Identification of the K efflux channel coupled to the gastric H-K-ATPase during acid secretion

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Lambrecht, Nils W. G., Iskandar Yakubov, David Scott, and George Sachs. Identification of the K efflux channel coupled to the gastric H-K-ATPase during acid secretion. Physiol Genomics 21: 81–91, 2005.doi:10.1152/physiolgenomics.00212.2004.—Genomic microarray analysis of genes specifically expressed in a pure cell isolate from a heterocellular organ identified the likely K efflux channel associated with the gastric H-K-ATPase. The function of this channel is to supply K to the luminal surface of the pump to allow H for K exchange. KCNQ1-KCNE2 was the most highly expressed and significantly enriched member of the large variety of K channels expressed in the gastric epithelium. The function of this K channel in acid secretion was then shown by inhibition of secretion in isolated gastric glands with specific KCNQ inhibitors and by colocalization of the channel with the H-K-ATPase in the secretory canaliculus of the parietal cell. KCNQ1-KCNE2 appears to be the K efflux channel that is essential for gastric acid secretion.

There are many cell-specific physiological pathways in various organs for which the actual proteins involved remain unknown. This is particularly true if such a pathway can be catalyzed by any member of a large family of proteins expressed in the organ and highly selective inhibitors are not available. mRNA expression analysis of one specific cell type compared with the original organ may identify a specifically enriched and highly expressed gene compared with all other members of a functional group. The protein encoded by this gene can then be studied functionally, and the results should demonstrate properties that are essential for the pathway in question. An example is the large variety of K channels expressed in different cell types of the stomach, one or more of which may be required for acid secretion by the gastric parietal cell.

Hence, we have purified rat parietal cells to homogeneity and screened for the expression of 82 members of the K channel family by microarray analysis. We found 10 different K channel subunit mRNAs that are expressed in the gastric epithelium, three of which are significantly enriched in parietal cells. Functional assays in gastric glands in the absence and presence of various K channel inhibitors allowed identification of the particular K channel that is required for acid secretion by the gastric H-for-K exchange ATPase.

The parietal cell of the human stomach is responsible for the regulated secretion of 1–2 liters of ~100 mM HCl per day. The gastric H-K-ATPase translocates cytoplasmic protons across the membrane of the secretory canaliculus of the stimulated parietal cell in exchange for exoplasmic K. Proton transport does not occur in the absence of luminal K. The absolute requirement of luminal K for gastric ATPase activity requires the establishment of a large cytoplasmic-to-luminal efflux of K during acid secretion that occurs along with stimulation of acid secretion by the ATPase. It has been shown that a K conductance is present and colocalized with the ATPase in vesicles derived from the canalicular membrane of stimulated parietal cells. This conductance is absent in vesicles prepared from resting parietal cells. Direct electrophysiological methods to elucidate the nature of the K-conducting pore at the canaliculus of the parietal cells have not been successful, mainly because of the infolding of the membrane structure deep inside stimulated parietal cells, as seen in electron microscopic images. This makes this membrane very difficult to access by patch-clamp methods. On the other hand, cell-attached patch-clamp measurements on the basolateral membranes of parietal cells have revealed two distinct classes of predominantly inward-rectifying K channels that are activated by either calcium or cAMP. These channels most likely accomplish the uptake of K from the blood driven by the ~60-mV serosal membrane potential to compensate for the ~15 mM K that is lost in gastric juice with acid secretion.

Hence, the nature of the channel responsible for the K efflux across the secretory membrane of parietal cells remains controversial. Four different members of the K channel family have been proposed as the principle K channel at this site (7, 10, 16, 18), largely based on immunohistochemical localization studies in the gastric mucosa. The present study combines a novel method of parietal cell purification to homogeneity and screened for the expression of 82 members of the K channel family by microarray analysis. We found 10 different K channel subunit mRNAs that are expressed in the gastric epithelium, three of which are significantly enriched in parietal cells. Functional assays in gastric glands in the absence and presence of various K channel inhibitors allowed identification of the particular K channel that is required for acid secretion by the gastric H-for-K exchange ATPase.

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7.8) containing 10 µg/µl Pronase E from Streptomyces griseus (Roche Diagnostics) was injected. All incubation steps were performed in a beaker containing 50 ml of solution at 37°C with continuous stirring. The stomachs were first incubated for 30 min in solution A, which allowed Pronase to diffuse from the serosal to the mucosal side of the gastric wall. The proteolytic activity during this incubation is low because of the presence of EDTA and the absence of Ca²⁺ or Mg²⁺ ions. The solution was discarded, and the stomachs were incubated for 10 min in solution B (same as solution A, but without EDTA and with 10 mM Ca²⁺ and 15 mM Mg²⁺, pH 7.4). The digest removes the top layers of the mucosa (mainly mucous cells). The stomachs were placed back into solution A and incubated for a second 30 min. After this solution was discarded, a final digestion was performed by placing the stomachs in solution B for 10 min to release most of the gastric mucosal cells. The cells were filtered through a nylon sieve, washed twice in solution C (140 mM NaCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 10 mM HEPES, 11 mM glucose, 100 mg/l DTT, and 0.5 g/l BSA, pH 7.4), and concentrated to a total volume of 8 ml. This suspension was injected into a zonal rotor of an elutriator (Beckman) spinning at 2,000 rpm with a counter flow rate of 13 ml/min. After stabilization of the suspension in the chamber of the rotor, the speed was reduced to 1,600 rpm with a concurrent gradual increase of flow rate to 22 ml/min to remove cell debris and small cells. Middle-to-large cells were collected by increasing the flow rate of the pump to 45–65 ml/min. Approximately 100–150 ml were collected. Cells were pelleted by centrifugation in a Sorvall centrifuge for 5 min at 850 rpm. Cells were washed twice in solution C and concentrated to a total volume of 2–3 ml. This suspension was loaded on top of a nycodenz (Accurand; Accurate Chemical and Scientific) gradient containing three layers of 13.75, 9.2, and 5% nycodenz in solution C (wt/vol) and spun at 1,100 rpm for 8 min in the Sorvall centrifuge. Cells on top of the 9.2% layer were collected, and the content of parietal cells (PCs) was determined by a counting of cells staining positive with a generous gift from Dr. A. Smolka) and standard immunohistochemical procedures, as outlined elsewhere (13).

