A Hybrid between Na\(^{+}\),K\(^{+}\)-ATPase and H\(^{+}\),K\(^{+}\)-ATPase Is Sensitive to Palytoxin, Ouabain, and SCH 28080*  

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Robert A. Farley‡§, Silvia Schreiber¶, Shyang-Guang Wang‡, and Georgios Scheiner-Bobis¶  

From the ‡Department of Physiology and Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, California 90033 and the ¶Institut für Biochemie und Endokrinologie, Fachbereich Veterinärmedizin, Frankfurter Strasse 100, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany  

Na\(^{+}\),K\(^{+}\)-ATPase is inhibited by cardiac glycosides such as ouabain, and palytoxin, which do not inhibit gastric H\(^{+}\),K\(^{+}\)-ATPase. Gastric H\(^{+}\),K\(^{+}\)-ATPase is inhibited by SCH28080, which has no effect on Na\(^{+}\),K\(^{+}\)-ATPase. The goal of the current study was to identify amino acid sequences of the gastric proton-potassium pump that are involved in recognition of the pump-specific inhibitor SCH 28080. A chimeric polypeptide consisting of the rat sodium pump α3 subunit with the peptide Gln\(^{895}\)–Val\(^{930}\) of the gastric proton pump α subunit substituted in place of the original Asn\(^{886}\)–Ala\(^{911}\) sequence was expressed together with the gastric β subunit in the yeast Saccharomyces cerevisiae. Yeast cells that express this subunit combination are sensitive to palytoxin, which interacts specifically with the sodium pump, and lose intracellular K\(^{+}\) ions. The palytoxin-induced K\(^{+}\) efflux is inhibited by the sodium pump-specific inhibitor ouabain and also by the gastric proton pump-specific inhibitor SCH 28080. The IC\(_{50}\) for SCH 28080 inhibition of palytoxin-induced K\(^{+}\) efflux is 14.3 ± 2.4 μM, which is similar to the K\(_{i}\) for SCH 28080 inhibition of ATP hydrolysis by the gastric H\(^{+}\),K\(^{+}\)-ATPase. In contrast, palytoxin-induced K\(^{+}\) efflux from cells expressing either the native α3 and β1 subunits of the sodium pump or the α3 subunit of the sodium pump together with the β subunit of the gastric proton pump is inhibited by ouabain but not by SCH 28080. The acquisition of SCH 28080 sensitivity by the chimera indicates that the Gln\(^{895}\)–Val\(^{930}\) peptide of the gastric proton pump is likely to be involved in the interactions of the gastric proton-potassium pump with SCH 28080.  

The sodium pump (Na\(^{+}\),K\(^{+}\)-ATPase)\(^1\) and the gastric proton pump (H\(^{+}\),K\(^{+}\)-ATPase) are ion-transporting ATPases, both hydrolyzing ATP to actively pump ions against their electrochemical gradients (1, 2). Unlike other ion-transporting ATPases, they both require a highly glycosylated smaller peptide, the β subunit, for their function, in addition to a larger ATP-recognizing α subunit. The α subunits of proton and sodium pumps have ~60% identical primary sequences and form 10 membrane-spanning domains, denoted M1–M10, with their amino and carboxyl termini localized in the cytosol (3–9). The β subunits are 30% identical and form a single membrane span with the amino terminus on the extracellular side of the membrane. Despite this limited identity of the primary structure, the tertiary structure of both types of β subunits must be very similar, since the proton pump β subunit was shown in numerous investigations to form functional complexes with the α subunit of the sodium pump when these proteins were expressed together (10–14).  

Both enzymes are pharmacological receptors for clinically relevant drugs. Thus, the pumping activity of the gastric proton pump is specifically inhibited by imidazopyridines like 8-benzyloxy-3-cyanomethyl-2-methyl-imidazo[1,2-β],[3,4-γ]pyridine (SCH 28080) or omeprazole (15–17). Derivatives of the latter are widely used clinically for controlling hyperacidity or peptic ulcer (18, 19). The sodium pump is specifically inhibited by a series of naturally occurring steroids, such as ouabain and digoxin (20, 21). Based on their clinical use, these substances are also referred to as cardiac glycosides or cardioactive steroids; their application helps to increase muscular contractility of the failing heart (22–24).  

