Gastric parietal cell secretory membrane contains PKA- and acid-activated Kir2.1 K⁺ channels

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Malinowska, Danuta H., Ann M. Sherry, Kirti P. Tewari, and John Cuppoletti. Gastric parietal cell secretory membrane contains PKA- and acid-activated Kir2.1 K⁺ channels. Am J Physiol Cell Physiol 286: C495–C506, 2004. First published November 5, 2003; 10.1152/ajpcell.00386.2003.—Our objective was to identify and localize a K⁺ channel involved in gastric HCl secretion at the parietal cell secretory membrane and to characterize and compare the functional properties of native and recombinant gastric K⁺ channels. RT-PCR showed that mRNA for Kir2.1 was abundant in rabbit gastric mucosa with lesser amounts of Kir4.1 and Kir7.1, relative to RT-PCR showed that mRNA for Kir2.1 was abundant in rabbit gastric mucosa with lesser amounts of Kir4.1 and Kir7.1, relative to

Kir7.1, relative to β-actin. Kir2.1 mRNA was localized to parietal cells of rabbit gastric glands by in situ RT-PCR. Resting and stimulated gastric vesicles contained Kir2.1 by Western blot analysis at ~50 kDa as observed with in vitro translation. Immunoconfocal microscopy showed that Kir2.1 was present in parietal cells, where it colocalized with H⁺-K⁺-ATPase and CIC-2 Cl⁻ channels. Function of native K⁺ channels in rabbit resting and stimulated gastric mucosal vesicles was studied by reconstitution into planar lipid bilayers. Native gastric K⁺ channels exhibited a linear current-voltage relationship and a single-channel slope conductance of ~11 pS in 400 mM K₂SO₄. Channel open probability (Pₒ) in stimulated vesicles was high, and that of resting vesicles was low. Reduction of extracellular pH plus PKA treatment increased resting channel Pₒ to ~0.5 as measured in stimulated vesicles. Full-length rabbit Kir2.1 was cloned. When stably expressed in Chinese hamster ovary (CHO) cells, it was activated by reduced extracellular pH and forskolin/IBMX with no effects observed in nontransfected CHO cells. Cation selectivity was K⁺ = Rb⁺ > Cs⁺ = Li⁺ = NMDG⁺. These findings strongly suggest that the Kir2.1 K⁺ channel may be involved in regulated gastric acid secretion at the parietal cell secretory membrane.

H⁺-K⁺-ATPase; hydrogen chloride secretion; parietal cell K⁺ channel

THE COORDINATED FUNCTION OF THE H⁺-K⁺-ATPase and K⁺ and Cl⁻ channels in the apical membrane of the gastric parietal cell results in acid secretion (10, 19, 46, 50, 65). A Cl⁻ channel identified to be CIC-2 by cloning and expression studies (35) has been characterized in rabbit gastric mucosal vesicles (6), which also exhibit H⁺-K⁺-ATPase activity (10). Regulation of CIC-2 by PKA and low extracellular pH is consistent with regulation of gastric HCl secretion by cAMP (4) and with an essential property of gastric apical membrane ion channels that they must be able to function at very low extracellular (or luminal) pH of 3 or lower. Both native and recombinant CIC-2 not only functioned at pH 3 but also were activated by low pH on the luminal side of the membrane (6, 35, 55), mediated by an extracellular pH sensor (56).

Less is known of the K⁺ channel. Luminal K⁺ is required as a substrate for the gastric H⁺-K⁺-ATPase. K⁺ movement across the apical membrane was first suggested to occur through K⁺ channels on the basis of experiments measuring conductive K⁺ transport in isolated rabbit gastric vesicles, using various approaches (8, 10, 46, 65), and on initial K⁺ channel current recordings in gastric vesicles (7). As with the gastric Cl⁻ channel, gastric K⁺ channel properties must be consistent with its physiological role and location. Minimally, the channel must be able to function at low extracellular pH for HCl secretion to occur. K⁺ channel activity has been measured by patch clamp in the basolateral membrane of the Necturus gastric oxyntic cell (57, 62) and the rabbit parietal cell (51), but not in the apical membrane, since access to this membrane is virtually impossible. Some initial tetraethylammonium (TEA)-sensitive K⁺ channel current recordings from stimulated gastric membrane vesicles fused to lipid bilayers have been reported (7). However, detailed molecular and functional studies of gastric apical membrane K⁺ channels have been lacking. Two studies recently appeared suggesting that KCNQ1 coupled with KCNE2 or KCNE3 (20) or Kir4.1 (18) is the parietal cell apical membrane K⁺ channel essential for gastric HCl secretion. Both studies have some strong supporting data, but they are in conflict with each other and both fail to prove an exclusive role for these proteins in K⁺ transport at the apical membrane.

Studies of native gastric apical membrane proteins and their regulatory mechanisms have utilized gastric H⁺-K⁺-ATPase-containing membranes isolated from the gastric mucosa of cimetidine-injected, nonsecreting (resting) and histamine-injected, secreting (stimulated) rabbits (6, 10). These resting and stimulated gastric mucosal membranes exhibit conductive K⁺ transport (10, 46, 65), K⁺ channel activity (7), Cl⁻ channel activity (6), H⁺-K⁺-ATPase activity (10), and HCl accumulation (10) consistent with the physiological state of the gastric mucosa from which they were derived. In the present study, Kir2.1 K⁺ channel mRNA was identified in rabbit gastric parietal cells by in situ reverse transcriptase-polymerase chain reaction (RT-PCR). Immunoconfocal microscopy of rabbit gastric glands was used to localize Kir2.1 and compare this with H⁺-K⁺-ATPase and CIC-2 Cl⁻ channel location. Some properties of K⁺ channel function of native rabbit gastric vesicles and recombinant Kir2.1 expressed in Chinese hamster ovary (CHO) cells and in Xenopus laevis oocyte membranes were characterized and compared. Regulation of native and recombinant channel function was also examined. The data suggest an important role for the Kir2.1 K⁺ channel in gastric

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HCl secretion at the secretory membrane of the parietal cell. Some of these findings have been reported in abstract form (9, 36).

