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pH Dependence of the Inwardly Rectifying Potassium Channel, Kir5.1, and Localization in Renal Tubular Epithelia*

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The physiological role of the inwardly rectifying potassium channel, Kir5.1, is poorly understood, as is the molecular identity of many renal potassium channels. In this study we have used Kir5.1-specific antibodies to reveal abundant expression of Kir5.1 in renal tubular epithelial cells, where Kir4.1 is also expressed. Moreover, we also show that Kir5.1/Kir4.1 heteromeric channel activity is extremely sensitive to inhibition by intracellular acidification and that this novel property is conferred predominantly by the Kir5.1 subunit. These findings suggest that Kir5.1/Kir4.1 heteromeric channels are likely to exist *in vivo* and implicate an important and novel functional role for the Kir5.1 subunit.

The ability of a cell to maintain an electrochemical imbalance across its plasma membrane is one of the most fundamental and essential biological processes. A large variety of integral membrane proteins exist to facilitate transport across membranes. One such example is the family of inwardly rectifying potassium (Kir) channels that are found in almost every cell type, where they play key roles in controlling membrane potential, cellular excitability, and K^+ fluxes (1, 2).

Kir channel subunits possess two transmembrane domains separated by the signature K^+-selective pore sequence and can assemble as both homotetramers and heterotetramers. Since the initial isolation of Kir1.1 by Ho *et al.* (3) in 1993, approximately fifteen distinct clones have been identified that can be divided into seven major subfamilies, Kir1.0–Kir7.0 (1, 2). Physiological roles have been either established or proposed for nearly all of these major subfamilies. However, the Kir5.0 family remains a notable exception. To date, only one member of this family (Kir5.1) has been identified (4).

We have previously reported the production of specific polyclonal antibodies against Kir5.1 and shown it to be highly expressed in spermatozoa and spermatogenic tissue (5). Kir5.1 mRNA has also been shown to be present in kidney, spleen, adrenal glands, liver, and in several brain regions (4). However, almost nothing is known about the specific cell types involved or the function of Kir5.1 in these tissues. This is primarily because of the inability of Kir5.1 to produce functional K^+ channel activity when heterologously expressed by itself, and even though it can form functional heteromeric K^+ channels with Kir4.1 (6) and Kir4.2 (7), its functional relevance is not yet clear.

Renal potassium channels play a critical role in several key tubular transport functions (8, 9). Tubular basolateral K^+ channels are principally responsible for the maintenance of the membrane potential, thus generating the driving force for electrogenic solute translocation across the apical membrane. K^+ secretion by the apical membrane is also an important source of K^+ excretion. Regulatory mechanisms for the cross-talk between the K^+ channels of the apical and basolateral membranes are therefore essential. Although some of the potassium channels responsible have been cloned, the molecular identity of many renal K^+ channels remains to be established. Given the high levels of expression of rat Kir5.1 mRNA in the kidney (4) we have further investigated the localization of Kir5.1 in rat renal tissues and the potential role of this subunit.

In this study we show that Kir5.1 subunits are highly expressed in the epithelial cells of the convoluted tubules of rat kidney, the same cells in which Kir4.1 is expressed (10). More importantly, we also show that mild intracellular acidification markedly inhibits heteromeric Kir5.1/Kir4.1 channel activity and that this property is conferred primarily by the Kir5.1 subunit. These findings suggest that pH-sensitive, heteromeric Kir5.1/Kir4.1 channels are likely to exist *in vivo* and identify Kir5.1 as a potentially important regulator of renal function.