In recent years, the work of many investigators has been focused on the identification of amino acids or peptides of the proton and sodium pumps involved in interaction with omeprazole and SCH 28080, or ouabain and related cardiac glycosides, respectively. Analysis of a series of sodium pump mutants helped to identify several amino acids involved in the recognition of ouabain (25–28), but little is known about amino acids or peptides of the gastric proton pump involved in the recognition of omeprazole or SCH 28080. Amino acids in both the amino- and carboxyl-terminal halves of the α subunit of gastric H\(^{+}\),K\(^{+}\)-ATPase have been suggested to participate in the binding sites for these inhibitors. Blostein et al. (29) expressed a chimeric polypeptide consisting of the amino-terminal 519 amino acids of gastric H\(^{+}\),K\(^{+}\)-ATPase and the COOH-terminal 507 amino acids of Na\(^{+}\),K\(^{+}\)-ATPase in LLC-PK1 cells and reported inhibition of potassium influx by SCH 28080. Munson et al. (30) labeled a peptide from the M1–M2 region of gastric H\(^{+}\),K\(^{+}\)-ATPase with a photoactive analog of SCH 28080. Labeling experiments have also identified Cys\(^{892}\) within the extracellular loop connecting the M7 and M8 membrane spans of the gastric proton-potassium pump α subunit as a component of the binding site for omeprazole (17). Since omeprazole binding to the proton pump can be inhibited by SCH 28080 (31), this result raises the possibility that amino acids within the COOH-terminal half of the gastric pump α subunit might also partic-
To test this hypothesis, we made use of the highly specific interactions between the sodium pump and palytoxin to investigate whether the M7/M8 extracellular peptide of the gastric proton pump α subunit is involved in SCH 28080 recognition. Palytoxin from marine corals of the genus Palythoa, like the cardioactive steroids, is a specific inhibitor of the sodium pump (32, 33). Unlike the cardioactive steroids, however, which inhibit ion flow through the pump, palytoxin converts the enzyme into an open channel that allows ions to flow down their concentration gradient (34). The palytoxin-induced channels have been found to have a single-channel conductance of about 100 pS (32, 33). Unlike the cardioactive steroids, however, which inactivate membrane-bound catalytic subunits and the gastric proton pump subunit, Palythoa palytoxin-induced channels are not inactivated by ouabain or SCH 28080.

A Hybrid Sensitive to Ouabain, Palytoxin, and SCH28080

To investigate whether SCH 28080 inhibits binding of [3H]ouabain to yeast membranes, the above experiment was repeated at 0, 1, and 10 μM ATP in the presence of 25 μM SCH 28080. All other conditions were the same as described before. To each of the wells 50 μl of a monomeric antibody (22E6) against the gastric proton pump β subunit were added at a 1:2000 dilution in PBS-T and incubation continued for 2 h at room temperature. Unbound antibody was then removed by four washes with PBS-T, and afterward 50 μl of an alkaline phosphatase-conjugated anti-mouse IgG was added to the wells. After 2 h of incubation at room temperature, the wells were washed four times with PBS-T, and then twice with 10 mM diethanolamine, pH 9.5, containing 5 mM MgCl₂. All further steps concerning the addition of the substrate p-nitrophenyl phosphate were carried out as described (42).

Ouabain Binding to Yeast Microsomal Preparations—A total of 250 μg of yeast microsomal protein was incubated for 1 h at 30 °C with 5 mM PO₄ (Tris form), 5 mM MgCl₂, 10 mM Tris/HCl, pH 7.3, and various concentrations of [3H]ouabain. The incubation volume was 250 μl. Thereafter, the protein was pelleted by centrifugation for 5 min at 12,000 × g. After washing twice with 1 ml of ice-cold water, the pellet was dissolved in 250 μl of 1 N NaOH by incubation for 15 min at 80 °C. The NaOH was neutralized with 250 μl of 1 M HCl, and the radioactivity was determined by scintillation counting after the addition of scintillation fluid.

ATP-promoted Binding of [3H]Ouabain—A total of 125 μg of microsomal protein was incubated for 5 min at 30 °C in 10 mM Tris/HCl, pH 7.5, 50 nM [3H]ouabain, 5 mM MgCl₂, 50 mM NaCl, and various concentrations of ATP (Tris form). The total volume of each sample was 250 μl. Thereafter, the protein was pelleted by centrifugation at 12,000 × g for 5 min, washed twice with H₂O at 4 °C, dissolved in 250 μl of 1 M NaOH, and processed as described at the end of the preceding paragraph.

To investigate whether SCH 28080 inhibits binding of [3H]ouabain to yeast membranes, the above experiment was repeated at 0, 1, and 10 μM ATP in the presence of 25 μM SCH 28080. All other conditions were the same as described before.

Palytoxin-induced K⁺ Efflux from Yeast Cells—Effect of SCH 28080 on the ATP-promoted Binding of [3H]Ouabain—Single yeast colonies were incubated at 30 °C in a cell incubator with vigorous shaking overnight in 5 ml of SD growth medium (6.7 g/liter Bacto Yeast nitrogen base without amino acids) supplemented with d-galactose (20 g/liter SD medium) as the only carbon source. The cell suspension was then transferred to 200 ml of SD medium and incubation continued for an additional 24 h under the same conditions. Thereafter, cells were collected by centrifugation for 1 min at 3000 × g, washed twice with 30 ml of NaGHBC (20 mM NaCl, 100 mM glucose, 10 mM HEPES, 0.5 mM boric acid, 1 mM CaCl₂, adjusted to pH 7.5 with Tris), and suspended in NaGHBC to a final concentration of 10⁶ cells/ml (A₅₄₀ = 6).