MATERIALS AND METHODS

K⁺ channel screening. Poly(A)⁺ mRNA was isolated from frozen rabbit gastric mucosa, large intestine, small intestine, and lung by using Tri reagent followed by the Oligotex-dt mRNA procedure. Isoform-specific PCR primers were designed from the known sequences of the inward rectifying Kir K⁺ channels 2.1 (rabbit; accession no. D21057), 4.1 (rabbit; accession no. ABO1007534), and 7.1 (human; accession no. AJ007557). Rabbit brain mRNA was used as a positive control, and mRNA from three rabbit epithelial tissues (large intestine, small intestine, and lung) was used to investigate tissue distribution. First-strand cDNA was synthesized and amplified by hot-start PCR, using TaKaRa ExTaq polymerase and Taqstart antibody. The same amount of cDNA was used for all the PCR reactions. A 669-bp fragment of β-actin was amplified to control for relative quantities of cDNA used. The PCR products were subcloned into pCRII-TOPO (Invitrogen) and sequenced by the dyeodeoxy chain termination method, using α-35S-labeled ATP and T7 DNA polymerase.

Cloning rabbit gastric Kir2.1. The full-length open reading frame of the rabbit gastric Kir2.1 K⁺ channel was cloned from rabbit gastric mucosal mRNA by using RT-PCR. First-strand cDNA was synthesized and then amplified by using hot-start PCR with primers flanking the coding region of the previously cloned rabbit heart Kir2.1 or RHIIK1 (30). The sense primer was 5'-GAAAGCTGGAAGGACGACTG-3' and the antisense primer was 5'-GGAGTCGTCACTGATCATCTTCT-GACTCTCGCCG-3'. PCR conditions consisted of 40 cycles of denaturing for 45 s at 94°C, annealing for 45 s at 64°C, and elongating for 2 min at 72°C. A 1,302-bp cDNA encoding the entire open reading frame of rabbit gastric Kir2.1 was obtained and sequenced.

Direct in situ RT-PCR. Adult rabbit stomachs were removed and washed in PBS. Gastric glands were isolated by collagenase digestion (4), were blocked for hot-start PCR, using TaKaRa ExTaq polymerase and Taqstart antibody. The same amount of cDNA was used for all the PCR reactions. A 669-bp fragment of β-actin was amplified to control for relative quantities of cDNA used. The PCR products were subcloned into pCRII-TOPO (Invitrogen) and sequenced by the dyeodeoxy chain termination method, using α-35S-labeled ATP and T7 DNA polymerase.

Western blot analysis of Kir2.1 in gastric vesicles. Resting and stimulated rabbit gastric vesicle proteins (10) were boiled for 30 s, separated by SDS-PAGE, and blotted to Hybond ECL nitrocellulose membrane (Amersham). The nitrocellulose replicates were stained with Blot FastStain and photographed before nonspecific sites were blocked for 1 h at 22°C with TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) containing 0.1% Tween 20, 5% blocking-grade nonfat dry milk (Bio-Rad), and goat anti-rabbit Fab fragments (1:130). Nitrocellulose replicates were incubated at room temperature with anti-Kir2.1 antibody (1:200) for 1 h, followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1,000) for 1 h, both diluted in TBS-0.1% Tween 20-1% milk. The ECL Western blotting analysis system from Amersham was then used for detection and visualization on autoradiographic film. For control, Kir2.1 antibody was blocked with its antigenic peptide.

In vitro translation. For in vitro translation, the TNT T7 Coupled Reticulocyte Lysate System (Promega) was used according to the manufacturer’s instructions. Full-length cDNA for rabbit Kir2.1 was used and [35S]methionine was used for detection. Reactions with or without cDNA and with and without microsomes were always performed. Proteins were separated on mini-SDS PAGE gels, stained with GelCode Blue Stain reagent, dried for 2 h, and then autoradiographed overnight.

Immunohistochemistry and confocal microscopy. Isolated, fixed, and permeabilized adult rabbit gastric glands were used (26). Rabbit gastric glands, isolated by collagenase digestion (4), were fixed for 20 min at 22°C in 4% paraformaldehyde. The glands were then permeabilized with 0.5% Triton X-100 in PBS for 20 min and washed three times in PBS before setting onto poly-l-lysine-coated coverslips (26). All reactions were carried out at room temperature. Glands were then blocked (1% BSA, 0.05% Tween 20 in PBS) for 30 min and incubated with rabbit anti-Kir2.1 antibody (1:200), chicken anti-ClC-2 antibody (1:300), or anti-H⁺-K⁺-ATPase α-subunit antibody (1:200,000) for 1 h. Samples were washed five times for 5 min with PBS containing 0.05% Tween 20 and then incubated with 1:1,000 goat anti-rabbit IgG (Kir2.1) labeled with Alexa Fluor 546 (red fluorescence) and goat anti-chicken (ClC-2) or goat anti-mouse (H⁺-K⁺-ATPase α-subunit) IgG labeled with Alexa Fluor 488 (green fluorescence). Controls included blocking anti-Kir2.1 antibody with its antigenic peptide and omitting the primary antibodies for Kir2.1, ClC-2, and H⁺-K⁺-ATPase α-subunit. Images of the stained gastric glands were obtained by using a laser scanning confocal microscope (LSM 510, Zeiss) equipped with argon and helium-neon lasers, an Axioplan upright microscope, and oil-immersion objectives. Digitalized images were processed using the LSM 510 software and Adobe Photoshop. Standard excitation/emission wavelengths for Alexa 546 and 488 were used as previously described (54).