Fluorescence-assisted cell sorting of 80% purified rat PCs. Purified PC suspensions (see above) were further purified using the UCLA Flow Cytometry Core and the FACStar PLUS service. Excitation wave length was 488 nM; sorting was achieved by gating cells, using an emission of 532 nM, and forward scatter (size of cells). Cells were pelleted by centrifugation in a Sorvall centrifuge for 5 min at 850 rpm. Cells were washed twice in solution C and concentrated to a total volume of 8 ml. This suspension was injected on top of a nycodenz (Accurand; Accurate Chemical and Scientific) gradient containing three layers of 13.75, 9.2, and 5% nycodenz in solution C (wt/vol) and spun at 1,100 rpm for 8 min in the Sorvall centrifuge. Cells on top of the 9.2% layer were collected, and the content of parietal cells (PCs) was determined by a counting of cells staining positive with a generous gift from Dr. A. Smolka) and standard immunohistochemical procedures, as outlined elsewhere (13).

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cRNA labeling and rat expression oligonucleotide microarray hybridization. Fresh resected rat stomachs were opened, and the oxyntic mucosa was scraped of the stomach wall by using the sharp edge of a slide. The fragments were filtered through a nylon sieve in 1.5 ml of RNAlater (Quiagen) and homogenized on ice. Total RNA from this homogenate, from purified suspensions off the nycodenz gradient or from cell suspensions obtained from fluorescence-assisted cell sorting (FACS), was isolated using a NucleoSpin RNA II kit (BD Biosciences). The typical RNA concentration was 400–500 ng/µl. The RNA was assessed regarding purity and stability, using a Bioanalyzer 2100 (Agilent Technologies).

Fluorescently labeled cRNA was generated, using a reverse transcriptase (RT) reaction with a poly(dT)-T7 promoter primer followed by T7 polymerase-based linear amplification in the presence of fluorophor-labeled nucleotides Cy3- or Cy5-CTP, according to the manufacturer’s protocol (Low RNA Input Fluor Linear Amp kit, Agilent Technologies). The final cRNA concentration, typically 300–500 ng/µl, and the Cy3- or Cy5-cytidine incorporation of 5–10 pmol/µg cRNA were determined using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies). Three and one-half micrograms of labeled cRNA from whole gastric mucosal epithelium were combined with labeled cRNA from each purified cell suspension and hybridized to a 22K rat oligonucleotide expression array (Agilent Technologies), according to the manufacturer’s protocol. Each set of experiments (n = 3) contained one dye swap experiment. A total of six arrays were hybridized. The microarray was scanned and the intensities normalized over background with the use of a microarray scanner from Agilent Technologies, including proprietary software. The microarray data were deposited for public access to the Gene Expression Omnibus National Center for Biotechnology Information database under accession numbers GPL1439, GSM30415, GSM30416, and GSM30417.

RT-real-time quantitative polymerase chain reaction. The same RNA sample used for labeled cRNA generation for microarray hybridization was also used in the RT-real-time quantitative polymerase chain reaction (qPCR). Two to four micrograms of total RNA were added to the RT reaction by use of Omniscript (Quiagen) RT and an oligo(dT) 12–18 primer (Invitrogen), according to the to the manufacturer’s protocol. Two microliters of RT product were then added to a qPCR reaction together with the Dynamo SYBR Green qPCR kit (Finnzymes, Espoo, Finland), according to the manufacturer’s protocol. The primers used are shown in Table 1.

Real-time qPCR was performed in six-well strips using a DNA Engine Opticon 2 unit (MJ Research). The cycle of threshold (Ct) is determined as the fluorescent signal (binding of SYBR Green to double-stranded cDNA) of one standard deviation over background. The efficiency of each primer pair was also measured by cloning each PCR product into pCR-4-TOPO cloning vector (Invitrogen) and amplification with known amounts of cdNA starting material (10 pg to 10 ng). The slope of the linear curve of Ct vs. cDNA starting material is used to calculate the efficiency of the primer pair [efficiency = 10⁻¹/slope].

