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.—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.

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 (33). This conductance is absent in vesicles prepared from resting parietal cells (23). 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 (12). 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 (17). 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 derived from the canalicular membrane of stimulated parietal cells (33). This conductance is absent in vesicles prepared from resting parietal cells (23). 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 (12). 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 (17). 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 followed by comparative (subtractive) oligonucleotide expression microarray analysis to find proteins that could represent the apical K secretory channel. One of these, KCNQ1, is then shown to be the likely candidate protein because of its high level of parietal cell expression and immunohistochemical localization and the effects of selective inhibitors of this K channel on acid secretion in the isolated, intact gastric gland.

EXPERIMENTAL PROCEDURES

Parietal cell purification from rat stomachs. Stomachs from four to five 200- to 250-g male Sprague-Dawley rats were resected and everted through an incision of the forestomach. Stomach contents were washed off, and ligations were placed at the gastric antrum and the forestomach. A volume of 1–1.5 ml of solution A (50 mM HEPES, 350 μM EDTA, 0.5 mM NaH₂PO₄, 1 mM Na₂HPO₄, 20 mM NaHCO₃, 70 mM NaCl, 20 mM KCl, and 11 mM D(+)-glucose, pH 8.1).
Fresh resected rat stomachs were opened, and the majority of cells were resuspended in 100–200 µl of 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 to ensure the 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 washed twice in solution C and concentrated to a total volume of 2–3 ml. This suspension was loaded on top of a nycodenz (Accudenz; Accurate Chemical and Scientific) gradient containing 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 on top of this layer were collected by increasing the flow rate of the pump 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 washed twice in solution C and concentrated to a total volume of 2–3 ml. This suspension was loaded on top of a nycodenz (Accudenz; 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 monoclonal antibody against the H-K-ATPase α-subunit (MAb 12.18; 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 sorted during a 4-h time period, pooled in an emission of 532 nM, and forward scatter (size of cells). 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 (Accudenz; 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 monoclonal antibody against the H-K-ATPase α-subunit (MAb 12.18; a generous gift from Dr. A. Smolka) and standard immunohistochemical procedures, as outlined elsewhere (13).

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RNA 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 (Qiagen) 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 Bioanalyser 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 (Qiagen) 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 (Finzymes, Espoo, Finland) and 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 | AS-CTCCGCGGTGATGCTGTATTCAAT, AS-CCGCCCTCTGATGGCAATCAAGA |
| Rat Kir4.1 | AS-AGGACTTTGCTGGAGCAACGCA, AS-AGTTTGATGTCAGAAACCTTCCT |
| Rat Kir4.2 | AS-ATTGGCTTATGGGGCCTTCC, AS-CCGGAGGAGGGCTTCCTTC |
| Rat Kir5.1 | AS-ATCGGCTTACTGGGGGCTTCC, AS-GAATCTTGGCGCTTCCCTCCCAGT |
| Rat Kir7.1 | AS-GTCTCAGCTTCTGCCAGGACACCA, AS-GTGGATGTGTCAGAAACCTTCCT |
| Rat KCNQ-1 | AS-GCGACACCCATTTCTGAG, AS-ATGGCTAGGATCTGACAGT |
| Rat KCNE1 | AS-ATTGGCTTATGGGGGCTTCC, AS-GAATCTTGGCGCTTCCCTCCCAGT |
| Rat KCNE2 | AS-AGGACTTTGCTGGAGCAACGCA, AS-AGTTTGATGTCAGAAACCTTCCT |
| Rat H-K-ATPase-α | AS-CCACGCACTGGCAAGATTGCTCAGG, AS-CATCATAGGATGGGCTTGTTG |
| Rat H-K-ATPase-β | AS-AGGACTTTGCTGGAGCAACGCA, AS-AGTTTGATGTCAGAAACCTTCCT |
| Rat pepsinogen C | AS-AAGACCCACACAAATATGACCCTGGC, AS-GTACCCAAGGGCCTTATCAG |
| Rat HDJ | AS-CCCGTCGCCCCCAGGTT |
| Rat GAPDH | AS-AGGACTTTGCTGGAGCAACGCA, AS-AGTTTGATGTCAGAAACCTTCCT |
| Rat Na-K-ATPase-α1 | AS-AGGACTTTGCTGGAGCAACGCA, AS-AGTTTGATGTCAGAAACCTTCCT |

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 Pfaff (20), using the formula

\[
\text{Ratio} = \frac{\text{Efficiency}_{\text{target}} \times \Delta C(T)_{\text{target}}}{\text{Efficiency}_{\text{ref}} \times \Delta C(T)_{\text{ref}}}
\]

**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 laparatomy was performed. A catheter was placed in the aorta and secured by a ligation. The animal was perfused with 2 liters of PBS with a flow rate of 100 ml/min. This 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 different sedimentation rates. 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.

**[14C]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) [14C]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 at 180 rpm. The glands were pelleted with a microcentrifuge at 10,000 rpm for 1 min. Eight hundred microliters of supernatant were removed and counted in 10 ml of scintillation fluid. The remainder of the supernatant was centrifuged at 600 rpm for 5 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.

**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 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 HeNe1 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.