Bilayer electrophysiology. The procedures and equipment were as described in detail previously (6, 35). Briefly, a GeneClamp 500 amplifier (Axon Instruments), a TL-1 DMA interface, and a Frequency Devices eight-pole Bessel function filter were used for these studies. Routinely, 100-s channel recordings were obtained at set holding potentials and filtered at 500 Hz. Multiple recordings (usually 3) and at least three reconstructions were obtained in each condition. Identification of events and determination of dwell times and amplitudes, as well as curve fitting, were carried out using the pCLAMP program versions 5.5 and 6. Lipid bilayers were formed across a 100-μm-diameter aperture in the wall of a Delrin cup by using a 3:1 mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-r-serine] (POPS) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 30
and 10 mg/ml in n-decane, respectively. Except when specified differently, all of the solutions contained 2 mM MgSO$_4$, 1 mM K-ATP (pH 7.4), and 10 mM K-EGTA (pH 7.4) and were buffered with either 10 mM K-HEPES (pH 7.4) or 10 mM K-citrate (pH 3.0). A fire-polished glass capillary tube was used to apply lipids to the aperture of the Delrin cup, followed by vesicles (1–2 mg/ml protein) to the planar lipid bilayer. Reconstitutions of the channels lasted from 10 min to several hours. Upon vesicle fusion to the lipid bilayer, channel orientation has been previously established such that the cis side corresponds to the cytosolic side of the channel and the trans side corresponds to the extracellular or luminal side (6). When specified, PKA catalytic subunit (50 U/ml) was added to the cis side of the bilayer. pH$_{cis}$ was 7.4 in all experiments. The electrical reference side of the bilayer was the trans side. In channel current recordings, the zero current level, which corresponds to the closed state of the channel, as well as the open state of the channel are indicated. When two open-state current levels were observed that were an even multiple of the lowest open-state current level, they were interpreted as reflecting the activity of two channels present in the bilayer. All channel open probability ($P_o$) values reported have been corrected for the number of channels present as previously described (37, 36). To increase the chances of observing the correct number of channels in the bilayer, we carried out multiple 100-s recordings in each reconstitution. For all the gastric vesicle work, recordings from one, two, and three channels were observed. The distribution of the observations was 75% for one channel, 50% for two channels, and 25% for three channels. This distribution was similar regardless of physiological state of the vesicles. The number of different membranes or reconstitutions of the channel ($n$) on which current recordings were performed is indicated with each data point. Current-voltage ($I/V$) relationships were fitted by linear regression. Slope conductance was determined in symmetric salt solutions by linear regression analysis of the $I/V$ relationship. In cases where estimates of errors in the reversal potentials (intercept of the x-axis) were required, records from individual membranes or groups of recordings from several membranes were analyzed separately, and reversal potential values were obtained. The mean ± SE for these values was then calculated. The K$^+$ equilibrium potential was calculated using Nernst’s equation (29), and activity coefficients were from Ref. 47.

Expression of Kir2.1 in CHO cells and patch-clamp measurements. Kir2.1 cDNA was stably transfected into CHO cells by using Lipofectamine as previously described (60). Whole cell K$^+$ currents were measured as previously described (11, 60). Measurements started 50–100 ms after 1,500-ms voltage-clamp pulses began. These pulses occurred in 20-mV increments between –30 mV. Patch pipettes (borosilicate glass) of 1.5 MΩ of resistance were prepared by using a two-stage Narashige puller. The bath solution contained 140 mM K-gluconate, 5 mM MgCl$_2$, 10 mM HEPES, 1 mM CaCl$_2$, and 10 mM glucose (pH 7.3). The pipette solution contained 140 mM K-gluconate, 5 mM MgCl$_2$, 10 mM HEPES, 5 mM EGTA, and 1 mM MgATP (pH 7.3). Data were acquired with an Axopatch CV-4 head stage with a Digidata 1200 digitizer and an Axon Instruments (Foster City, CA), Lotus 123 (Microsoft), and Origon (Microcal) were used for data analysis. For $I/V$ curves, the currents were normalized to cell capacitance (pA/pF). Statistical significance of the difference between two means was determined by using the Student’s $t$-test by using $n$ as the number of cells. To determine the cation selectivity, symmetric 140 mM K-gluconate was used to start with, and then the bath solution was changed to 28 mM K-gluconate plus 112 mM N-methyl-D-glucamine (NMDG$^+$)-Cl, NaCl, RbCl, CsCl, or LiCl. $I/V$ curves were plotted, and the reversal potentials were obtained.

Statistical analysis. Statistical significance of the difference between two means was determined by using the Student’s $t$-test to compare the means of two small samples ($n < 30$) from normal populations with unknown variances not assumed to be equal. We used $n$ in all calculations of SE and statistical significance.

Expression of Kir2.1 in Xenopus laevis oocytes. Kir2.1 K$^+$ channel cRNA was made by using the T7 mMessage mMachine Kit and was expressed in Xenopus laevis oocytes as previously described for the CIC-2 Cl$^-$ channel (35, 55). Oocytes were isolated, injected with 10 ng of channel cRNA per oocyte, and left to express for 4 days. Oocyte plasma membranes were then prepared (35), reconstituted into planar lipid bilayers, and characterized electrophysiologically.

Materials. POPs and POPE were obtained from Avanti Polar Lipids and dissolved in n-decane (Aldrich). HEPES, citric acid, PKA catalytic subunit, and AP-conjugated goat anti-rabbit antibody were from Sigma. Tri reagent was from Molecular Research Center, Oligotex-dt mRNA kit was from Qiagen, rabbit brain poly(A)$^+$ mRNA and Taqstart antibody were from Clontech, Tag polymerase was from Pan Vera, the TA cloning kit was from Invitrogen, and T7 DNA polymerase (Sequenase v.2) was from United States Biochemical. The T7 mMessage mMachine kit was from Ambion. DIG-11-dUTP, AP-conjugated anti-DIG antibody, and NBT/BCIP were from Boehringer Mannheim. Goat anti-rabbit Fab fragments were from Jackson ImmunoResearch; rabbit polyclonal antibody to Kir2.1 was from Alomone; mouse anti-H$^+-$K$^+$-ATPase α-subunit antibody and Biotin FastStain were from Chemicon. The TNT T7 Coupled Reticulocyte Lysate System was obtained from Promega, [35S]methionine was from Amersham, and GelCode Blue Stain reagent was from Pierce. Alexa Fluor-labeled IgGs were from Molecular Probes. The chicken anti-CIC-2 antibody (from Dr. Carol Blaisdel) was made against a CIC-2 COOH-terminal fusion protein, and its specificity has been well characterized (38). All other reagents were of the highest grade available.