MATERIALS AND METHODS

Molecular Biology

Gene-specific primers based upon rat Kir5.1 (5′-CGCAAGGCAGTGGCCAAAG-3′ and 5′-CATCTGGGATTCCATGGAGAT-3′) were used to amplify the correct 3′ end of the gene from rat genomic DNA by PCR.[1] The amplified fragment was used to reconstruct the correct full-length rat Kir5.1. Sequences were confirmed on both strands by automated sequencing. The full and corrected sequence of this clone has been deposited in the GenBank™ data base (AF249676). NCBI-BLAST searches and sequence alignments were performed using the Omiga 2.0 program (Oxford Molecular, Oxford, UK). The C-terminal truncation of Kir5.1 (Kir5.1ΔC49) was created by PCR truncation. All channel subunits were subcloned into the oocyte expression vector pBF, which provides 5′ and 3′ untranslated regions from the *Xenopus* β-globin gene flanking a polylinker containing multiple restriction sites. *In vitro* mRNAs were generated using SP6 polymerase. Kir subunits were joined in tandem as described previously (6).

Electrophysiology

Two-electrode Voltage Clamp Recording—Xenopus laevis care and handling were in accordance with the highest standards of institutional

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF249676.

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[1] The abbreviations used are: PCR, polymerase chain reaction; DAB, 3,5′-diaminobenzidine; ECF, extracellular fluid.

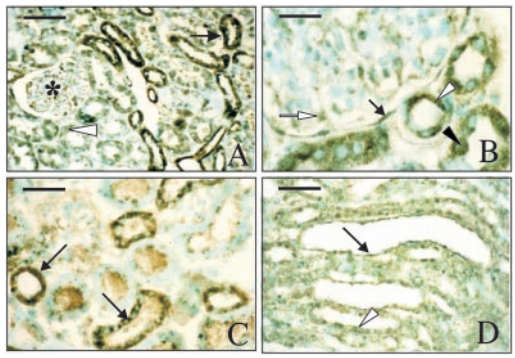

FIG. 1. **Immunolocalization of Kir5.1 in the kidney.** *A*, positive ABC immunoperoxidase staining shows the presence of the Kir5.1 subunit in proximal convoluted tubules (*open arrowhead*) and in distal convoluted tubules (*closed arrow*). The glomeruli (*) are poorly stained. *B*, localization of Kir5.1 at a higher magnification showing that labeling is present in the endothelial cells surrounding glomerular capillaries (*open arrows*) and in the flattened parietal layer of Bowman's capsule (*closed arrow*). The epithelial cells of both proximal (*open arrowhead*) and distal (*closed arrowhead*) convoluted tubules are also positively stained. *C*, more transverse sections at high magnification showing abundant expression of Kir5.1 in tubular epithelia (*closed arrow*). *D*, longitudinal sections through the outer medullary zone confirmed the presence of Kir5.1 in the epithelium of proximal and distal tubules and show positive immunostaining along the thin segment of the loop of Henle (*closed arrow*) and in the epithelium of collecting tubules (*open arrowhead*). Scale Bar = 50 μm (*A* and *D*), 10 μm (*B*), and 25 μm (*C*).

guidelines. Frogs underwent no more than two surgeries, separated by at least three weeks. Frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester. Standard recording solution contained 90 mM KCl, 3 mM $MgCl_2$, 10 mM HEPES (pH 7.4) unless otherwise stated. Intracellular acidification was achieved using a potassium acetate buffering system (11). Microelectrodes were filled with 3M KCl and had resistances of 0.1–0.5 megohms. Recordings were performed at 22 °C 18–48 h after injection with a GeneClamp 500 amplifier (Axon Instruments) interfaced to a Power Macintosh 7200/90 computer with an ITC-16 computer interface (InstruTECH Corp., Port Washington, NY). Currents were evoked by voltage commands from a holding potential of −10 mV, delivered in −10-mV increments from 50 to −120 mV, unless otherwise stated. Unless otherwise stated oocytes were injected with Kir4.1 and Kir5.1 (or Kir5.1ΔC49) mRNAs in a 1:5 ratio as described previously (6).