For the measurement of the palytoxin-induced K⁺ loss from yeast cells, 450 μl of the cell suspension were incubated with various concentrations of palytoxin in the absence or presence of ouabain or SCH 28080 for 2 h at 30 °C. Since SCH 28080 was dissolved in dimethyl sulfoxide:ethanol (1:1, v/v), the content of the solvent was kept constant in all samples; the final volume of each sample was 500 μl. To determine the total cellular K⁺ content (100% value), 450 μl of the yeast cell suspension were incubated for 1 h at 70 °C with 50 μl of 1% lithium dodecyl sulfate (w/v). Afterward, cells were centrifuged at 12,000 × g for 2 min in a bench-top centrifuge. The supernatants were collected and measured for their K⁺ content by flame photometry.

Initial rates of palytoxin-induced K⁺ efflux were measured using a flow ionometer. Yeast cells were suspended at a concentration of 15 × 10⁶ cells/ml in NaGHBC buffer containing 0.2, 0.5, or 5 μM palytoxin for cells expressing NKh3/HHK; 0.5, 2, or 5 μM palytoxin for cells expressing NKh3/HHK; and 1, 2.5, or 5 μM palytoxin for cells expressing the NGH26/HHK hybrid. The total volume of the solution was 1 ml. Using

The efflux from yeast cells that express a hybrid sodium pump consisting of a sodium pump α subunit and a gastric proton pump β subunit? Does a similar phenomenon occur when yeast cells express a chimeric sodium pump α subunit and a gastric proton pump β subunit? Does a similar phenomenon occur when yeast cells express a chimeric sodium pump α subunit and a gastric proton pump β subunit? Does a similar phenomenon occur when yeast cells express a chimeric sodium pump α subunit and a gastric proton pump β subunit?
FIG. 1. Scatchard analysis of [3H]ouabain binding. Membranes isolated from yeast cells expressing the NK3/NKβ1 (○) or either of the chimeric proteins NK3/HKβ (■) and NGH26/HKβ (▲) were incubated in the presence of 5 mM phosphate, 5 mM MgCl2, and various concentrations of [3H]ouabain for 60 min at 30 °C as described under "Experimental Procedures." Afterward, bound [3H]ouabain was measured by scintillation counting. Nonspecifically bound [3H]ouabain was determined by including 3 mM nonradioactive ouabain in the assay. The figure shows one representative experiment.

RESULTS

Binding of [3H]ouabain—In the presence of phosphate (P) and Mg2+, Na+-K+-ATPase (E2) becomes phosphorylated (E2-P) and binds ouabain with high affinity [E2-P-Ouabain]. Use of radioactive ouabain enables one to determine the affinity of the enzyme for ouabain under these conditions. Thus, as expected, the rat wild-type sodium pump α3 and β1 subunits (NK3/NKβ1) in microsomal yeast membranes bind [3H]ouabain with high affinity (Fig. 1). The Kd determined from a Scatchard plot is 7.7 ± 0.3 nM (Table II). A similar affinity for ouabain was obtained with membranes from cells expressing the NK3/HKβ subunits (Kd of 6.4 ± 1.4 nM). The Bmax for ouabain binding to the NK3/HKβ hybrid (0.85 ± 0.07 pmol/mg) is slightly reduced compared with that of the wild-type sodium pump (1.1 ± 0.02 pmol/mg) (Table II). Ouabain binding to microsomal membranes from cells expressing the chimeric sodium pump α subunit (NGH26) together with the sodium pump β1 subunit (NKβ1) is almost undetectable (data not shown).

Replacement of the sodium pump βa by the proton pump β subunit results in the formation of NGH26/HKβ complexes capable of binding ouabain (Fig. 1). The equilibrium dissociation constant Kd for ouabain binding to the NGH26/HKβ heterodimer (21.1 ± 2.5 nM; Table II) is slightly increased compared with complexes assembled with NK3, indicating a reduction in affinity. In addition, the maximum amount of ouabain bound by NGH26/HKβ is also reduced (0.18 ± 0.01 pmol/mg), accounting for only a fraction (17%) of the total binding obtained with the wild-type sodium pump (Fig. 1; Table II).

Expression of NK3, NGH26, and HKβ Subunits in Yeast—To investigate the relative level of expression of the α3 and NGH26 subunits, microsomal proteins isolated from transformed yeast were probed in a Western blot with a monoclonal antibody raised against the sodium pump α subunit. Fig. 2 shows that both subunits are expressed in the yeast in comparable quantities. As shown in the same figure, the antibody does not recognize any protein of ~100 kDa corresponding to the α3 or NGH26 subunit in microsomes isolated from nontransformed yeast cells.

