Identification by Differential Display of a Hypertonicity-inducible
Inward Rectifier Potassium Channel HighlyExpressed in Chloride
Cells*

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By using differential mRNA display to monitor the molecular alterations associated with adaptation of eu-
ryhaline eels to different salinities, we identified a cDNA fragment strongly induced in seawater eel gills.
Cloning of a full-length cDNA and its expression in COS-7 cells indicated that the clone codes for an inward
rectifier K\(^+\) channel (eKir) of 372 amino acid residues, which has two transmembrane segments and a typical
pore-forming region (H5). Only low sequence similarities are present, except the H5 region, compared with
other members of the inward rectifier K\(^+\) channel family (Kir). Consistent with this divergence in the amino acid
sequence, a phylogenetic analysis indicated early divergence and independence of eKir from other
members; it is only distantly related to the Kir5.0 subfamily members. RNase protection analysis showed
that eKir is highly expressed in the seawater eel gill, kidney, and posterior intestine but very weakly in freshwater
eels. Immunohistochemistry of gill sections revealed dense localization of eKir in the chloride cells. Immuno-
electron microscopy indicated that eKir is mainly present in the microtubular system in the chloride cell.
This location and its salt-inducible nature suggest that the eKir channel cloned here is a novel member of the Kir5.0
subfamily of the Kir family and is implicated in osmoregulation.

Euryhaline fishes such as eel, salmon, tilapia, and flounder can live both in freshwater and seawater. To toler-
ate a wide range of salinities and move between freshwater and seawater, they developed special structures and mechanisms for osmo-
regulation, one of which is the chloride cells in the gill. The chloride cells or the ionocytes are rich in mitochondria and
actively absorb salt in freshwater and pump out excess salt in seawater to help maintain body fluid homeostasis.
Understanding the molecular basis of these extraordinary abilities of euryhaline species has long been a major goal in animal phys-
iology. Previous studies were mainly focused on the morpho-
logical, electrophysiological, and hormonal aspects. Although
such conventional approaches have revealed a number of in-
teresting facts concerning the osmoregulatory processes, de-
tailed mechanisms and the molecules involved remain to be
clarified. We employed, in the present study, the recently de-
veloped RNA arbitrary primed polymerase chain reaction
(RAP-PCR)\(^1\) to identify the mRNA species whose expressions
are regulated by the environmental salinities. RAP-PCR is a
method closely related to the differential display method that
provides an attractive tool for the isolation of differentially
expressed genes (1, 2). Our attempt using mRNA preparations
from freshwater and seawater eels resulted in identification of
several mRNA species that exhibit markedly altered expres-
sion. One of them was, as detailed below, a K\(^+\) channel
that belongs to the inward rectifier K\(^+\) channel family and shows a
unique tissue distribution as being confined to osmoregulatory
organs such as the gill, kidney, and intestine.

K\(^+\) channels are a diverse group of membrane proteins. Recent molecular cloning has established the presence of two structurally and functionally distinct families of the channel as
follows: 1) the voltage-gated K\(^+\) channels (Kv) that have six transmembrane spans within each subunit and are activated
by depolarization in a steeply voltage-dependent manner, and
2) the inward rectifier K\(^+\) channels (Kir) that have only two transmembrane segments and conduct inward current more
readily than outward current. Although the members of these
K\(^+\) channel families are different in their subunit structures,
electrophysiological properties, and mechanisms of activation,
they are considered to be assembled with multiple (probably four) homologous or heterologous subunits to make up the
K\(^+\)-selective pore (3–6). All of the channel subunits cloned so
far including those of animals (7–10) and even microorganisms
(11) share a highly conserved sequence of about 17 amino acid
residues called the pore region or H5, which has been shown to
be part of the K\(^+\)-selective pore (12–14). The presence of this
H5 sequence is therefore considered the signature of a K\(^+\)
channel protein.

Although the function of the voltage-gated K\(^+\) channels
is relatively simple and well established as being responsible for
action potential repolarization and frequency encoding, the
function of the inward rectifier K\(^+\) channels is quite diverse.
The inward rectifier K\(^+\) channels are found in a wide variety of

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1 The abbreviations used are: RAP-PCR, RNA arbitrary primed po-

lymerase chain reaction; eKir, eel inward rectifier K\(^+\) channel; Kv,
voltage-gated K\(^+\) channel; Kir, inward rectifier K\(^+\) channel; MBP, mal-
tose-binding protein; bp, base pair(s); Pipes, 1,4-piperazinediethanesul-
fonic acid; Mops, 4-morpholinepropanesulfonic acid.