Patch Clamp Recording—Patch clamp recordings were performed as described previously (6) using an Axopatch 200B amplifier (Axon Instruments). Oocytes were bathed in a cytoplasmic solution containing 120 mM KCl, 10 mM EGTA, 5 mM HEPES, 0.1 mM dithiothreitol, pH 7.2. Recording electrodes were pulled from borosilicate glass, dipped in sticky wax (Kerr, Emoryville, CA) prior to polishing, and had resistances of 3–5 megohm. The pipette solution contained 120 mM KCl, 5 mM HEPES, 200 μM $CaCl_2$, pH 7.2. Patch records were obtained in the cell-attached and inside-out configuration by stepping the holding potential to various test potentials for 20–60 s. Current traces at each holding potential were filtered at 500 Hz and digitized at 5 kHz.

Immunohistochemistry

The production and specificity of the anti-Kir5.1 antibody has previously been described in detail (5). Sections of kidney from 60-day-old Harlan Sprague-Dawley rats were prepared, fixed, and stained as described previously (5). Antibody staining was visualized using 3,5′-diaminobenzidine (DAB), and tissues were counterstained with methylene blue. To confirm the specificity of these Kir5.1 antibodies, kidney sections were stained with antibodies preabsorbed to the immunogenic peptide (5) and with the secondary antibody alone. In both cases no DAB staining could be visualized (not shown).

RESULTS AND DISCUSSION

We have previously generated and purified highly specific polyclonal antibodies for Kir5.1. Using these antibodies we immunostained rat kidney sections. Fig. 1 shows intense ABC immunoperoxidase staining in the cortex and inner and outer zones of the medulla. In particular, Kir5.1 appears to be abundantly expressed in the epithelial tissues of the proximal and distal convoluted tubules. Positive staining was also observed in the endothelial cells surrounding glomerular capillaries, in the flattened parietal layer of Bowman's capsule, in the thin segment of the loop of Henle, and in the collecting tubule epithelial cells.

Although Kir5.1 does not appear to form functional homomeric channels, coexpression with Kir4.1 (6) generates novel heteromeric channels. However, the functional relevance of these channels is unclear. Kir4.1 has been shown to be expressed in renal distal tubular epithelial cells, where it is confined to the basolateral membrane (10). Therefore, because Kir5.1 is also expressed in these cells and can form a functional heteromeric channel with Kir4.1, it is not unreasonable to assume that these heteromeric channels exist *in vivo*.

The inability of Kir5.1 to produce functional K^+ channel activity when expressed alone could mean that the channel is present, but inactive, in the plasma membrane, or it could mean that the protein fails to reach the plasma membrane. Kir6.2 is also incapable of independent functional expression because of the presence of an endoplasmic reticulum retention sequence in the C terminus that prevents the subunit from reaching the plasma membrane (12, 13). We therefore examined the role of the C terminus of Kir5.1. Alignment of the rat sequence with the mouse sequence of Kir5.1 (14) revealed significant differences in the C-terminal sequences. A BLAST search of the sequence data base with the rat Kir5.1 sequence identified a novel human homolog of Kir5.1 in a large chromosome 17 clone (GenBank™ accession number AC005298; 107283–108341 base pairs). Identification of this human homolog confirms the chromosomal localization proposed by Mouri *et al*. (14), who localized mouse Kir5.1 to the murine chromosome 11, which shares extensive homology with human chromosome 17.

Closer examination of these three Kir5.1 sequences revealed that the original rat clone contained an error at position 1079 that introduces a frameshift and results in premature truncation. Using gene-specific primers we PCR-amplified the 3′ end of the rat Kir5.1 gene from rat genomic DNA and reconstructed the correct sequence. The corrected amino acid sequence of the C terminus of rat Kir5.1 then aligned correctly with the murine and human homologs (not shown) and demonstrates that the C-terminal sequence of Kir5.1 is highly conserved between the species.