The expression levels of the gastric proton pump β subunit (HKβ) were measured in yeast membrane preparations from cells expressing either NK3/HKβ or NGH26/HKβ using a monoclonal antibody raised against the gastric proton pump β subunit. The Western blot presented in Fig. 3A shows that the antibody recognizes two protein bands of about 40 and 43 kDa in membranes from cells expressing the NK3/HKβ or NGH26/HKβ heterodimers. Since corresponding bands are not detected in membranes from nontransformed cells, these bands at 40 and 43 kDa are gastric pump β subunits, possibly glycosylated to various extents. It is apparent from the figure that the abundance of the HKβ subunit in membrane preparations from cells expressing the NK3/HKβ complex is higher than in membranes from cells expressing the NGH26/HKβ hybrid.

Quantification of the HKβ subunit expression levels in yeast microsomes from cells expressing either the NK3/HKβ or the NGH26/HKβ hybrid cannot easily be addressed by the Western blotting method. This was done instead by applying a variation of an antigen capture assay (42). In this assay the antigen (here HKβ) is affixed to the wells of a microtiter plate. This affixed antigen is then incubated with an antibody specific for the antigen (here antibody 22E6 against HKβ). Subsequently, an alkaline phosphatase-conjugated secondary antibody (here an anti-mouse IgG) is added to the well. With sufficient washes between additions, the antigen, the primary antibody, the secondary antibody, and alkaline phosphatase should be present in equal amounts. Thus, the amount of p-nitrophenyl phosphate hydrolyzed by the alkaline phosphatase is directly pro-
duced by the alkaline phosphatase can be used as measure of
and, therefore, the amount of the
preparations at saturating levels of ATP are nearly identical
Mg2+

FIG. 3. Immunodetection of HKβ subunits. A, microsomal pro-

teins from untransformed yeast (lane 1), or yeast expressing either
NGH26/HKβ (lane 2) or NKa3/HKβ (lane 3) heterodimers were sepa-
rated by SDS-polyacrylamide gel electrophoresis and then electrotrans-

tferred onto nitrocellulose membranes. Expressed proteins were visual-

ized by using the monoclonal antibody 2/2E6 against HKβ and the
enhanced chemiluminescence Western blot analysis system. B, a total
of 35 µg of microsomal protein was attached to the bottom of the wells
of microtiter plates and was incubated first with the 2/2E6 antibody
against HKβ and then with an alkaline phosphatase-conjugated sec-

ondary antibody. After either 40 or 60 min of incubation with
-p-ri

trophosphosphate, the p-nitrophosphate anion was determined by meas-

uring the absorbance at 405 nm using a molar absorbance coefficient
ε = 18,500 liters/mol/cm. Microsomal protein from nontransformed cells
served as a control, and values obtained with the control microsomes
were subtracted from the rest.

porational to the number of HKβ subunits present in each well,
and, therefore, the amount of the p-nitrophosphate anion pro-
duced by the alkaline phosphatase can be used as measure of
the relative abundance of HKβ subunits.

Fig. 3B shows the results of this experiment. After either 40
or 60 min of incubation with substrate, the amount of the
p-nitrophosphate anion formed in samples containing the HKβ
from cells expressing the NGH26/HKβ heterodimers accounts
for only 13–17% of the value obtained from the microsomes from
cells expressing the NKa3/HKβ subunit combination.

Binding of [3H]Ouabain in the Presence of ATP, Na+, and
Mg2+—The sodium pump can be phosphorylated either by
inorganic phosphate in the presence of Mg2+ (the conditions of
the experiment in Fig. 1) or by ATP in the presence of Na+ and
Mg2+ (44). In both cases, ouabain binds to the E2·P confor-
mational state of the enzyme and forms a stable and easily measur-
able [E2·P·Ouabain] complex. By measuring the formation of the
[E2·P·Ouabain] complex as a function of the ATP concentra-
tion, one can determine whether ATP is hydrolyzed to yield
the phosphoenzyme, and, if so, it is possible from these experi-
ments to obtain an EC50 value indicating the relative affinity
of the enzyme for ATP (43, 45).

Fig. 4 shows that ATP promotes binding of [3H]Ouabain to
membranes containing the NKa3/NKβ1 heterodimer with an
EC50 of 3.9 ± 0.16 µM. Similarly, ATP promotes binding to the
NKa3/HKβ heterodimer with an EC50 of 1.05 ± 0.16 µM. The
Rmax values for [3H]Ouabain binding to these two membrane
preparations at saturating levels of ATP are nearly identical
(0.76 pmol/mg). The EC50 for [3H]Ouabain binding to yeast
membranes containing the NGH26/HKβ heterodimer in an
ATP-dependent reaction is 1.13 ± 0.51 µM; the maximum
amount of ouabain bound, however, −0.13 pmol/mg, is 17% of
the amount bound by either NKa3/NKβ1 or NKa3/HKβ.