Table 1. RT-qPCR primer pairs

| Rat Kir2.1 | S-CTGCCGGTGGAATCGCTAGTAAT, AS-CCCTCCCATGCTAACAGCA |
| Rat Kir6.1 | S-GGCTCCTGGCCACACACAG, AS-ATGCTCAAGGCTGTTCATT |
| Rat Kir6.2 | S-ATTGCAATGCGGCGCTC, AS-CCGGAGGAGGCTGCTCTTC |
| Rat Kir5.1 | S-ATCCGCTCTGACATTTGACAGTC, AS-GACTTCGAGCTTTCCCTACAAGTC |
| Rat Kir7.1 | S-CTCCATGCTGGAGAACATTAC, AS-GTGATATGTTGACAGAATC |
| Rat KCNQ-1 | S-GGACACCCCATTTTCTGAG, AS-ATGCGCTAGACGTGTC |
| Rat KCNE1 | S-ATCCGCTAGTGGCCTGGGGCTTGTTATC, AS-CTAGGCGGCTGGGTCTCATTAAT |
| Rat KCNE2 | S-GGACAGGCTGCGGAGCT, AS-CCGCTGGGCCCGGGTTT |
| Rat KCNE3 | S-GGACAGGCTGCGGAGCT, AS-AGGGAGGCTGCGGAGCT |
| Rat H-K-ATPase-α | S-CCGCTGAGTCTGGCAGGAGAT, AS-ACTTATGAGGCTCTGAG |
| Rat H-K-ATPase-β | S-CCGCTGAGTCTGGCAGGAGAT, AS-ACTTATGAGGCTCTGAG |
| Rat peptisinogen C | S-AAGACCCACAAATATGACCTGGG, AS-GTACCGGCTGATACCTGAG |
| Rat HDC | S-CTGGCTCTGCTGGTACAA, AS-CCGCTGAGGAGAT |
| Rat GAPDH | S-GGCTGCTGCTGGTACAA, AS-CCGCTGAGGAGAT |

RT-qPCR, RT-real-time polymerase chain reaction.
Target primer pair amplifications were compared with reference primer pair amplifications (Gap-DH) in the same experiment for each RT product tested. All reactions were carried out in duplicates and 4 separate amplifications for each primer pair were performed. The relative expression ratio of the target gene compared with the reference (ref) gene was calculated according to Pfaffl (20), using the formula

$$\text{Ratio} = \left( \frac{\text{Efficiency}_{\text{target}} \times D_{\text{target}} \times \text{sample}}{\text{Efficiency}_{\text{ref}} \times D_{\text{ref}} \times \text{sample}} \right)$$

Isolation of intact rabbit gastric glands. Because large amounts of isolated gastric glands cannot be isolated from rats, male New Zealand White rabbits weighing 2 kg were used. Glands were prepared with slight modifications of the original procedure (2). The rabbit was anesthetized with 1.5 ml of pentobarbital sodium intravenously (50 mg/ml). After loss of corneal reflex, a laparotomy was performed. A catheter was placed in the aorta and secured by a ligature. The animal was perfused with 2 liters of PBS with a flow rate of 100 ml/min. These results in severe edema of the gastric wall. The stomach was removed and opened, and food particles were washed off. The mucosa was stripped off the submucosal tissue by scraping, cut into small fragments, placed into solution D (140 mM NaCl, 1.2 mM MgSO4, 1 mM CaCl2, 10 mM HEPES, 11 mM glucose, and 2 g/l BSA, pH 7.4), washed twice, and pelleted in a Sorvall centrifuge at 600 rpm for 5 min. The pelleted mucosal fragments were suspended in 50 ml of solution D containing 0.6 mg/ml collagenase B (Boehringer Mannheim, Mannheim, Germany) and incubated in an orbital shaker at 37°C and 150 rpm for 30 min. The digest was pelleted in a Sorvall centrifuge at 600 rpm for 5 min and washed twice with fresh solution D. The resulting solution contains single cells, gastric glands, and larger undigested mucosal fragments that were separated using difference in sedimentation rate. Undigested larger fragments, which settle immediately on the bottom of the tube, were removed. Glands were then allowed to settle for 10 min. The supernatant, containing single cells, was removed and glands were mixed with solution D. This procedure was repeated 2–3 times until the supernatant appeared clear, indicating only minor contamination of single cells.

[^1C14]:aminopyrine uptake. These experiments were carried out as published previously (2); 1.5 ml bullet tubes were weighed, and 1 ml of freshly isolated gastric glands was added containing ~80,000 counts/min (cpm) [^1C14]:aminopyrine in the presence of histamine-IBMX (1 μM) and 1 μl of a 1:1000 stock solution of various inhibitors (see RESULTS). The tubes were placed into an orbital shaker and incubated for 30 min at 37°C. The solution D containing 0.6 mg/ml collagenase B was added, and the tubes were washed with solution D. The resulting solution contains single cells, gastric glands, and larger undigested mucosal fragments that were separated using difference in sedimentation rate. Undigested larger fragments, which settle immediately on the bottom of the tube, were removed. Glands were then allowed to settle for 10 min. The supernatant, containing single cells, was removed and glands were mixed with solution D. This procedure was repeated 2–3 times until the supernatant appeared clear, indicating only minor contamination of single cells.

PMT1 detected the signal below 545 nm after passage through a 505- to 530-nm band-pass filter, and PMT2 detected the signal above 545 nm after passage through a 560-nm long-pass filter. This setup can detect simultaneously in two channels green and red fluorescence of nonstacked or stacked acridine orange, respectively. Images were recorded after a total incubation of 10 min to allow equilibration of the dye.

Immunohistochemical colocalization studies. Male Sprague-Dawley rats weighing 200–300 g were fasted overnight and pretreated 30 min before being euthanized with either pentagastrin (100 μg/kg iv) or cimetidine (100 μg/kg iv). The stomachs were removed, washed, and immediately fixed in 10% formaldehyde overnight. The stomachs were sectioned and embedded in paraffin, and 3-μm sections were serially cut using standard histology methods. Slides with tissue sections were deparaffinized (3 × 100% xylene, 5 min) and rehydrated (1 × 100% ethanol (EtOH), 1 × 95% EtOH, 1 × 85% EtOH, 1 × 70% EtOH, 5 min each). Sections were rinsed twice in PBS, immersed in antigen retrieval buffer (0.01 M sodium citrate buffer, pH 6), and microwaved for 2 min. Sections were rinsed twice in PBS and incubated with protein-free blocking solution (Dako) for 30 min. Polyclonal antibody (rabbit) against KCNQ1 (Chemicon) was added in a 1:100 dilution and incubated for 1 h. Slides were washed for 15 min in PBS and incubated for 30 min in a solution containing anti-rabbit FITC conjugate (American Qualex, 1:100) and an anti H-K-ATPase α-subunit monoclonal antibody directly conjugated to rhodamine (Mab 95, 1:100). Slides were washed for 15 min in PBS and mounted in 70% glycerol in PBS. Slides were placed under a confocal microscope (Zeiss, LSM510) which was setup using multitrack mode and a 100 × objective. The first track used the Argon laser (excitation 488 nm) and a filter set to detect FITC emission (505–530 BP); the second track used the NeHe laser (excitation 543 nm) and a filter set to detect rhodamine emission (565 LP). This setup prevents detection of fluorescence stemming from FITC in the rhodamine photomultiplier tube.