Fig. 2, *A-C* shows that expression of the correct rat Kir5.1 clone in *Xenopus* oocytes still fails to elicit functional K^+ currents but that Kir5.1 is still capable of forming novel heteromeric channels when coexpressed with Kir4.1. These novel channels are characterized by an instantaneous component that is followed by a time-dependent increase in the current and appears identical to those channels we have previously reported for Kir4.1 and Kir5.1 coexpression (6). It is therefore unlikely that the distal C terminus of Kir5.1 has any effect on the ability of the subunit to form heteromeric channels with Kir4.1. In our previous study (6) we also reported that the most accurate way to record currents from these heteromeric channels is in a fixed 2:2 stoichiometry by expression of tandemly linked Kir4.1-Kir5.1 dimers. This ensures that the currents recorded are not only from channels of a fixed stoichiometry but also that there are no contaminating currents from Kir4.1 homotetramers. We therefore linked the correct rat Kir5.1 sequence in tandem with Kir4.1 and expressed this dimeric Kir4.1-Kir5.1 construct in *Xenopus* oocytes. Expressing the full-length Kir4.1-Kir5.1 dimer generated currents almost identical to those formed by expression of individual mRNAs, and Fig. 2*D* shows that the rectification properties of these Kir4.1-Kir5.1 currents do not differ from those previously re-

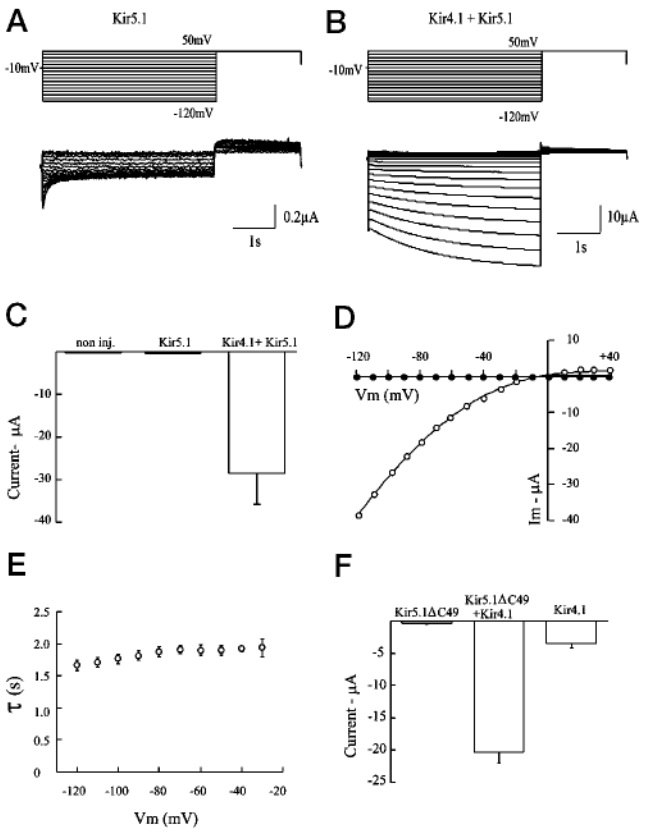

FIG. 2. **Functional consequences of the C terminus of Kir5.1.** Representative current families from oocytes expressing (*A*) the full-length Kir5.1 and (*B*) Kir4.1 + Kir5.1 mRNAs. The voltage protocols are shown on top. *C*, current amplitudes recorded at −100 mV for non-injected (*non inj.*) Kir5.1 and Kir4.1 + Kir5.1-injected oocytes. A constant amount of Kir5.1 or Kir4.1 + Kir5.1 mRNA was injected. *Bars* represent the mean ± S.E. of 4–6 cells. *D*, steady-state current-voltage relationship for the oocytes expressing Kir5.1 (*closed circles*) and the Kir4.1-Kir5.1 dimer (*open circles*) mRNAs. *E*, the slow component of activation for the Kir4.1-Kir5.1 dimer was fitted with a single exponential function, and the time constants are plotted as a function of the command potential. The data points are the mean ± S.E. ($n = 4$). *F*, current amplitudes recorded at −100 mV for Kir4.1, Kir5.1ΔC49, and Kir4.1 + Kir5.1ΔC49-injected oocytes. *Bars* represent the mean ± S.E. of 6 cells.