Palytoxin-induced Potassium Efflux from Yeast Cells—Yeast
cells expressing the NKa3/NKβ1, NKa3/HKβ4, or NGH26/HKβ
subunits were incubated for 2 h with various concentrations of
palytoxin. Thereafter, cells were removed by centrifugation and K+
in the supernatant was determined by flame photometry.
As shown in Fig. 5, the interaction of palytoxin with the
NKa3/NKβ1, NKa3/HKβ, and NGH26/HKβ complexes results
in a loss of up to 80% of cytosolic K+ levels in yeast. The EC50
value for the palytoxin-induced K+ efflux from cells expressing
the NKa3/NKβ1 subunits is 136 nM. The corresponding value
obtained with cells expressing the NKa3/HKβ subunits is approx-
imately 2-fold higher (313 nM), and the EC50 obtained with
cells expressing the NGH26/HKβ subunits is about 6-fold
higher (822 nM) than for NKa3/NKβ1. Palytoxin has no effect
on nontransformed yeast cells and only a small loss of K+
is observed from yeast cells that express the chimera NGH26
subunit together with the rat sodium pump β1 subunit (data

not shown).

To estimate the initial rates of the palytoxin-induced efflux,
cells expressing either NKa3/NKβ1, NKa3/HKβ, or NGH26/
HKβ complexes were incubated at various concentrations of
palytoxin (see “Experimental Procedures”) and K+ was mea-

sured in the extracellular medium using a flow ionometer.
During the period of recording, the initial rate of K+ efflux (V0)
at each palytoxin concentration was linear and increased with
increasing palytoxin concentrations. The rate of efflux can be
described by the formula

\[
V0 = kN \cdot k \cdot V_{max}
\]

where k is the single channel conductance, N is the number of channels and is
equal to the Bmax for ouabain binding, and P0 is the probability
that the channels are open. P0 = kA, where A is the concen-
tration of palytoxin, the exponent x is the number of palytoxin
molecules required to open the channel, and k is a constant of
proportionality. A plot of \( V_{in} \) versus logA showed a straight
line with the slope near 1 for all subunit combinations, indic-
ating that the channels are opened by the binding of a single
palytoxin molecule. Substituting \( k' = k \cdot k' \cdot k \cdot N \) \( V_{in} \)
versus A resulted in a straight line for each heterodimer with
a slope of k′N. Values of k′N for a given αβ heterodimer were not
shown in Fig. 7, only cells expressing the NGH26/HKβ heterodimer were incubated for 120 min with various concentrations of palytoxin as described under “Experimental Procedures.” Cells were then centrifuged, and the K⁺ concentration in the supernatant determined by flame photometry. Percentage of total refers to [K⁺] calculated as a percentage of [K⁺] measured in samples treated with lithium dodecyl sulfate.

Inhibition of Palytoxin-induced Potassium Efflux by Ouabain and SCH 28080—The palytoxin-induced K⁺ efflux from yeast cells expressing α1/β1 subunits of the sodium pump has been shown to be completely inhibited by ouabain (37). Similarly, the palytoxin-induced K⁺ efflux from yeast cells expressing any of the subunit combinations NKα3/NKβ1, NKα3/HKβ, or NGH26/HKβ is also inhibited by ouabain (Fig. 6). At 400 nM palytoxin, ouabain inhibits the K⁺ efflux from cells expressing the NKα3/NKβ1 heterodimer with IC₅₀ = 26.5 ± 0.1 μM (Table II). Comparable results are obtained with cells expressing either NKα3/HKβ or NGH26/HKβ heterodimers; ouabain inhibits the palytoxin-induced K⁺ efflux with IC₅₀ values of 23.1 ± 3.4 μM and 27.6 ± 1.9 μM, respectively (Table II).

To investigate the effect of the gastric proton pump-specific inhibitor SCH 28080, yeast cells expressing NKα3/NKβ1, NKα3/HKβ, or NGH26/HKβ complexes were incubated at 400 nM palytoxin with various concentrations of SCH 28080. After an incubation period of 2 h, K⁺ in the supernatant was determined as described under “Experimental Procedures.” As shown in Fig. 7A, only cells expressing the NGH26/HKβ complexes are sensitive to SCH 28080. The palytoxin-induced K⁺ efflux from these cells is almost completely inhibited by 50 μM SCH 28080 with an IC₅₀ of 14.3 ± 2.4 μM (Fig. 7B).

