylation level in the absence of SCH 28080 and in its presence. In mutants D824N, E834Q, E837Q, and D839N the amount of specific phosphorylated intermediate was not significantly different from zero. In all preparations a considerable amount of mutated H+,K+-ATPase protein (0.8—4.1% of total) was produced, indicating that the lack of activity of these mutants is not due to a lack of biosynthesis. These findings suggest that each of the D824, E834, E837, and D839 residues is essential for the enzyme to become phosphorylated. It might be that these residues are involved in H+ binding which is essential for ATP phosphorylation.

The two mutants E795Q and E820Q, which show an SCH 28080 sensitive phosphorylated intermediate, were studied in more detail. The effect of preincubation with either K+, or SCH 28080, or ouabain or vanadate on the AT32P-phosphorylation level is shown in the autoradiograms of the SDS-PAGE gels (Fig. 1). In the SF9 membranes both a 100- and a 140-kD phosphorylated protein are found. The 140-kD protein is also present in membranes of uninfected cells and does not originate from

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FIGURE 1. Autoradiogram of SDS-PAGE of the AT32P phosphorylated Sf9 membranes infected with wild-type and mutated baculoviruses. Membranes isolated from Sf9 cells infected with either wild-type virus or mutant viruses E795Q and E820Q were phosphorylated at 0°C with 0.1 μM [γ-32P]ATP in the presence of 1 mM MgCl₂ and 20 mM Tris-acetic acid (pH 6.0) after preincubation for 60 minutes at 0°C with the following compounds: 100 pM SCH 28080, 10 mM KCl, 1 mM ouabain, and 1 mM vanadate. The acid-quenched samples were solubilized and subjected to SDS-PAGE at pH 6.5. Purified pig gastric H+,K+-ATPase was used as a control.

H⁺,K⁺-ATPase. Formation of the 100-kD phosphorylated intermediate of mutant E820Q is hardly affected by each of the used ligands in contrast to both the wild-type enzyme and mutant E795Q, where these ligands except ouabain prevent formation of the 100-kD band. The K⁺ sensitivity of mutant E795Q (I₅₀ = 0.45 ± 0.10 mM; n = 3) is similar to that of the wild-type enzyme (I₅₀ = 0.38 ± 0.04 mM; n = 5). The I₅₀ value of mutant E820Q, however, is 10 times higher (I₅₀ = 4.5 ± 1.2 mM; n = 4), and complete inhibition is not reached at 30 mM. This mutant has additionally a 100 times lower sensitivity towards SCH 28080. An I₅₀ value of 1.7 ± 0.6 mM (n = 4) was found, whereas the wild-type enzyme has an I₅₀ value of 14 ± 3 nM (n = 4) and mutant E795Q an I₅₀ value of 8 ± 3 nM (n = 4).

Dephosphorylation studies show that the phosphorylated intermediate of the wild-type enzyme is K⁺ sensitive and ADP insensitive. The E795Q mutant demonstrated similar behavior. The phosphointermediate of the E820Q mutant, however, was insensitive towards added K⁺ up to 100 mM. The E820Q mutant also showed no sensitivity for ADP, suggesting that this mutation did not lead to blockade of the E₁-P → E₂-P conversion, which would have resulted in an ADP-sensitive phosphorylated intermediate. The phosphorylated intermediate of the wild-type enzyme and of mutants E795Q and E820Q showed similar hydroxylamine sensitivity, indicating that the K⁺-insensitive mutant E820Q had also formed an acyl-phosphate.

The overall ATPase activity, determined at 10 μM ATP, of both the wild-type enzyme and mutant E795Q could be activated by K⁺ (K₀.₅ = 0.2 mM). In the membranes of uninfected Sf9 cells and mutant E820Q, no activation by K⁺ of the ATPase activity was observed.

The aforementioned findings suggest that Glu795 is not involved in K⁺ binding, but binding of extracellular K⁺ to Glu820 is essential in the long range of conforma-
tional changes that enhance the hydrolysis rate of the phosphorylated intermediate at Asp386.

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