RESULTS

Purification of gastric PCs to homogeneity. We obtained 60–80% pure PC suspensions by use of a combination of counterflow elutriation and density centrifugation, as previously described (3, 24). A significant amount of cells in these suspensions (20–40%) do not stain with the specific PC antigen H-K-ATPase α-subunit, as shown in Fig. 1. Cells emphasized with arrows in Fig. 1B (bright field) are not present in Fig. 1A (anti H-K-ATPase α-subunit staining). Counting the cells demonstrated only ~80% PC in this fraction. This is insufficient purity for allocation of any K channels specifically to PCs by genomic analysis.

PCs stain deeply eosinophilic on hematoxylin and eosin sections of the gastric mucosa because of the high density of mitochondria. In addition, microarray expression analysis of 80% pure cell suspensions showed high levels of mitochondrial gene transcripts (data not shown). One of the main products of mitochondrial metabolism is NADH, which has a fluorescent excitation of 488 nm with an emission spectral peak at 532 nm. In the presence of 10 mM glucose, PCs show strong fluorescence at 532 nm, which can be used as a marker in FACS. Figure 2 shows the flow chamber analysis of prepurified (80%) PC suspensions.

Figure 2A shows fluorescence at 532 nm (y-axis) and forward scatter (size, x-axis). There are two separate cell populations present. The population with a high fluorescent signal (green dots) is present in cells of medium-to-large size and medium-to-high granularity, as seen in Fig. 2B side scatter (granularity, y-axis) and forward scatter (x-axis). The fluores-
cent signal is not present at longer wavelengths (592 nm, y-axis, and forward scatter, x-axis; Fig. 2C). The cells with high fluorescence are then sorted. The resulting cell suspension shows a homogenous population of PCs (>99%), as shown in Fig. 3.

Virtually all cells in the field now stain positively with the monoclonal antibody against the H-K-ATPase $\alpha$-subunit (12.18), and it is difficult to find any cells not staining with this PC-specific marker. The final purity was 99 ± 1% ($n = 5$), as determined by counting unstained cells (homogeneity).

Analysis of mRNA expressed by partially purified and homogenous PC suspensions. Total RNA from prepurified PC suspensions (80%) or homogenous rat PC suspensions (>99%) as well as from crude gastric mucosal epithelium (stomach) was isolated and checked regarding stability and purity, using Agilent’s bioanalyzer, and fluorescently labeled cRNA was generated. The latter represents messages from all cell types of the gastric epithelium, including endocrine cells (A, G, D, X), enterochromaffin-like (ECL) cells, chief cells, mucous cells, and PCs. Because PCs make up 20–30% of all cells in the gastric mucosa, a purification factor for PC-specific mRNA of 3.3- to 5-fold is expected for the homogenous PC suspensions (>99%) and 2.6- to 4-fold for the less pure PC suspensions (80%). Labeled cRNA from the purified cells and from total gastric epithelium was combined at equal concentrations and hybridized onto a 22K rat oligonucleotide expression microarray (Agilent Technologies). This array contains 60-mer oligonucleotides representing over 20,000 well-characterized rat transcripts.

Fig. 1. Purity assessment of parietal cell (PC) suspensions after counterflow elutriation and cell density purification. A: staining with MAb 12.18 against H-K-ATPase $\alpha$-subunit. B: light microscopic image. Arrows in both panels point to cells not labeled with the antibody.

Fig. 2. Flow chamber analysis during fluorescence-assisted cell sorting (FACS). Green-labeled cells are gated during FACS and represent PCs. A: 532-nm emission (y-axis) vs. forward scatter (cell size, x-axis). B: side scatter (cell granularity, y-axis) vs. forward scatter. C: 592-nm emission (y-axis) vs. forward scatter. FL, fluorescence; FSC, forward scatter; SSC, side scatter.
Figure 4 shows a typical composite image of the signal of the hybridized PC cRNA probe (green) and crude gastric mucosal cRNA probe (red). The area in the circle shows the oligonucleotide spot for KCNQ1. Figure 4, inset, shows a higher magnification of this area, with the arrow pointing to this spot. The intensity of the colors is proportional to the quantity of hybridized cRNA. As expected, most genes show equal hybridization (yellow). These are therefore equally present in PCs compared with other cells of the gastric epithelium. Of the 21,133 genes, there are only 625 genes with significantly higher expression compared with the whole gastric epithelium (ANOVA, $P < 0.001$; green). We performed three independent RNA isolation-labeling procedures for both the prepurified and the homogenous PC suspensions compared with whole gastric epithelial message expression (one RNA isolation and labeling).

The quality of the array data was assessed by analysis of the levels of commonly used housekeeping genes and of genes known to be specifically present in the various cell types of the gastric mucosa (Table 2). Table 2, column 3, shows the average of normalized fluorescent signals of whole gastric epithelial cRNA of all six array experiments. Table 2, columns 4 and 5, shows the average of normalized fluorescent signals of cRNA of the three experiments with prepurified PCs or homogenous PCs, respectively.