DISCUSSION

Because of their importance as receptors for clinically relevant drugs, much effort has been put into identifying sequences of the proton and sodium pumps that participate in the recognition of omeprazole, SCH 28080, and ouabain and related cardiac steroids. Analysis of the properties of sodium pump mutants allowed for the identification of several sites involved in recognition of ouabain (27, 28). Comparatively little is known, however, about amino acids or peptides involved in the binding of omeprazole or SCH 28080 to the gastric proton pump. The omeprazole binding site contains Cys⁸⁰⁶ within the extracellular loop connecting the M7 and M8 membrane spans of the proton pump α subunit (17). Since omeprazole binding to the proton pump can be inhibited by SCH 28080 (31), it is possible that the binding sites for both substances are identical or in close proximity to each other. Thus, the M7/M8 extracellular peptide of the gastric pump α subunit might be part of the binding site for SCH 28080. This peptide corresponds to a sequence of the sodium pump α subunit that has been shown to contain amino acids involved in the binding of ouabain (28). The corresponding region of the rat gastric proton pump α subunit contains the peptide Gin⁹⁰⁵–Val⁹³⁰, which is involved...
in assembly with the gastric proton pump \( \beta \) subunit (13), much as the corresponding Asn\(^{866-\text{Ala}^{911}} \) peptide of the sodium pump \( \alpha \) subunit is involved in assembly with the sodium pump \( \beta \) subunit (46).

To investigate a possible involvement of the M7/M8 extracellular peptide of the gastric proton pump \( \alpha \) subunit in SCH 28080 recognition, wild-type sodium pump \( \alpha \) subunits or \( \alpha \) subunit chimeras containing Gln\(^{805-\text{Val}^{830}} \) of the gastric proton pump \( \alpha \) subunit were coexpressed with either sodium or proton pump \( \beta \) subunits in yeast (Table I) and were investigated with respect to their ability to recognize either the sodium pump-specific inhibitors ouabain and palytoxin or the gastric proton pump-specific inhibitor SCH 28080.

**Ouabain Binding**—Microsomal membranes prepared from yeast cells expressing the various subunit combinations bind \(^{3}H\)ouabain with high affinity (Fig. 1; Table II). The NGH26/HK\( \beta \) heterodimer was characterized by a reduced ouabain binding affinity (\( K_d = 21.1 \pm 2.5 \) nM; Table II), and a lower maximum ouabain binding capacity (0.18 \( \pm \) 0.01 pmol/mg) than the NKo3/NKβ complex. The ouabain binding capacity of the NGH26/HK\( \beta \) heterodimer is only 17% of the total binding obtained with the wild-type sodium pump (Fig. 1; Table II), and is similar to the expression level that has previously been reported for the NGH26/HK\( \beta \) complex (13). To investigate the reasons for the lower ouabain binding capacity, the expression levels of the NKo3, NGH26, and HK\( \beta \) were measured in microsomal preparations by Western blots and antibody capture assays. Fig. 2 shows that the level of expression of the NGH26 subunit is approximately the same as the level of expression of the \( \alpha \) subunit. Fig. 3, however, indicates that HK\( \beta \) is present in microsomal membranes from yeast cells expressing the NGH26/HK\( \beta \) complex at lower levels than HK\( \beta \) is present in microsomes from yeast expressing NKo3/HK\( \beta \). Results of the antibody capture assay indicate that only about 13–17% of the amount of \( p \)-nitrophenolato obtained from NKo3/HK\( \beta \) was formed by membranes containing the NGH26/HK\( \beta \) complex (Fig. 3B). Thus, it appears that the reduced expression level of HK\( \beta \) in membranes from cells expressing the NGH26/HK\( \beta \) heterodimer limits the number of NGH26/HK\( \beta \) complexes, and the low level of ouabain binding shown in Fig. 1 is a measure of the number of these complexes in the membranes. The limiting effect of HK\( \beta \) expression on the number of assembled pumps can be explained by the requirement of the expression system for two different plasmids to direct the synthesis of the \( \alpha \) and \( \beta \) subunits independently in the yeast cells. Transformation of yeast with the two plasmids results in cells with different copy numbers for the two plasmids.

| Vector used for transformations | Protein expressed in yeast | Combined with vector | Enzyme expressed in yeast |
|---------------------------------|---------------------------|----------------------|-------------------------|
| YEprNGH26                       | NGH26 = rat sodium pump \( \alpha \) subunit | pGIT-rβ1             | NKo3/NK\( \beta \)      |
| pGIT-rβ1                        | NKβ1 = rat sodium pump \( \beta \) subunit    | YEprNGH26            | NGH26/NK\( \beta \)     |
| pGIT-HK\( \beta \)              | HK\( \beta \) = rat gastric proton pump \( \beta \) subunit | YEprNGH26            | NKo3/HK\( \beta \)      |