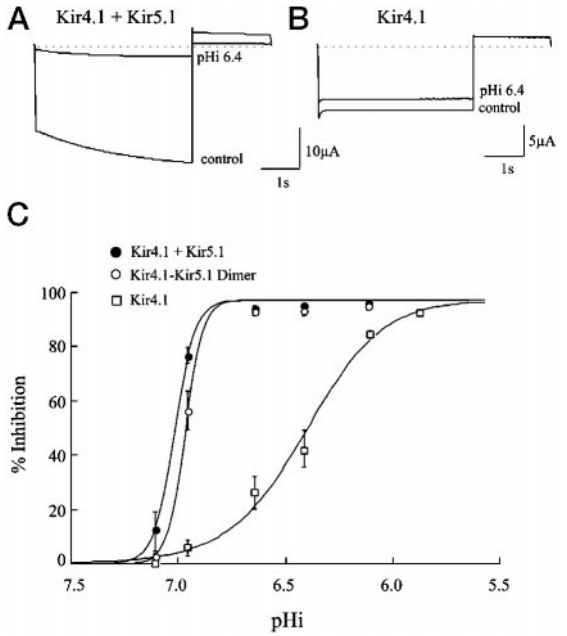

FIG. 3. **pH sensitivity of Kir4.1 and Kir4.1/Kir5.1 channels.** Representative currents recorded from oocytes expressing (*A*) Kir4.1 + Kir5.1, and (*B*) Kir4.1 mRNA. Currents were recorded in control conditions and during the perfusion of a membrane-permeable potassium acetate buffer that reduces the oocyte intracellular pH to the indicated value (11). Currents were recorded at −100 mV. *C*, the presumed intracellular pH_i *versus* current inhibition for Kir4.1 + Kir5.1 (*closed circles*), the Kir4.1-Kir5.1 dimer (*open circles*), and Kir4.1 (*squares*) currents. Data points are the mean ± S.E. of 6–8 oocytes. The *solid line* shows the fit with the equation, $1/[1 + ([H^+]_i/K)^n]$, from which the apparent pK_a values were calculated.

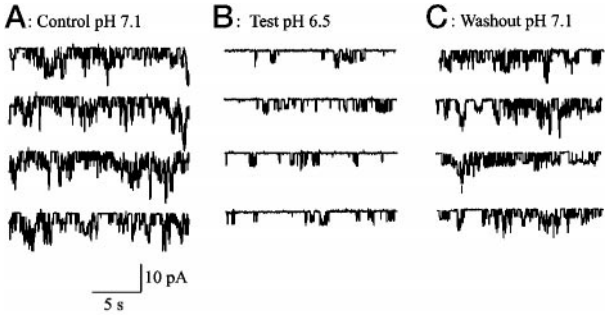

FIG. 4. **Sensitivity of Kir4.1-Kir5.1 channels to intracellular pH.** Representative inside-out patch recordings from oocytes injected with Kir4.1-Kir5.1 dimer mRNA. Channel activity was evoked by voltage commands to −100 mV in (*A*) control condition (pH 7.1), (*B*) during patch perfusion with a test solution at pH 6.5, and (*C*) during return to control solution at pH 7.1. The solutions were applied through a fast flow system.

ported (6). In addition, the time-dependent component was well fitted by a single exponential function that yielded time constants not different from the previously reported truncated dimer (Fig. 2*E*) (6). Therefore, the biophysical properties of these channels appear identical to those we have previously reported and indicate that the distal C terminus of this subunit does not affect the ability of Kir5.1 to form novel heteromeric channels with Kir4.1.