In contrast to the prepurified PC suspension, which shows significant increases of message levels of genes known to be specific for ECL cells (HDC and VMAT2) and G cells (gastrin), the homogenous PC suspension shows a significant decrease of these messages, consistent with the high degree of purification of only PCs. This is also indicated by further reduction of message levels of highly expressed genes in cells other than PCs, like pepsinogen (chief cells), mucins 1 (mucous cells), ghrelin (A cells), and somatostatin (D cells) in homogenous PC suspensions compared with prepurified PC suspensions. The array contains two genes that are known to be specific for PCs, the M3 cholinergic receptor and the H2 histaminergic receptor. Both gene messages are present in significantly higher levels ($P < 0.001$) in prepurified and homogenous PC suspensions compared with crude gastric mucosa.
The array did not contain valid oligonucleotides for the PC-specific H-K-ATPase α- or β-subunit expression. Therefore, we used RT followed by real-time qPCR to probe the same RNA samples used for cRNA generation and microarray screening to quantify message levels for this ATPase in the prepurified PC suspensions compared with crude gastric mucosa. The results show the high degree of purity of the PC suspensions and the presence or absence of key gene expressions of the different cell types of the gastric mucosa. Because the primary purpose of this work was to attempt to identify the K channel associated with acid secretion, we compared the level of K channel gene expression in the fundic epithelium and the 80 and >99% pure PC suspensions.

Comparative oligonucleotide expression microarray analysis and real-time qPCR of K channel expression. Table 3 shows the results of the analysis of 23 of the 82 K channel subunits present on the microarray, 4 of which were previously suggested to be present in PCs (7, 10, 16, 18).

The microarray data show that some of these K channel proteins are expressed at significantly higher levels in PCs (enrichment) compared with the gastric epithelium. In addition, the total level of expression for each transcript is also shown. The K channel subunit with the highest level of expression and Table 3. Potassium channel expression