Values in Table II were obtained by taking the recording period represents a steady state between K\(^+\) efflux through the palytoxin-induced channel and K\(^+\) influx through the yeast TRK1 and TRK2 uptake pathways. At the end of the incubation period.
period with saturating palytoxin, the steady-state levels of K\(^+\) in the cells expressing different \(\alpha\beta\) complexes were the same. The concentration of palytoxin required for half-maximal loss of K\(^+\) under these conditions, however, is different for each complex (Table II). The EC\(_{50}\) for the palytoxin-induced K\(^+\) efflux from cells expressing the NKo3/NK\(\beta_1\) subunits is 136 nM. The corresponding value obtained with cells expressing the NKo3/HK\(\beta_1\) subunits is approximately 2-fold higher (313 nM), and the EC\(_{50}\) obtained with cells expressing the NGH26/HK\(\beta_1\) subunits is about 6-fold higher (822 nM) than for NKo3/NK\(\beta_1\). These differences in EC\(_{50}\) values may be explained in several ways. First, the affinity of the complexes containing HK\(\beta_1\) for palytoxin may be reduced compared with NKo3/NK\(\beta_1\) due to distortion of the palytoxin binding site by assembly of the \(\alpha\) subunits with HK\(\beta_1\). Alternatively, the rate of K\(^+\) ion conduction through heterodimers containing different combinations of \(\alpha\beta\) subunits may be different. Finally, the different EC\(_{50}\) values may reflect the different numbers of functional complexes present in the cells expressing different heterodimers. Although it was not possible to test for reduced palytoxin binding affinity since radiolabeled palytoxin is not available, analysis of the initial rates of K\(^+\) efflux from the cells at various palytoxin concentrations shows that a difference in the number of channels is sufficient to explain the data. From the data presented in Table II it can be seen that dividing \(k^'N\) by the number of channels (\(B_{max}\)) yields nearly identical values of the apparent single-channel conductance \(k^'\) for all of the \(\alpha\beta\) heterodimers. These values are 0.23 ± 0.04 for NKo3/NK\(\beta_1\) (0.17 ± 0.09 for NKo3/HK\(\beta_1\), and 0.27 ± 0.02 for NGH26/HK\(\beta_1\). The similarity of these values indicates that the product of the single channel conductance (\(k^'N\)) and the constant of proportionality \(k\) between palytoxin concentrations and the open channel probability is the same. Although values for each of these constants cannot be determined independently from these measurements, it is possible that the single-channel conductance and the open probability are similar for all of the heterodimers.

**Inhibition of Palytoxin-induced Potassium Efflux by Ouabain and SCH28080**—The palytoxin-induced K\(^+\) efflux from yeast cells expressing any of the subunit combinations NKo3/NK\(\beta_1\), NKo3/HK\(\beta_1\), or NGH26/HK\(\beta_1\) is completely inhibited by ouabain (Fig. 6). At 400 nM palytoxin, ouabain inhibits the K\(^+\) efflux from cells expressing the different heterodimers with IC\(_{50}\) values of 23–28 \(\mu\)M (Table II). The small differences between the IC\(_{50}\) values for ouabain inhibition of palytoxin-induced K\(^+\) efflux in the different heterodimers is similar to the pattern of \(K_d\) values for ouabain binding to the heterodimers (13).

As expected from the known insensitivity of Na\(^+\),K\(^+\)-ATPase to SCH28080, there was <10% inhibition by SCH28080 of K\(^+\) efflux induced by 400 nM palytoxin from yeast cells expressing either the NKo3/NK\(\beta_1\) or the NKo3/HK\(\beta_1\) subunits (Fig. 7A). In contrast, the palytoxin-induced K\(^+\) efflux from yeast cells expressing the NGH26/HK\(\beta_1\) subunits is almost completely inhibited by 50 \(\mu\)M SCH28080. The IC\(_{50}\) value for the SCH28080 inhibition of the palytoxin-induced K\(^+\) efflux is 14.3 ± 2.4 \(\mu\)M (Fig. 7B). This value should be compared with the IC\(_{50}\) values determined for the inhibition of proton transport into porcine gastric vesicles (0.5 \(\mu\)M) or for SCH28080 inhibition of aminopyrine uptake in rabbit gastric glands (0.2 \(\mu\)M) (16). Thus, sensitivity to SCH28080 can be conferred upon Na\(^+\),K\(^+\)-ATPase by substitution of 26 amino acids from gastric H\(^+\),K\(^+\)-ATPase (Hn\(^{905}-\)Val\(^{930}\)) for the homologous residues in the extracellular loop connecting transmembrane segments 7 and 8 of Na\(^+\),K\(^+\)-ATPase a3 subunit.