RESULTS

K$^+$ channel screening, epithelial tissue distribution, and cloning from rabbit gastric mucosa. To investigate the molecular nature of the gastric K$^+$ channel, we screened gastric mRNA by RT-PCR for three different K$^+$ channels: Kir2.1 (1, 30, 39, 44), 4.1 (2, 58), and 7.1 (33, 43), which appeared to have one or more characteristics similar to those of native gastric vesicles. Primers were designed to be isoform specific, and β-actin was used as a control for the amount of cDNA used. Brain mRNA was the positive control, because in other species, brain has been found to have a major amount of all three K$^+$ channels (15, 30, 33, 43, 44, 58). Rabbit large intestine, small intestine, and lung mRNAs were examined in parallel. The results are shown in Fig. 1. Brain had large and

![Fig. 1. Screening for K⁺ channels by RT-PCR. Isoform-specific primers were used to amplify cDNA fragments of Kir2.1 (1,304 bp), Kir4.1 (635 bp), Kir7.1 (425 bp), and β-actin (669 bp) from brain, gastric mucosa, large (LG) intestine, small (SM) intestine, and lung mRNA. Two controls were carried out: control 1 contained no mRNA in the RT reaction, and control 2 contained no DNA in the PCR reaction. Shown are ethidium bromide-stained agarose gels (1.2% for Kir2.1; 2% for Kir4.1, Kir7.1, and β-actin) of the PCR products.](http://www.ajpcell.org/doi/fig/10.1152/ajpcell.0100104)
similar amounts of mRNA for all three K⁺ channels relative to actin. The gastric mucosa had a major amount of Kir2.1 mRNA, with much less Kir4.1 and just a trace of Kir7.1 relative to actin. Large intestine, small intestine, and lung also had major amounts of Kir2.1 mRNA, with differing amounts of Kir4.1 and Kir7.1. The large intestine and lung had substantial amounts of Kir4.1 mRNA, with much smaller amounts of Kir7.1 mRNA, whereas the small intestine had the reverse. Because Kir2.1 was the most abundant, the full-length cDNA (2,285 bases with 1,281 bases open reading frame) was obtained by RT-PCR from rabbit gastric mucosal mRNA and sequenced. Its nucleotide sequence was identical to that cloned from rabbit heart (30).

**Localization of Kir2.1 K⁺ channel mRNA.** Isolated rabbit gastric glands were used to localize Kir2.1 mRNA (Fig. 2) by direct in situ RT-PCR. Staining mitochondria of gastric glands with an NBT-succinic dehydrogenase-linked assay identified the parietal cells (highly enriched in mitochondria) as dark blue cells (Fig. 2A). This staining is similar to that observed when glands are immunostained with the parietal cell-specific anti-H⁺-K⁺-ATPase β-subunit antibody (5). The use of direct in situ RT-PCR (Fig. 2Ba) showed that strong staining occurred mainly in the parietal cells, which was specific upon comparison with controls. No ExTaq polymerase (Fig. 2Bb), no primers (Fig. 2Bc), no DIG-11-dUTP (Fig. 2Bd), and RNase-pretreated (Fig. 2Be) samples had little or no staining throughout the glands. No DNase pretreatment (Fig. 2Bf) resulted in more intense staining throughout the glands, indicating amplification of genomic DNA as well as cDNA for Kir2.1 in all cells (22, 42). Therefore, Kir2.1 mRNA was specifically localized to parietal cells of gastric glands.

**Immunolocalization of the Kir2.1 K⁺ channel protein.** If the Kir2.1 K⁺ channel is involved in acid secretion, it is essential to localize not only the mRNA but, in particular, the protein to the parietal cell and to study how its localization compares with the H⁺-K⁺-ATPase and with CIC-2 Cl⁻ channels known to be present in the parietal cell apical secretory membrane (54). Before immunomicroscopy of Kir2.1 in gastric glands was carried out, Western blots of rabbit gastric vesicles were used to investigate specificity of the polyclonal peptide anti-Kir2.1 antibody and to investigate whether Kir2.1 is present in both resting and stimulated vesicles. The results are shown in Fig. 3A. An ~50-kDa protein band was evident in resting and stimulated gastric vesicles, and this protein band was blocked when the antibody was preincubated with its antigenic peptide. When in vitro translation was performed with the Kir2.1 cDNA (Fig. 3B), a major band of ~50 kDa was observed without microsomes, the expected size for Kir2.1. With microsomes present there did not appear to be a major increase in size, suggesting low or no posttranslational modification by glycosylation in agreement with reports that Kir2.1 is not glycosylated (53). The Kir2.1 protein observed on Western blots would therefore seem to be the unglycosylated form. The anti-Kir2.1 antibody was blocked by peptide. Arrowheads indicate a specific protein of ~50 kDa detected by anti-Kir2.1 antibody and blocked by peptide. B: TNT T7 Coupled Reticulocyte Lysate System was used to transcribe rabbit Kir2.1 cDNA with [³⁵S]methionine present. Proteins separated on mini-SDS-PAGE gels were dried and autoradiographed. Reactions with (+) or without (−) microsomes are shown, and reactions without cDNA were performed as control. Arrowhead indicates that a major protein of ~50 kDa was made.

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**Fig. 2.** Localization of Kir2.1 mRNA in gastric glands by direct in situ RT-PCR. A: parietal cells (dark blue) were identified by staining mitochondrial succinic dehydrogenase with a nitro blue tetrazolium (NBT)-linked assay. B: direct in situ RT-PCR for Kir2.1 was carried out on isolated gastric glands: a, contained all of RT-PCR components; b–f, controls: no Ex-Taq polymerase (b), no primers (c), no digoxigenin-11-2'-deoxyuridine-5'-triphosphate (DIG-dUTP) (d), RNase-pretreated samples (e), and no DNase pretreatment (f). Scale bars: 10 μm (A), 20 μm (B).