**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 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 included 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 HeNe1 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.

**Acridine orange accumulation into acid spaces of intact rabbit gastric glands.** Freshly isolated gastric glands were incubated in the presence or absence of HMR-1556 or SCH-28080 in glass-bottom petri dishes (MatTek) and placed under a confocal microscope (Zeiss, LSM510) using a heated stage at 37°C; 1 μM acridine orange and 1 μM histamine-IBMX (Sigma) were added. Excitation was at 488 nm, and the emission signal was split by use of a 545-nm dichroic mirror.
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 α-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.
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.

Fig. 3. Purity assessment of PC suspensions after FACS. A: staining with MAb 12.18 against H-K-ATPase α-subunit. B: light microscopic image. All cells seen in the light microscopic image are also emitting fluorescent signal (PC homogeneity).

Fig. 4. Typical 22K oligonucleotide microarray cohybridized with Cy3-labeled cRNA from PCs (green) and Cy-5 labeled cRNA from gastric epithelium (red). Inset: magnification of the area containing the oligonucleotide spot for KCNQ1 (arrow).
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 rRNA generation and microarray screening to quantify message levels for this ATPase in the PC-specific H-K-ATPase

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>
<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 (99% PC/ST)</th>
<th>Real-Time Ratio (80% PC/ST)</th>
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<tr>
<td>296642_Rn</td>
<td>Voltage-gated channel, KvLQT1 (Kcnq1)</td>
<td>24,861.22</td>
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<td>NM_01729</td>
<td>Inwardly-rectifying channel Kcnj5 (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_03162</td>
<td>Inwardly-rectifying channel Kcnj10 (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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<td>NM_13332</td>
<td>Inwardly-rectifying channel Kcnj15 (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 Kcnj16 (Kir5.1)</td>
<td>542.89</td>
<td>2,171.46</td>
<td>2,723.16</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 Kcnj8 (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>
<td>ND</td>
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<tr>
<td>219616_Rn</td>
<td>Inwardly-rectifying channel Kcnj11 (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>
<td>ND</td>
</tr>
<tr>
<td>260865_Rn</td>
<td>Inwardly-rectifying channel Kcnj13 (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>
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<tr>
<td>NM_05380</td>
<td>Inwardly-rectifying channel Kcnk4 (KCTD1)</td>
<td>413.10</td>
<td>588.24</td>
<td>281.15</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 KCNQ2 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). This inhibitor has 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 [14C]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 [14C]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 labeled with a monoclonal anti-H-K-ATPase α-subunit antibody (covalently conjugated to rho-
Inhibitor is therefore warranted. A continued effort to develop a better secretion, with the median pH rising from 1.4 to 3.0 over a 24-h period (11). Proton pump inhibitors have a relatively slow onset of action and do not provide complete inhibition of acid antagonists do not provide complete acid inhibition, and the inhibition of K binding to the pump. The H2 receptor antagonists do not provide complete inhibition of acid secretion. There are currently two main therapeutic drug classes available. Cimetidine and similar drugs inhibit the stimulation of the gastric mucosa, with PCs exhibiting canalicular spaces that are strongly and linearly stained with the anti-H-K-ATPase antibody (Fig. 7A). Only in this compartment is there definite colocalization of H-K-ATPase and KCNQ1 detected (Fig. 7C). In nonstimulated regions, the impression gleaned is that there is separate localization of H-K-ATPase and KCNQ1, as suggested by recent work in transgenic mice (19).

**DISCUSSION**

The main treatment of gastric acid-related diseases like peptic ulcer disease and gastroesophageal reflux disease is the effective therapeutic inhibition of acid secretion. There are currently two main therapeutic drug classes available. Cimetidine and similar drugs inhibit the stimulation of PCs via inhibition of the H2 receptor, and proton pump inhibitors like omeprazole inhibit the H-K-ATPase directly by covalent binding and inhibition of K binding to the pump. The H2 receptor antagonists do not provide complete acid inhibition, and the proton pump inhibitors have a relatively slow onset of action with oral dosing and do not provide complete inhibition of acid secretion, with the median pH rising from 1.4 to ~3.0 over a 24-h period (11). A continued effort to develop a better inhibitor is therefore warranted.

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 canaliculus, 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 IKS 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

![Confocal microscopy of stimulated (A–C) and resting (D–F) rat gastric mucosal sections.](http://physiolgenomics.physiology.org/)
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 abundance 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 cell 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 canalicus. 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 heteroexamers 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 canalicus 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 aminopyrine secretion using the well-established model of aminopyrine (EC50 found a 100-fold increased apparent affinity for XE-991 (15). We KCNE3, an Isk isomer conferring voltage insensitivity to KCNQ1, increased the apparent affinity of XE-991 14-fold (31). In contrast, addition of KCNE1 (minK, Isk), which confers voltage sensitivity to KCNQ1 homotetramers in the oocyte expression system, XE-991 (DuPont). Linopirdine has a 10-fold increase in apparent affinity for XE-991 (14). 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 KCNE2, another Isk isomer conferring voltage insensitivity to KCNQ1 heteroexamers (27). This suggests that the principal K channel at the secretory canalicus 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 canalicus 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