It is not known whether the extracellular loop connecting transmembrane segments 7 and 8 is directly involved in binding SCH28080 or whether the binding site is induced elsewhere in the protein as a result of chimera formation. If the extracellular loop is part of the binding site, it is likely that the binding site also consists of additional parts of the polypeptide. Using chimical ATPases, Blostein et al. (29) concluded that the amino-terminal half of the gastric proton pump must be involved in SCH28080 binding, and this is in good agreement with the results of Munson et al. (30), who labeled a 9.9-kDa tryptic peptide starting at Gln\(^{104}\) of the gastric proton pump a subunit with a photoactivated derivative of SCH28080. An amino-terminal localization of the SCH28080 binding site was further supported by the observation that the rat gastric proton pump peptide Val\(^{115}\)-Ile\(^{126}\) from the M1 membrane-spanning domain confers SCH28080 sensitivity to the sodium pump (11). In that report, however, SCH28080 did not inhibit palytoxin-induced K\(^+\) efflux from yeast cells expressing this chimera. The results of that investigation have recently been questioned by Asano et al. (48), however, who found that replacement of the Val\(^{115}\)-Ile\(^{126}\) sequence of gastric H\(^+\),K\(^+\)-ATPase with the corresponding region of Na\(^+\),K\(^+\)-ATPase a subunit did abolish SCH28080 sensitivity. A possible explanation for this apparent discrepancy is suggested by the possibility that SCH28080 binding is induced indirectly by chimera formation and that the different chimeras have different properties because of their different sequences. Thus, mutations within Val\(^{115}\)-Ile\(^{126}\) do not necessarily have to preclude interactions of SCH28080 with the protein, since the other points of attachment are possibly enough to stabilize binding. Asano et al. implicated Thr\(^{265}\) and Pro\(^{323}\) as possible determinants of the affinity of the enzyme for SCH28080 (48), together with Gly\(^{326}\) in the M6 membrane-spanning domain of gastric H\(^+\),K\(^+\)-ATPase (49).

The finding that the extracellular peptide Gln\(^{905}-\)Val\(^{930}\) of the proton ATPase confers SCH28080 sensitivity to Na\(^+\),K\(^+\)-ATPase is consistent with previous findings demonstrating that SCH28080 competes with omeprazole and that omeprazole reacts with Cys\(^{892}\) from the M7/MS connecting loop (31, 50). A recent investigation suggested that the binding sites for omeprazole and SCH28080 are not identical but do overlap (51). Taking our results into consideration, one can imagine that the interaction of SCH28080 with the Gln\(^{905}-\)Val\(^{930}\) peptide reduces the accessibility of Cys\(^{892}\) for omeprazole. Furthermore, conformational changes induced by either inhibitor binding or chimera formation may easily account for participation of other benzimidazole-reactive cysteines in SCH28080 binding.

### Table II

**Summary of results determined for yeast expressing various sodium/proton pump subunit combinations described in text**

| \(K_d\), ouabain | \(B_{max}\), ouabain | EC\(_{50}\), ATP | EC\(_{50}\), palytoxin | \(k^'N\) | IC\(_{50}\), ouabain | IC\(_{50}\), SCH28080 |
|-----------------|---------------------|----------------|---------------------|---------|----------------|------------------|
| NKo3/NK\(\beta_1\) | 7.7 ± 0.3 | 1.10 ± 0.02 | 3.90 ± 0.16 | 136.4 | 0.25 ± 0.02 | 26.5 ± 0.1 |
| NKo3/HK\(\beta_1\) | 6.5 ± 1.4 | 0.85 ± 0.07 | 1.05 ± 0.16 | 313.3 | 0.14 ± 0.02 | 23.1 ± 0.4 |
| NGH26/HK\(\beta_1\) | 21.1 ± 2.5 | 0.18 ± 0.01 | 1.18 ± 0.51 | 822.0 | 0.05 ± 0.01 | 27.6 ± 1.9 |

\(\pm\) indicates very low affinity.
and Cys892, the fact that both SCH 28080 and omeprazole inhibit H\(^+\) secretion catalyzed by the proton pump, and the demonstration that SCH 28080 inhibits the palytoxin-induced K\(^+\) efflux from cells expressing the NGH26/HK1 heterodimer implicates the M7/M8 loop of a subunit in ion conduction by the proton pump. The homologous region of the sodium pump has also been suggested to be involved in ion binding, occlusion, or translocation (34, 45, 52). Further investigation of this possibility and the identification of additional peptides and amino acids within the Gln205–Val330 sequence that are important for interactions between the protein and the transported ions, will require additional experiments with new chimeras and mutants. The approach developed in this study may be useful, therefore, in elucidating the mechanisms that lead to SCH 28080 inhibition of H\(^+\) secretion by the gastric proton pump.

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A Hybrid between Na\(^+\),K\(^+\)-ATPase and H\(^+\),K\(^+\)-ATPase Is Sensitive to Palytoxin, Ouabain, and SCH 28080

Robert A. Farley, Silvia Schreiber, Shyang-Guang Wang and Georgios Scheiner-Bobis

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