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**Fig. 3.** Western blot of Kir2.1 in resting (R) and stimulated (S) rabbit gastric vesicles and in vitro translation of Kir2.1. A: rabbit gastric vesicle proteins separated by 10% SDS-PAGE were transblotted to nitrocellulose, probed with anti-Kir2.1 antibody (1:200) for 1 h followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1,000) for 1 h, and visualized using chemiluminescence. For control, Kir2.1 antibody was blocked with its antigenic peptide. Arrowheads indicate a specific protein of ~50 kDa detected by anti-Kir2.1 antibody and blocked by peptide. B: TNT T7 Coupled Reticulocyte Lysate System was used to transcribe rabbit Kir2.1 cDNA with [³⁵S]methionine present. Proteins separated on mini-SDS-PAGE gels were dried and autoradiographed. Reactions with (+) or without (−) microsomes are shown, and reactions without cDNA were performed as control. Arrowhead indicates that a major protein of ~50 kDa was made.
antibody was then used to immunolocalize the channel in gastric glands as shown in Fig. 4. Kir2.1 K⁺ channel protein was localized mainly to parietal cells, and this staining was absent when the antibody was blocked with the peptide antigen. Staining was also absent in the absence of primary antibody and in the absence of secondary antibody (data not shown). In Fig. 5, gastric glands were stained for Kir2.1 and the H⁺-K⁺-ATPase or for Kir2.1 and ClC-2. Yellow fluorescence indicates colocalization of respective pairs of proteins. Kir2.1 colocalized with not only the H⁺-K⁺-ATPase but also with ClC-2, suggesting that Kir2.1 is present in the same membranes as the H⁺-K⁺-ATPase and the ClC-2 Cl⁻ channel, where it is physiologically relevant and essential for HCl secretion.

**Measurement of acid-activated K⁺ channel activity in native stimulated gastric membranes:** conductance, I/V relationship, open probability, and ion selectivity. A K⁺ channel that is involved in HCl secretion must be present and active in stimulated gastric vesicles that accumulate HCl and have active ClC-2 Cl⁻ channels. Minimally, this K⁺ channel must be able to function at pHtrans 3. Therefore, to detect the relevant K⁺ channel, we reconstituted stimulated vesicles into a planar lipid bilayer with symmetric 400 mM K₂SO₄ (800 mM K⁺) solutions and asymmetric pHcies 7.4 (intracellular) and pHtrans 3.0 (extracellular). Figure 6A shows current recordings obtained at different holding potentials and corresponding amplitude histograms. A channel was evident and very active with open probabilities around 0.5 irrespective of holding potential (0.52 ± 0.06, n = 6). The I/V relationship of the channel currents at pHtrans 3.0 (Fig. 6B) was linear with a slope conductance of 10.8 ± 0.2 pS (n = 16). To determine the ion selectivity, we recorded channel currents in stimulated gastric membranes at pHtrans 3 with an asymmetric fivefold reduction of the K₂SO₄ concentration on the trans side from 400 mM K₂SO₄ to 80 mM K₂SO₄ (800/160 mM K⁺, cis:trans). The I/V relationship was linear (Fig. 6B). There was a significant (P < 0.01) leftward shift of the reversal potential from zero under symmetric conditions to −29.1 ± 0.7 mV (n = 3) under gradient conditions. This is as expected for a highly K⁺-selective channel, because the K⁺ equilibrium potential in these conditions was calculated to be −29.2 mV using Nernst’s equation (29) and known activity coefficients (47). Na⁺ was virtually impermeant through the channel (data not shown), as also shown in earlier work (10, 46). Regulation of this channel was then investigated by using gastric vesicles isolated from resting gastric mucosae.
channel was virtually closed with a few short openings. This is in contrast to the channels in stimulated gastric membranes that are open with a \( P_o \) of 0.4–0.5 (see Fig. 6A). In Fig. 7A, trace \( b \), \( \text{pH}^{\text{trans}} \) was reduced to 3.0 and further recordings were obtained. The channel opened to a \( P_o \) of about 0.20. PKA catalytic subunit (50 U/ml) was then added to the \( \text{cis} \) side, and in Fig. 7A, traces \( c \) and \( d \) show sequential recordings of the channel. The channel opened further, with the \( P_o \) increasing to about 0.30 and then to about 0.50. Corresponding amplitude histograms are shown to the right of these traces. Figure 7B shows a summary of the data obtained from four to eight experiments. At -80 mV and \( \text{pH}^{\text{trans}} \) 7.4, resting \( P_o \) was virtually closed. Reducing \( \text{pH}^{\text{trans}} \) to 3.0 significantly increased resting channel \( P_o \) to \( 0.25 \pm 0.03 \) \((n = 8; P < 0.001)\), and addition of PKA to the \( \text{cis} \) side further significantly increased it to \( 0.46 \pm 0.03 \) \((n = 8; P < 0.01)\), similar to the \( P_o \) of channels in stimulated gastric vesicles at \( \text{pH}^{\text{trans}} \) 3.0 \( (0.53 \pm 0.05; n = 5)\). Therefore, with low \( \text{pH}^{\text{trans}} \) and treatment with PKA, \( K^+ \) channels in resting vesicles appear to open to the stimulated level. The \( I/V \) relationship of channels in resting vesicles was linear and the slope conductance was 10.7 \pm 0.2 pS \((n = 17)\) at both \( \text{pH}^{\text{trans}} \) 7.4 and 3.0 and with or without treatment with PKA, similar to that measured for \( K^+ \) channels in stimulated vesicles, supporting the view that the same \( K^+ \) channel is present in both resting and stimulated vesicles. These findings suggest that \( K^+ \) channel activity is regulated by PKA phosphorylation and by low extracellular \( \text{pH} \), both of which activate the channel.