\(1\) The nucleotide sequence(s) reported in this paper has been submitted to
the GenBank\(^{TM}\)/EBI Data Bank with accession number(s)
AB009669.

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tissues and cell types where they are involved not only in the maintenance of the resting membrane potential and control of cell excitability but also in processes not usually associated with electrically excitable membranes such as hormone secretion, K⁺ excretion, and buffering extracellular K⁺ concentrations or recycling K⁺. Several inward rectifier K⁺ channels have recently been cloned from a number of tissues and species by expression cloning (7, 8), low stringency hybridization (15), and PCR methods (10, 17, 18). Their sequencing and characterization have demonstrated structural and functional diversities of the channel proteins, and currently the molecular determinants of rectification are being identified. The next step will be to clarify the physiological significance of the channel subtypes. Here we report cloning of a unique member of the Kir family whose expression is strongly induced in eels, especially in the chloride cells of the gill, during adaptation to seawater and therefore considered to play an essential role in osmoregulation.

EXPERIMENTAL PROCEDURES

Differential Display—Differential display was carried out as described (9). The cDNA was preamplified to a 1:1000 dilution with Taq polymerase (Takara). The mixture was cycled first at 94°C for 1 min, 36°C for 5 min, and 72°C for 30 min followed by 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. An aliquot of each amplification mixture was subjected to electrophoresis in a 7.5% polyacrylamide gel, and DNA was visualized by ethidium bromide staining. Differentially expressed bands of interest were extracted from the gel, and DNA was visualized by ethidium bromide staining. Subsequently, the differential bands were excised from the gel, and DNA was eluted using a QiAQuick gel extraction kit (Qiagen Inc.). The PCR conditions were as follows: 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, 35 cycles. The amplified fragments were subcloned into pBluescript II vector, and their sequences were determined.

Cell Culture and Plasmid Transfection—Cos-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 10 mM Hepes, penicillin (50 units/ml), and streptomycin (50 mg/ml) at 37°C in 5% CO₂. The pcDL-SRα296 vector and the COS-7 cells using DOSPHER liposomal transfection reagent (Boehringer Mannheim). Two days after the transfection, the cells were used for electrophysiological experiments.

Electrophysiology—For electrophysiological experiments, the whole-cell patch clamp technique (22) was employed. The EPC-9 amplifier (HEKA) was used to record the membrane current. The patch electrode solution contained (in mM) 95 potassium aspartate, 40 KCl, 1 MgCl₂, 5 EGTA (K salt) and 20 Hepes (sodium salt, pH 7.4). The electrode resistance ranged between 3 and 5 MΩ. The position of the extracellular solution was (in mM) 140 KCl, 1 MgCl₂, 10 Hepes (sodium salt, pH 7.4). Extracellular solutions containing 40 mM K⁺ and 20 mM K⁺ were made by substituting KCl with isosomotic NaCl.

RNase Protection Assay—A 590-bp PCR fragment was subcloned into the pBluescript II vector and linearized by digestion with BstXI and was used as a template to generate a 300-nucleotide antisense RNA probe. In vitro transcription was carried out using an RNA transcription kit (Stratagene) with [α-32P]UTP. Template DNA was then digested with restriction enzyme EcoRI and purified from the reaction mixture by ethanol precipitation. The probe was labeled with 10 μCi of [α-32P]UTP. The labeled probe (10⁶ cpm/sample) was mixed with 10 μg of total RNA from various eel tissues and hybridized overnight in 80% formamide, 40 mM Pipes, pH 6.4, 1 mM EDTA, and 0.4 M NaCl. RNase digestion was performed for 2 h at 37°C with a mixture of RNase A (40 μg/ml) and RNase T₁ (2 μg/ml). Protected fragments were electrophoresed on 5% polyacrylamide, 8 μa urea denaturing gels and visualized by autoradiography.

Expression and Purification of MBP-eKir Fusion Protein—To express the eKir-MBP (maleose-binding protein) fusion protein, a 590-bp fragment of eKir (nucleotides 544–1135) was prepared by PCR and cloned into the pMAL-p vector (New England Biolabs). Escherichia coli strain XLI-Blue harboring the recombinant plasmid was grown in TB medium in the presence 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h, and harvested by centrifugation. After cell lysis, the lysate was centrifuged, and the supernatant was subjected to affinity chromatography on amylose resin as described by the manufacturer.