<table>
<thead>
<tr>
<th>GenBank ID</th>
<th>Description</th>
<th>Stomach</th>
<th>80% PC</th>
<th>&gt;99% PC</th>
<th>Ratio (80% PC/ST)</th>
<th>Ratio (&gt;99% PC/ST)</th>
<th>Real-Time Ratio (80% PC/ST)</th>
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<tbody>
<tr>
<td>2906642_Rn</td>
<td>Voltage-gated channel, K(v)QT1 (Kcn1)</td>
<td>24,862.1</td>
<td>103,724.0</td>
<td>124,534.8</td>
<td>4.17 ± 1.33</td>
<td>5.01 ± 1.19</td>
<td>7.66 ± 0.88</td>
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<tr>
<td>NM_13332</td>
<td>Voltage-gated channel, K(v)QT-like (Kcn2)</td>
<td>1748.34</td>
<td>1711.81</td>
<td>2250.20</td>
<td>1.01 ± 0.63</td>
<td>1.28 ± 0.98</td>
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<tr>
<td>NM_03159</td>
<td>Voltage-gated channel, K(v)QTT-like (Kcn3)</td>
<td>119.83</td>
<td>128.05</td>
<td>67.63</td>
<td>1.06 ± 0.82</td>
<td>0.56 ± 0.38</td>
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<td>AP299748</td>
<td>Voltage-gated channel, K(v)QT-like (Kcn5)</td>
<td>246.29</td>
<td>213.01</td>
<td>197.68</td>
<td>0.90 ± 0.83</td>
<td>0.80 ± 1.62</td>
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<tr>
<td>261282_Rn</td>
<td>Voltage-gated channel, K(v)QT-like (Kcn5)</td>
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<td>522.87</td>
<td>291.85</td>
<td>1.43 ± 0.17</td>
<td>0.80 ± 0.11</td>
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<td>264567_Rn</td>
<td>Voltage-gated channel Isk-1 (Kcne1)</td>
<td>2945.48</td>
<td>2341.78</td>
<td>2747.07</td>
<td>0.80 ± 0.93</td>
<td>0.93 ± 0.97</td>
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<td>NM_13360</td>
<td>Voltage-gated channel Isk-2 (Kcne2)</td>
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<td>6088.49</td>
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<td>2.24 ± 3.99</td>
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<td>2.84 ± 0.76</td>
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<tr>
<td>221142_Rn</td>
<td>Voltage-gated channel Isk-3 (Kcne3)</td>
<td>2171.22</td>
<td>618.49</td>
<td>443.50</td>
<td>0.49 ± 0.19</td>
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<td>NM_01702</td>
<td>Inwardly-rectifying channel Kcn1 (Kir1.1)</td>
<td>79.66</td>
<td>204.06</td>
<td>39.35</td>
<td>2.56 ± 6.67</td>
<td>0.49 ± 2.26</td>
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<tr>
<td>NM_03160</td>
<td>Inwardly-rectifying channel Kcn2 (Kir1.2)</td>
<td>232.20</td>
<td>284.23</td>
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<td>1.38 ± 1.58</td>
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<td>233019_Rn</td>
<td>Inwardly-rectifying channel Kcn2 (Kir1.2)</td>
<td>392.74</td>
<td>286.90</td>
<td>437.29</td>
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<td>1431.99</td>
<td>1591.43</td>
<td>879.96</td>
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<td>0.61 ± 0.21</td>
<td>ND</td>
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<td>NM_03161</td>
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<td>2605.02</td>
<td>2775.75</td>
<td>0.61 ± 0.18</td>
<td>0.65 ± 0.23</td>
<td>ND</td>
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<tr>
<td>NM_01319</td>
<td>Inwardly-rectifying channel Kcn6 (Kir3.2)</td>
<td>18104.1</td>
<td>11556.51</td>
<td>12348.04</td>
<td>0.64 ± 0.92</td>
<td>0.68 ± 0.01</td>
<td>ND</td>
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<tr>
<td>NM_05383</td>
<td>Inwardly-rectifying channel Kcn9 (Kir3.3)</td>
<td>175.59</td>
<td>219.64</td>
<td>154.49</td>
<td>1.25 ± 1.37</td>
<td>0.88 ± 0.41</td>
<td>ND</td>
</tr>
<tr>
<td>NM_01729</td>
<td>Inwardly-rectifying channel Kcn5 (Kir3.4)</td>
<td>184.34</td>
<td>174.62</td>
<td>128.15</td>
<td>0.95 ± 0.29</td>
<td>0.70 ± 0.44</td>
<td>ND</td>
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<tr>
<td>NM_03160</td>
<td>Inwardly-rectifying channel Kcn10 (Kir4.1)</td>
<td>106.41</td>
<td>157.59</td>
<td>84.33</td>
<td>1.48 ± 2.10</td>
<td>0.79 ± 0.82</td>
<td>1.37 ± 1.89</td>
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<tr>
<td>NM_13332</td>
<td>Inwardly-rectifying channel Kcn15 (Kir4.2)</td>
<td>232.20</td>
<td>284.26</td>
<td>244.92</td>
<td>1.22 ± 2.73</td>
<td>1.05 ± 0.86</td>
<td>1.84 ± 0.75</td>
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<tr>
<td>NM_05331</td>
<td>Inwardly-rectifying channel Kcn16 (Kir5.1)</td>
<td>542.89</td>
<td>2171.46</td>
<td>2723.16</td>
<td>4.00 ± 10.83</td>
<td>5.02 ± 6.07</td>
<td>4.61 ± 0.97</td>
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<tr>
<td>NM_01709</td>
<td>Inwardly-rectifying channel Kcn8 (Kir6.1)</td>
<td>304.17</td>
<td>135.46</td>
<td>209.57</td>
<td>0.45 ± 0.24</td>
<td>0.69 ± 0.37</td>
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<tr>
<td>219616_Rn</td>
<td>Inwardly-rectifying channel Kcn11 (Kir6.2)</td>
<td>322.72</td>
<td>284.34</td>
<td>364.01</td>
<td>0.88 ± 0.96</td>
<td>1.13 ± 1.03</td>
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<tr>
<td>260865_Rn</td>
<td>Inwardly-rectifying channel Kcn13 (Kir7.1)</td>
<td>764.27</td>
<td>510.62</td>
<td>292.62</td>
<td>0.67 ± 0.60</td>
<td>0.38 ± 0.03</td>
<td>1.54 ± 0.58</td>
</tr>
<tr>
<td>NM_05380</td>
<td>Inwardly-rectifying channel Kcn4 (KT4.1)</td>
<td>413.10</td>
<td>508.24</td>
<td>261.15</td>
<td>1.42 ± 1.47</td>
<td>0.68 ± 0.26</td>
<td>ND</td>
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Ratio values are means ± SD.
enrichment in PCs is KCNQ1 [K(v)LQT1]. In addition, KCNE2 and Kir5.1 are also highly expressed, and their level of expression is increased in PCs, albeit at a 10-fold lower expression level compared with KCNQ1. Message levels for KCNE1, KCNE3, KCNQ2, Kir2.3, Kir3.1, Kir3.2, and Kir7.1 are present in significant amounts but show the same or lower expression levels in PCs compared with whole gastric epithelium. All other K channels do not show significant message levels or an increase of message levels. Some of the more important message levels have been confirmed using quantitative real-time qPCR, as shown in Table 3, column 8.

The results clearly indicate an abundance and specificity of KCNQ1 expression in the genome of PCs with coexpression of KCN2 but not KCNE1 or KCNE3. The only other K channel gene highly expressed and enriched was the inward rectifier Kir5.1. All the other channels decreased in expression when purity went from 80 to 99% PC content or were only weakly expressed.

Function of KCNQ1 in acid secretion. The above results of the genomic screen prompted us to investigate the functional role of KCNQ1 in PC acid secretion using the well-established isolated rabbit gastric gland model (2).

Recent advances in K channel inhibitor development have provided several relatively selective inhibitors for KCNQ1. Previously, two separate groups had shown significant inhibition of the KCNQ1–KCNE2 heterohexamer expressed in HEK293 and COS cells by the KCNQ1-specific blocker 293B (Aventis) with an IC50 of 1.8 μM, similar to the inhibition of the heart KCNQ1–KCNE1 channel complex [K(v)LQT1–IsK; Refs. 4, 10]. However, this inhibitor also showed inhibitory effects against cystic fibrosis transmembrane conductance regulator and other channels (1). A more specific inhibitor, HMR 1556, was developed on the basis of the structure of 293B (Aventis). This inhibitor has an EC50 of 10–40 nM on isolated ventricular myocytes and does not inhibit other K channels except at high concentrations (>10 μM; Refs. 8, 9, 26). We also tested a new, even more potent M channel (KCNQ2–3 heterotetramer) inhibitor, XE-991, which was recently developed by DuPont using linopirdine as the lead chemical (34).

We measured acid secretion in intact isolated rabbit gastric glands by the uptake of the weak base [C14]aminopyrine (pKa ~4.0) (2) and by the accumulation of another weak base, acridine orange (5), into acidic spaces of the gland visualized by confocal microscopy. The glands showed sustained histamine-stimulated acid secretion in the presence of the phosphodiesterase inhibitor IBMX (all at 1 μM concentrations), resulting in an uptake ratio (gland supernatant) of [C14]aminopyrine of 64.79 ± 8.41.