**Functional expression of the recombinant Kir2.1 \( K^+ \) channel.** Kir2.1 was expressed in CHO cells to examine effects of forskolin/IBMX and low extracellular \( \text{pH} \). Figure 8A shows whole cell currents obtained at extracellular (bath) \( \text{pH} \) \((\text{pH}_b)\) of 7.4 and 6.0. Addition of 5 \( \mu \text{M} \) forskolin with 20 \( \mu \text{M} \) IBMX resulted in a large increase in the current at \( \text{pH}_b \) 7.4. When the medium \( \text{pH}_b \) was lowered to 6.0, the \( K^+ \) current increased, but only at negative holding potentials. Upon addition of forskolin/IBMX, the \( K^+ \) current increased at both negative and positive holding potentials. The data from three to four experiments are plotted as \( I/V \) curves after the currents were normalized to cell capacitance. At \( \text{pH}_b \) 6.0, the \( K^+ \) channel clearly showed inward rectification, but upon addition of forskolin/IBMX, the \( I/V \) curve became virtually linear. Also shown are the currents and \( I/V \) curves of nontransfected CHO cells. No significant responses to either low extracellular \( \text{pH} \) or forskolin/IBMX occurred. This finding indicates that the currents measured in the transfected CHO cells are due to Kir2.1. Figure 8B summarizes the effect of forskolin/IBMX and \( \text{pH}_b \) 6 on normalized channel conductance \((\text{at} -140 \text{mV holding potential})\) in Kir2.1-transfected and nontransfected CHO cells. Significant increases in normalized conductances were observed with forskolin/IBMX at \( \text{pH}_b \) 7.4 \((P < 0.05)\), when \( \text{pH}_b \) was lowered to 6 \((P < 0.02)\), and when lowered \( \text{pH}_b \) was followed by forskolin/IBMX addition \((P < 0.05)\) in transfected, but not in nontransfected, cells. Therefore, Kir2.1 \( K^+ \) channel activity appears to be regulated by PKA phosphorylation and low extracellular \( \text{pH} \), as observed with native gastric vesicles. To further characterize recombinant Kir2.1, we examined its cation selectivity, and the results are shown in Fig. 9. From the \( I/V \) curves, the cation selectivity of Kir2.1 was shown to be \( K^+ = Rb^+ \) \( \gg Na^+ = Cs^+ = Li^+ = NMDG^+ \). \( Na^+ \), \( Cs^+ \), \( Li^+ \), and \( NMDG^+ \) were all less permeant through Kir2.1, as previously reported for some of these cations \((49, 68)\). Kir2.1 is highly \( K^+ \) selective and virtually impermeant to \( Na^+ \), correlating well with the \( K^+ \) channels in native gastric vesicles and with previous studies \((10, 46)\).
Recombinant Kir2.1 was also expressed in *Xenopus laevis* oocytes. Oocyte plasma membranes expressing Kir2.1 were isolated, fused to planar lipid bilayers, and examined for K⁺ currents by using symmetric 400 mM K₂SO₄, pHₘₐₚ 3.0, and PKAₜᵣₑₐₚ, conditions under which the gastric K⁺ channel is optimally functional. Figure 10 shows current recordings obtained at different holding potentials with corresponding amplitude histograms (A) and the IV relationship (B). Three very active channels were evident. The Pₒ was about 0.5–0.7 irrespective of holding potential. The IV relationship was linear, and the slope conductance was 11.6 ± 2.5 pS (n = 26), which is not significantly different from the conductance of native K⁺ channels present in resting and stimulated gastric vesicles. The ability to function at pHₘₐₚ 3.0 is similar to native gastric K⁺ channels. However, the kinetics of channel opening and closing appear somewhat different from those observed with native gastric vesicles.

**DISCUSSION**

The purpose of the present study was to identify and localize K⁺ channels in the gastric mucosa, in particular, the parietal cells, to characterize and compare electrophysiological properties of native (from resting and stimulated rabbit gastric mucosae) and recombinant K⁺ channels, and to investigate whether/how they are regulated. K⁺ channels in gastric mucosal membrane vesicles, which also exhibited H⁺-K⁺-ATPase activity, were suggested to transport K⁺ from the cytosol to the lumen of the gastric parietal cell to provide an essential continuous supply of K⁺ to the H⁺-K⁺-ATPase during stimulated HCl secretion (8, 10, 46, 65). RT-PCR showed that Kir2.1 K⁺ channel mRNA was the very abundant in rabbit gastric mucosa compared with Kir4.1 and Kir7.1, which were present at low levels relative to β-actin. These studies therefore focused on Kir2.1. Kir2.1 mRNA was localized to parietal cells of gastric glands by in situ RT-PCR. Immunofluorescence microscopy showed that Kir2.1 was localized to the parietal cell and intracellular membranes. It also colocalized with H⁺-K⁺-ATPase and ClC-2 in the parietal cell, strongly supporting the suggestion that Kir2.1 is a candidate K⁺ channel involved in HCl secretion at the parietal cell apical membrane.

Native gastric K⁺ channels present in gastric mucosal membrane vesicles described in the present studies have different electrophysiological characteristics from those described in the basolateral membrane of the *Necturus oxyntic* cell (57, 62) and the rabbit parietal cell (51) as studied by patch clamp. However, these native gastric K⁺ channels showed many characteristics in common with Kir2.1. Both native and recombinant channels were highly K⁺ selective and virtually impermeant to Na⁺. Regulation of both channels was similar. They were both activated by low extracellular pH and intracellular PKA. Slope conductance of native K⁺ channels was similar to that of Kir2.1 expressed in oocyte membranes, although channel kinetics appeared somewhat different.

In the experimental conditions used, rectification was not observed in native K⁺ channels, although Kir2.1 has been described as a strongly inwardly rectified channel that, in the absence of Mg²⁺, becomes linear (1, 30, 34, 39, 44, 63, 67). However, recently this rectification has been shown to be not an intrinsic gating property of the channel as first thought but, rather, due to a very high affinity of the channel for intracel-
lular cations such as Mg$^{2+}$, polyamines, and trace contaminants in organic buffers (e.g., HEPES) (23–25). High K$^+$ concentrations also reduce rectification by displacing the cations, as do chelators such as EDTA, ATP, and EGTA, which chelate Mg$^{2+}$ to greater or lesser extents. Because experiments with native channels were performed with intracellular ATP, HEPES, EGTA, and Mg$^{2+}$ present in Ca$^{2+}$-free conditions and a high K$^+$ concentration of 800 mM, lack of rectification is not surprising. I/V curves of recombinant Kir2.1 expressed in CHO cells were also linear (without and with forskolin/IBMX) but appeared inwardly rectified when extracellular pH was reduced in the absence of forskolin/IBMX.