Intriguingly, the newly revealed distal C terminus of Kir5.1 possesses a sequence (RRRSFSAVA) with partial homology to the region containing the arginine-rich endoplasmic reticulum retention sequence found in Kir6.2 (13). We therefore examined whether this motif prevents independent functional expression of Kir5.1. We created a C-terminal truncation of Kir5.1 that deleted amino acids 371–419 (Kir5.1ΔC49) and therefore removed this motif. However, injection of Kir5.1ΔC49 mRNA into *Xenopus* oocytes did not produce K^+ channel activity (Fig. 2*F*). By contrast, coexpression of Kir5.1ΔC49 with Kir4.1 clearly demonstrates that this truncation has no effect on the ability of the subunit to form heteromeric channels with Kir4.1 (Fig. 2*F*). This suggests that either Kir5.1 is only able to form functional channels as a heteromer or that it is lacking an accessory regulatory subunit or agonist.

One of the most important renal functions is the homeostatic regulation of extracellular fluid (ECF) volume, pH, and osmolality (8, 9). These processes are primarily dependent on the regulation of Na^+ balance, which itself is dependent on the relative K^+ concentrations. K^+ balance is also critical because of the impact of ECF K^+ concentrations on the resting membrane potential of tubular epithelia. Mechanisms for the regulation of K^+ secretion and recycling in renal epithelia are therefore vitally important. One such example is thought to be the pH-dependent regulation of the renal secretory K^+ channel, Kir1.1. This channel is found in the apical membrane of the tubular epithelia (15), where it is inhibited by intracellular, but not extracellular, acidification with a p*K* of 6.8 (16). Mutations in Kir1.1 that alter its pH sensitivity are responsible for inherited forms of antenatal Bartter's syndrome (17). We therefore tested

whether extracellular and intracellular pH changes were able to regulate Kir4.1/Kir5.1 heteromeric channel activity.

Extracellular acidification of oocytes was unable to modify the whole-cell amplitude of Kir4.1/Kir5.1 currents recorded by two-electrode voltage clamp (not shown). To assess the effects of intracellular acidification we used a well established potassium acetate buffering system that has been shown to modify the intracellular pH of oocytes (11). Fig. 3*A* shows that perfusion of oocytes with a potassium acetate buffer, which has been shown to reduce the pH_i to 6.4 (11), causes almost complete inhibition of Kir4.1/Kir5.1 currents. However, Fig. 3*B* shows that only a small inhibition of Kir4.1 currents is observed at this predicted pH_i. To assess the effects of pH_i on Kir4.1/Kir5.1 heteromeric channels we made use of the Kir4.1-Kir5.1 tandemly linked dimer. As well as ensuring a fixed stoichiometry this also excludes contaminating currents from Kir4.1 homotetramers (6). Fig. 3*C* shows the current inhibition/pH_i relationship for the Kir4.1 + Kir5.1 currents, as well as for the Kir4.1-Kir5.1 dimer and Kir4.1. The heteromeric Kir4.1-Kir5.1 channels generated by this dimeric construct exhibited the same sensitivity to intracellular acidification as the Kir4.1/Kir5.1 channels formed by individual subunit expression. The pK_a values calculated from these data were 6.9 ± 0.02 for Kir4.1 + Kir5.1, and 6.8 ± 0.02 for the Kir4.1-Kir5.1 dimer, as opposed to 6.1 ± 0.04 for Kir4.1. The latter value is in agreement with those previously calculated for Kir4.1 (17, 18). These results clearly demonstrate that Kir4.1-Kir5.1 heteromeric channels are very sensitive to changes in pH_i within the physiological range and that this dimeric construct is a valid tool for assessing the effects of pH on these heteromeric channels. These results also demonstrate that this extreme pH_i sensitivity is primarily conferred by the Kir5.1 subunit, because at pH_i 6.6, Kir4.1/Kir5.1 channels are almost completely inhibited, whereas Kir4.1 currents are almost fully active at this pH. Indeed, one report suggests that Kir4.1 exhibits maximal activity at pH 6.6 (18) and is therefore unlikely to contribute significantly to the pH-dependent regulation of Kir4.1/Kir5.1 channels within the physiological range.