Figure 5 shows the dose-response curve of inhibition of uptake ratio by SCH-28080, a specific K–competitive inhibitor of the H–K–ATPase with an EC50 of 525 nM, similar to previously reported data in the same system (29). The EC50 of the H2 receptor antagonist cimetidine was 957 nM. Similar dose-response curves are obtained in the presence of HMR-1556 and XE-991, with EC50 values of 452 and 282 nM, respectively. Linopirdine was much less effective in inhibiting acid secretion, with an EC50 of 22.98 μM.

The similarity of the inhibition curves for SCH-28080, HMR-1556, and XE-991 suggests that KCNQ1 is the K channel required for acid secretion by the H–K–ATPase in isolated rabbit glands (Fig. 5).

Acridine orange (AO) accumulates in isolated intact rabbit gastric glands during acid secretion. Upon accumulation into acid space, AO undergoes a metachromatic shift of fluorescence emission resulting from stacking of the dye. It is bright red in the acidic space and green in the cytoplasm (5). Using confocal microscopy that allows observation of intact gastric glands, we can visualize changes in pH in the PC secretory canaliculus and the gland lumen in real time in isolated living gastric glands. Confocal images of glands stimulated by 1 μM histamine-IBMX are shown in Fig. 6, left.

Figure 6 shows the bright-red staining of the gland lumen as well as several PC secretory canaliculi deep inside the cell body. There is no red fluorescence seen in experiments where histamine-IBMX (1 μM)-stimulated glands are preincubated with 10 μM HMR-1556, as shown in Fig. 6, middle. A similar result is seen if the glands are preincubated with the H–K–ATPase inhibitor SCH-28080 (Fig. 6, right). These results again illustrate the functional requirement of KCNQ1 for acid secretion in isolated rabbit glands.

Immunohistochemical localization of KCNQ1 in sections of rat gastric mucosa. We pretreated rats with pentagastrin (stimulation) or cimetidine (resting) to perform immunohistochemical localization studies. Figure 7 shows confocal images of rat gastric mucosal sections colabeled with a monoclonal anti-H–K–ATPase α-subunit antibody (covalently conjugated to rho-
The human stomach secretes 1–2 liters of ~100 mmol/l hydrochloric acid per day. The enzyme responsible for this extraordinary function is the H-K-ATPase, which translocates protons (outward in the form of hydronium ions) in exchange for K ions (inward) (23). This process is electroneutral. The absence of external K arrests the pump cycle in the E2 conformation (21, 30).

Regulation of H-K-ATPase activity appears to depend on pump location in the secretory canalicular membrane, substrate availability (ATP), and access to luminal K as the counter ion for transport (22). During stimulation of acid secretion, a major morphological change occurs in the PC, involving translocation of the ATPase into the membrane of the secretory canalculus, resulting in association of the pump with a K and Cl conductance (33). Inhibition of either conductance would be as effective as inhibition of the ATPase, since either K could not cross the membrane with a K conductance inhibitor or inhibition of Cl conductance would block K efflux due to the generation of a large, outside, positive potential.

A variety of candidate proteins for the secretory K pathway in PCs have been postulated in the last few years, based mainly on immunohistochemical localization studies in gastric mucosal sections using polyclonal antibodies and colocalization with the staining pattern of the H-K-ATPase in confocal microscopic images. In addition, some groups used RT-PCR or Northern blot analysis to compare RNA levels of some but not all members of the K channel family (7, 10, 16, 18). None of these studies was done on purified PCs. Hence, these signals could be derived from other cell types of the mucosa. In addition, the studies did not compare expression levels of other members of the K channel family on either genomic or proteomic levels, and hence a variety of conflicting data have been published (7, 10, 16, 18).

One of these channels, the cardiac voltage-gated K channel KCNQ1 (also known as KvLQT1), displays more convincing evidence of its role in acid secretion, stemming from an unexpected observation in a knockout mouse model for the human congenital long QT syndrome. KCNQ1 or its regulatory subunit KCNE1 (MinK, Isk), responsible for the slow delayed rectifier cardiac current $I_{KS}$ during the cardiac action potential sequence, is mutated, resulting in a long QT interval and cardiac arrhythmias. However, KCNQ1 knockout mice did not show the expected cardiac effects but instead showed gastric enlargement with mucous neck cell hyperplasia and PC aplasia (14). The histological changes seen in this knockout mouse are strikingly similar to the ones found in the H-K-ATPase β-subunit knockout mouse model (6). This seems to indicate that KCNQ1 may be functionally coupled to the H-K-ATPase in the rodent stomach.
The fact that any voltage-gated cardiac K channel would be present in the canalicular secretory membrane of PCs, which has most likely a constant membrane potential of \(-30-40\) mV, is surprising given that a constant outward flux of K is required to supply the outside face of the H-K-ATPase for acid secretion. However, in the case of KCNQ1, the recent characterization of KCNE1 (MinK) isoforms (KCNE2–5) led to the identification of at least one K channel (KCNQ1-KCNE2) that is voltage insensitive and, in addition, luminal pH activated as shown in whole cell patch clamp experiments with HEK293 cells expressing KCNQ1-KCNE2 heterodimers and placed in neutral (pH 7.4) or acidic (pH 5.5) extracytoplasmic pH (4, 10). Hence, a KCNQ1-KCNE2 heterohexamer would be a promising candidate for the canalicular K-conducting channel, which could provide K to the luminal side of the H-K-ATPase and activate acid secretion and would show the properties expected for a K conductance in this region of the PC.