**Fig. 8.** Effect of forskolin/IBMX (F/I) and extracellular pH (pH$_e$) 6.0 on whole cell K$^+$ currents in Chinese hamster ovary (CHO) cells expressing rabbit recombinant Kir2.1 K$^+$ channels. Representative scans (left) and I/V curves (right) in Kir2.1-transfected CHO cells (A) and nontransfected CHO cells (B) are shown. Currents were measured before and after addition of forskolin (5 μM) and IBMX (20 μM) at pH$_e$ 7.4 or after reduction of pH$_e$ to 6.0. For the I/V curves, 3–11 experiments were combined and currents were normalized to capacitance. Data for A and B at −140-mV holding potential are summarized in C. Data are plotted as means ± SE with no. of experiments indicated in parentheses. V_m, membrane potential. *P < 0.05; **P < 0.02; #P < 0.01.
Native gastric K\(^+\) channels and recombinant Kir2.1 showed significant activation by low extracellular pH. As opposed to voltage-gated K\(^+\) and Na\(^+\) channels, which are nonconductive in their fully protonated state (27, 28), mouse Kir2.1 channel remained highly permeable to K\(^+\) when fully extracellularly protonated, although the conductance was 30–38% of the initial level (48). However, caution is needed when comparing channels of different species because a single amino acid change can exert a major effect on function. It should be noted that rabbit (and human) Kir2.1 have one additional externally facing glutamic acid (E118) compared with mouse and rat and that a single additional negatively charged glutamic acid (E419) on the external surface of CIC-2 is the key to major channel activation by low pH (56). Native K\(^+\) channels (whether stimulated or resting) had a linear I/V relationship with a slope conductance of 11 pS, and lowering pH\(_{o}\) had no significant difference on channel conductance. Because the proteins of the parietal cell secretory membrane are in direct contact with the primary gastric secretion HCl (6), at the very least, the K\(^+\) channel must be functional at acid pH on the extracellular (luminal or trans) side. With the use of native stimulated vesicles, the K\(^+\) channel was able to function at low pH. K\(^+\) channels of resting gastric vesicles at pH\(_{trans}\) 7.4 were virtually closed (\(P_o \approx 0.02\)) and acidic pH\(_{trans}\) activated the channels to \(P_o \approx 0.25\).

Histamine activation of acid secretion is mediated by increased intracellular cAMP leading to PKA activation (4) and an increase in intracellular Ca\(^{2+}\) (3). In the present studies K\(^+\) channel currents were routinely recorded in Ca\(^{2+}\)-free conditions in the presence of EGTA, consistent with the established findings that HCl accumulation by these vesicle preparations does not require Ca\(^{2+}\) (10, 65) and indicating that these K\(^+\) channels are Ca\(^{2+}\)-independent. PKA addition to the cis (intracellular) side of native resting K\(^+\) channels resulted in activation of the \(P_o\) to \(\sim 0.5\), a level similar to that measured using stimulated vesicles. Similar I/V characteristics and conductance of resting and stimulated K\(^+\) channels suggest that the same channel is present in gastric mucosal membranes from resting and stimulated gastric mucosa. This suggestion is supported by the Western blotting of resting and stimulated gastric vesicles, which both showed the presence of Kir2.1. Resting K\(^+\) channel activation by PKA phosphorylation at the cytosolic side and by low pH on the extracellular side is consistent with the channel playing a physiological role in HCl secretion. When Kir2.1 was expressed in CHO cells, significant increases in normalized currents and conductances were observed with forskolin/IBMX at pH 7.4, when pH\(_{o}\) was lowered to 6, and when lowered pH\(_{o}\) was followed by forskolin/IBMX addition in transfected, but not in nontransfected, cells. Thus, as observed with native gastric vesicles, Kir2.1 K\(^+\) channel activity is regulated by PKA phosphorylation and low extracellular pH. PKA regulation of Kir2.1 has been controversial in studies by others, with effects varying from inhibition (64) to activation (17) and lack of effect (31). Further studies are needed to fully understand the structural basis of rectification and activation by extracellular pH and PKA.

Two other K\(^+\) channels (Kir4.1 and KCNQ1) have been localized to the gastric parietal cell and shown to colocalize with H\(^+\)-K\(^+\)-ATPase (18, 20). Fujita et al. (18) found Kir4.1, Kir4.2, and Kir7.1 (but not Kir2.1) in rat gastric mucosa by using RT-PCR. Kir4.1 was immunolocalized to the rabbit gastric parietal cell, where it appeared to colocalize with the \(\beta\)-subunit of the H\(^+\)-K\(^+\)-ATPase. Although the data were not shown, Fujita et al. stated that immunoreactivity of Kir4.2 was localized to surface mucous cells and that Kir4.2 was absent from the epithelium but was detected in “neural regions” of the gastric mucosa. Others (13, 40) have shown that Kir7.1 is located in the basolateral membrane in the kidney and is thought to be involved with providing K\(^+\) to the Na\(^+\)-K\(^+\)-ATPase. Functional data in the study by Fujita et al. were minimal, limited to Ba\(^{2+}\) inhibition of \([^{14}C]\)aminopyrine uptake in intact isolated parietal cells, where effects on basolateral K\(^+\) channels were not eliminated or taken into consideration. Previous work (8) briefly reported that Ba\(^{2+}\) and TEA inhibited K\(^+\)-dependent Cl\(^-\) uptake by isolated stimulated rabbit gastric vesicles. TEA inhibited \([^{14}C]\)aminopyrine uptake.
Fig. 10. Functional expression of Kir2.1 in Xenopus oocytes. Plasma membranes of oocytes expressing rabbit Kir2.1 were isolated, fused to planar lipid bilayers, and examined for K⁺ currents using symmetric 400 mM K₂SO₄, pH₄, 3.0, and PKA-γ (50 U/ml), conditions under which the gastric K⁺ channel is optimally functional. A: current recordings and corresponding amplitude histograms (right) obtained at the indicated holding potentials. Medium also contained 2 mM MgSO₄, 1 mM K-ATP (pH 7.4), 10 mM EGTA (pH 7.4), and 10 mM K-citrate (pH 3). c− indicates the closed state and c− alone indicates the open state of the channel. B: I/V curve obtained from data in A. Data are plotted as means ± SE