To confirm the effect of intracellular acidification on these channels we recorded currents in excised membrane patches from oocytes expressing the Kir4.1-Kir5.1 dimer. Fig. 4 shows that exposure of the intracellular surface of the patch to a solution at pH 6.5 rapidly reduced the activity of these channels by approximately 80%. This reduction in pH_i did not appear to modify the single channel amplitude and is clearly reversible upon return to control solution (Fig. 4*C*). Taken together these results clearly demonstrate that intracellular acidification directly inhibits these heteromeric channels.

In conclusion, our immunolocalization studies show Kir5.1 to be abundantly expressed in the epithelial cells of the proximal and distal convoluted tubules. The identification of Kir5.1 in the same cells in which Kir4.1 is also expressed suggests that heteromeric Kir4.1/Kir5.1 channels exist *in vivo*. Furthermore, the extreme sensitivity of these heteromeric channels to intracellular acidification implicates a novel role for the Kir5.1 subunit in pH-dependent regulation of renal K^+ buffering and recycling.

REFERENCES

1. Nichols, C. G., and Lopatin, A. N. (1997) *Annu. Rev. Physiol.* **59,** 171–191
2. Reimann, F., and Ashcroft, F. M. (1999) *Curr. Opin. Cell Biol.* **11,** 503–508
3. Ho, K., Nichols, C. G., Lederer, W. J., Lytton, J., Vassilev, P. M., Kanazirska, M. V., and Hebert, S. C. (1993) *Nature* **362,** 31–38
4. Bond, C. T., Pessia, M., Xia, X-M., Lagrutta, A., Kavanaugh, M. P., and Adelman, J. P. (1994) *Receptors Channels* **2,** 183–191
5. Salvatore, L., D'Adamo, M. C., Polishchuk, R., Salmona, M., and Pessia, M. (1999) *FEBS Lett.* **449,** 146–152
6. Pessia, M., Tucker, S. J., Lee, K., Bond, C. T., and Adelman, J. P. (1996) *EMBO J.* **15,** 2980–2987
7. Pearson, W. L., Dourado, M., Schreiber, M., Salkoff, L., and Nichols, C. G. (1999) *J. Physiol.* (*Lond.*) **514,** 639–653
8. Kawahara, K., and Anzai, N. (1997) *Jpn. J. Physiol.* **47,** 1–10
9. Wang, W., Hebert, S. C., and Giebisch, G. (1997) *Annu. Rev. Physiol.* **59,** 413–436
10. Ito, M., Inanobe, A., Horio, Y., Hibino, H., Isomoto, S., Ito, H., Mori, K., Tonosaki, A., Tomoike, H., and Kurachi, Y. (1996) *FEBS Lett.* **388,** 11–15
11. Choe, H., Zhou, H., Palmer, G., and Sackin, H. (1997) *Am. J. Physiol.* **273,** F516-F529
12. Tucker, S. J., Gribble, F. M., Zhao, C., Trapp, S., and Ashcroft, F. M. (1997) *Nature* **387,** 179–183
13. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) *Neuron* **22,** 537–548
14. Mouri, T., Kittaka, N., Horio, Y., Copeland, N. G., Gilbert, D. G., Jenkins, N. A., and Kurachi, Y. (1998) *Genomics* **54,** 181–182
15. Xu, J. Z., Hall, A. E., Peterson, L. N., Bienkowski, M. J., Eessalu, T. E., and Hebert, S. C. (1997) *Am. J. Physiol.* **273,** F739-F748
16. Fakler, B., Schultz, J. H., Yang, J., Sculte, U., Brandle, U., Zenner, H. P., Jan, L. Y., and Ruppersberg, J. P. (1996) *EMBO J.* **15,** 4093–4099
17. Schulte, U., Hahn, H., Konrad, M., Jeck, N., Derst, C., Wild, K., Weidemann, S., Ruppersberg, J. P., Fakler, B., and Ludwig, J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 15298–15303
18. Yang, Z., and Jiang, C. (1999) *J. Physiol.* **520,** 921–927