Definitive identification of the protein responsible for the K gradient during acid secretion can be performed if PCs can be purified to homogeneity and the abundance and function of a specific channel proven. The present study shows that we are now able, for the first time, to achieve purification of PCs to

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**Fig. 7.** Confocal microscopy of stimulated (A–C) and resting (D–F) rat gastric mucosal sections. A and D: detection of rhodamine fluorescent signal due to staining of the H-K-ATPase α-subunit (MAb 95). B and E: detection of FITC fluorescent signal due to staining of KCNQ1 (polyclonal antibody). C and F: composite image showing colocalization of H-K-ATPase and KCNQ1 (yellow). Bar = 10 μm.
virtual homogeneity (>99%) using NADH-induced autofluorescence in the presence of glucose followed by FACS. The purity was not only confirmed by immunohistochemical staining of cells with antibodies against the H-K-ATPase, but also by careful evaluation of mRNA transcripts coding for proteins that are known to be specific for PCs and other possible contaminating cells of the gastric mucosa. All of these transcripts showed the expected behavior in terms of presence or absence in PC suspensions, or gradual decrease to absence in the two purification steps (80% to homogeneity, Table 2). These results not only validate the purity of the purified PC suspension but also the practical use of the microarray platform, which showed good agreement with real-time qPCR quantitation of the transcripts in all but two oligonucleotide hybridizations. Therefore, this method can compare and identify transcripts in a qualitatively and quantitatively meaningful manner.

Comparison of all K channels present on the array revealed only three K channel subunits, KCNQ1, KCNE2, and Kir5.1, as being present at significantly increased levels in purified PCs, at three to five times the levels seen in the gastric epithelium. In addition, the level of expression of KCNQ1 is 10–50 times higher compared with KCNE2 and Kir5.1, indicating a much more active transcription of this subunit.

Kir5.1 is an inward rectifier, which would tend to allow K uptake rather than efflux, a counterintuitive concept for the K channel responsible for the K efflux that is necessary for sustained activity of the gastric H-K-ATPase in the secretory canaliculus. Furthermore, although this channel is sensitive to Ba2+, it is insensitive to HMR-1556 (25). This channel probably allows K entry into PCs to compensate for the K loss during acid secretion, where gastric juice K concentration is ~15 mM (28).

Secretory function for KCNQ1-KCNE2 heterohexamers in acid secretion was shown by equivalent inhibition of the KCNQ1-KCNE1-specific inhibitor HMR-1556 (Aventis) compared with the H-K-ATPase inhibitor SCH-28080. Both inhibitors (1 μM) abolished acid secretion shown by the absence of AO accumulation in the secretory canaliculus using confocal microscopy to visualize the stacked form of the dye, which emits bright-red fluorescence at pH <3 (Fig. 6). We determined the apparent affinity (EC50) of these inhibitors on acid secretion using the well-established model of amonopyrine uptake into rabbit gastric glands (2). We found an EC50 of SCH-28080 of 525 nM, similar to previously reported studies in this system (29). The EC50 of HMR-1556 was determined to be 425 nM, a similar pharmacological effectiveness compared with SCH-28080.

In addition, we tested the effect of two other inhibitors of KCNQ-type channels, linopirdine and a new compound based on this structure, XE-991 (DuPont). Linopirdine has a 10-fold lower apparent affinity compared with XE-991 on the inhibition of KCNQ1 homotetramers in the oocyte expression system (32). However, the addition of KCNE1 (minK, Isk), which confers voltage sensitivity to KCNQ1, decreased the apparent affinity of XE-991 14-fold (31). In contrast, addition of KCNE3, an Isk isomer conferring voltage insensitivity to KCNQ1, increased the apparent affinity of XE-991 (15). We found a 100-fold increased apparent affinity for XE-991 (EC50 = 282 nM) on acid secretion compared with linopirdine (EC50 = 23 μM). This significant increase in apparent affinity of XE-991 suggests that the candidate KCNE isoform is KCNE3 or a similar subunit conferring voltage insensitivity to KCNQ1. PCs did not show increased levels of KCNE3 expression but instead showed greatly increased expression of KCNQ2, another Isk isomer conferring voltage insensitivity to KCNQ1 heterohexamers (27). This suggests that the principal K channel at the secretory canaliculus responsible for the K gradient during acid secretion is a KCNQ1-KCNE2 complex.

Colocalization studies in confocal images obtained from rat stomachs either stimulated with pentagastrin or resting with the use of cimetidine and fasting, doubly labeled with the anti-KCNQ1 antibody and a monoclonal antibody against the H-K-ATPase α-subunit, showed a significant change in morphology of the PCs and a change in distribution of the H-K-ATPase in PC subcellular compartments (Fig. 7). Whereas most of the anti H-K-ATPase staining is evenly distributed in a vesicular (dot like) pattern throughout resting PCs, which might represent tubulovesicles, stimulated PCs exhibit canalicular spaces, which are strongly and linearly stained with the anti H-K-ATPase antibody. Only in this compartment is definite colocalization of H-K-ATPase and KCNQ1 detected. The KCNQ1 localization in resting cells is apparently distinct from the localization of the H-K-ATPase.

In summary, microarray analysis of mRNA expressed by pure PCs shows abundant and specific expression of KCNQ1 and KCNE2 mRNAs. Colocalization with the gastric H-K-ATPase in the canaliculus of stimulated PCs but not the tubulovesicular compartment of resting PCs and inhibition of acid secretion by specific KCNQ1 inhibitors suggest that KCNQ1 is probably coassembled with KCNE2 to generate the voltage-independent K efflux necessary for acid secretion in the gastric PC.

GRANTS

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REFERENCES