as well as H⁺-K⁺-ATPase-related ATP hydrolysis by stimulated rabbit parietal cells in which the basolateral membrane was permeabilized by digitonin. NH₄Cl overcame TEA inhibition of both K⁺-dependent Cl⁻ uptake and H⁺-K⁺-ATPase-related ATP hydrolysis, indicating the presence of a K⁺ channel in parietal cell secretory membranes. However, Ba²⁺ and TEA are general, nonspecific K⁺ channel inhibitors and do not indicate the molecular identity of the channel. Fujita et al. also showed that recombinant Kir4.1 K⁺ currents were not affected by reduction of extracellular pH, a property essential for an apical K⁺ channel. Detailed functional comparison between Kir4.1 and native gastric K⁺ channel function was lacking.

Grahammer et al. (20) reported that KCNQ1, KCNE2, and KCNE3 mRNAs were present in human stomach and that KCNQ1 localized in human parietal cells together with the H⁺-K⁺-ATPase. Chromanol 293B, presumed by Grahammer et al. to be a specific KCNQ1 inhibitor, inhibited acid secretion in the rat perfused stomach, dog Heidenhain pouch, and intact rats. Moreover, KCNE2, but not KCNE3, in COS cells resulted in extracellular acidic pH activation of K⁺ currents. Thus Grahammer et al. suggested that KCNQ1, when coassembled with KCNE2 or KCNE3, is the K⁺ channel complex involved in acid secretion. Others have shown that KCNQ1/KCNE3 can be activated by PKA (52), but this was not examined by Grahammer et al. In contrast, H⁺ transport by H⁺-K⁺-ATPase-containing vesicles from stimulated rabbits is insensitive to chromanol 293B, and K⁺ channel currents measured in CHO cells expressing recombinant Kir2.1 were activated by 293B (12) with no effect observed in nontransfected CHO cells. These findings strongly argue against involvement of KCNQ1 in K⁺ recycling and suggest that chromanol 293B has an alternative, unidentified target in the parietal cell.

Nevertheless, it seems that Kir2.1, Kir4.1, and KCNQ1 are all present in the gastric parietal cell and seem to colocalize with H⁺-K⁺-ATPase and, in the case of Kir2.1, also with CIC-2. K⁺ channel diversity is well established, and not only have there been different channels from the same family expressed in the same tissue (45) but also a single tissue, for example, rat pituitary, has been reported to express transcripts for 21 different Kv channel α-subunits [including Shaker-related Kv, ether-à-go-go (eag), and KCNQ1–3], 9 Kir channel α-subunits, and 7 auxiliary subunits (e.g., Kvβ1–3, minK, sulfonylurea receptor SUR1–2) (66). Such large diversity of K⁺ channels has also been described in cardiac cells (41). The clonal pituitary tumor cell line GH3/B6 has transcripts for 9 Kv, 6 eag, 3 KCNQ, and 5 Kir α-subunits and no auxiliary subunits (66), suggesting that if the proteins are expressed, then these channels are all present in the same cell. These channels cannot be distinguished from each other on the basis of electrophysiology or pharmacology. Heterooligomeric assembly of different Kir K⁺ channels has been studied and shown to result in different functional/electrophysiological properties compared with homooligomeric assembly of four subunits, suggesting interactions between different Kir K⁺ channel subunits (14, 16, 32, 41, 59, 67). Coexpression of auxiliary subunits with α-subunits can affect function and in some cases can be essential for
functional channel activity (e.g., Kir.6.1 with SUR1) (21). Kir.2.1 is not affected by SUR1 (21). These types of interactions can result in K⁺ current inhibition, activation, altered kinetics, rectification, and pH sensitivity; have been shown to be specific and occur between small domains in the cytoplasmic region of the channels; and may depend in part on the ratio of the amounts of various subunits present. Which K⁺ channel subunits are present together in a single cell type is very important and can have major functional consequences. KCNE1 expressed with KCNQ1 increases the K⁺ current, slows activation, and shifts voltage dependence toward more positive potentials (52). In contrast, KCNE2 expressed with KCNQ1 results in a greatly decreased K⁺ current and altered gating properties compared with KCNQ1 alone (61), whereas KCNE3 expressed with KCNQ1 results in a constitutively open K⁺ channel that can be further activated by PKA (52). KCNE3 suppresses KCNQ4 currents (52).

Whether Kir2.1, Kir4.1, and KCNQ1 (with KCNE2 or KCNE3) coexist side by side in the gastric mucosal membranes in parietal cells and are activated at appropriate times by appropriate activators or whether they assemble and/or function cooperatively with each other or other subunits, including auxiliary ones, remains to be examined. Such interactions might explain the difference in the gating kinetics observed between native gastric vesicles and Kir2.1 expressed in oocyte membranes. Detailed functional studies and comparison of behavior and kinetics of native gastric K⁺ channels with recombinant K⁺ channels separately and coexpressed in CHO cells might lead to a further understanding of how K⁺ is moved from cytosol to lumen to enable acid secretion to occur. Although care must be used in transposition of the results of studies on isolated membranes to the native environment of the cell, the present study provides electrophysiological, biochemical, and immunological evidence for a K⁺ channel, Kir2.1, in gastric mucosal membranes with the required properties for involvement in acid secretion and that colocalizes with both H⁺/K⁺-ATPase and CIC-2 Cl⁻ channels. Similar in vivo effects of histamine on a voltage-dependent rabbit gastric Cl⁻ channel, CIC-2, present in resting and stimulated gastric membranes, have been previously reported (6, 35). In these studies, both native gastric resting and recombinant CIC-2 Cl⁻ channels are activated by PKA and low pH. Protein kinase action and extracellular pH provide the means by which to control both K⁺ and Cl⁻ efflux across the parietal cell secretory membrane. The findings of the present studies strongly suggest that Kir2.1 is present in gastric mucosal membranes of the parietal cell and may be involved in acid secretion.

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