Preparation of Antiserum and Western Blotting—Antiserum to eKir was raised in a Japanese White rabbit. The MBP-eKir fusion protein was emulsified with Freund’s adjuvant and injected several times. Western blotting was used to establish the specificity of the antiserum. Antibody-labeled membranes were then peroxidase-conjugated to 100 ng/ml Tris buffer containing 0.9% NaCl, 0.2 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml leupeptin. The homogenates were centrifuged at 5000 x g for 20 min, and the pellets were resuspended in the same buffer. These procedures were repeated three times at 4°C. The membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a membrane. After blocking in the buffer containing 5% nonfat milk, the membranes were incubated with 0.1% of the eKir antiserum (1:3000). The channel-antibody complexes were visualized using alkaline phosphatase-conjugated secondary antibody with substrates for alkaline phosphatase.

Immunohistochemistry—Frozen sections were cut in a cryostat, washed in phosphate-buffered saline (100 mM NaCl, 10 mM NaH₂PO₄, pH 7.4), and fixed in acetone containing 5% H₂O₂ to inactivate endogenous peroxidase. These sections were stained using the avidin-biotin complex (ABC) method. After an overnight incubation with anti-eKir antiserum (1:1000), they were incubated with biotinylated goat anti-rabbit antiserum (1:3000), and then washed with PBS. Rabbit antiserum to eKir IgG was applied at a 1:1000 dilution. The channel-antibody complexes were then visualized using 3,3'-diaminobenzidine tetrahydrochloride and 0.02% H₂O₂ in 50 mM Tris-HCl, pH 7.4.

Electron Microscopy—Eels, adapted in seawater for 14 days, were perfused under anesthesia through the ventral aorta with a fixative containing 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. After the perfusion, gill arches were dissected out and immersed for 3 h in the same fixative at ice-cold temperature and rinsed in phosphate-buffered saline containing 8% sucrose. The fixed tissues were cryoprotected through a range of increasing sucrose concentrations up to 30% and then frozen, and cut on a cryostat at 10 μm. The eKir on the sections was detected by use of the pre-embedding 1-nm gold particle-silver enhancement method (23) slightly modified as described (24). Briefly, sections were incubated for 48 h with the anti-eKir (1:500) at 4°C and then incubated overnight with a goat anti-rabbit IgG labeled with 1-nm gold particles (1:100) (Nanogold, Nanoprobes Inc.) at 4°C. Tissue-bound gold particles were enhanced by incubation with a silver deposit.
RESULTS

Identification of Differentially Expressed Genes by RAP-PCR—In an attempt to isolate genes that may be responsible for freshwater or seawater adaptation of euryhaline fishes, we carried out RAP-PCR, a recently developed technique for detecting altered gene expression, using poly(A)-rich RNA preparations obtained from freshwater- and seawater-adapted eel gills. This method is based on the use of arbitrary primers to generate fingerprints by polymerase chain reaction from closely matched RNA populations. The RAP-PCR products were separated by agarose gel electrophoresis and compared for differences in their band patterns. Although both mRNA samples showed very similar display patterns, a number of bands were affected by the changes in salinity. We therefore isolated, from among the differentially displayed bands, the following three major ones and characterized them as follows: a band of about 220 bp that was amplified to a greater degree in freshwater samples (FW220) and two seawater-specific bands of about 250 and 300 bp (SW250 and SW300). DNA sequencing followed by a data base search using BLAST indicated that the major component of FW220 is an eel homolog of P450 (~70% similar); SW250 represents Rho-type GTPase-activating protein (p190RhoGAP, ~70% similar to mammalian homologs), a potent GTPase activator for various Rho-type GTPases, and SW300, an inward rectifier K⁺ channel (eKir). Fish P450s have been demonstrated to undergo marked up-regulation in response to water pollution regardless of osmotic environments (25); therefore, we did not analyze the FW220-derived clone any further. Gross morphological changes of the chloride cells in the gill have been shown to occur when euryhaline fishes face osmotic challenges (26), and the control of the actin cytoskeleton and cell morphology appear to be mediated by members of the Rho family (27, 28). In this context, the p190RhoGAP clone contained in the SW250 band seemed to be very interesting, but its characterization will be the subject of another report. The third clone, termed eKir, was selected for further characterization.

**FIG. 1.** Differential expression of eKir mRNA in gills of seawater- and freshwater-adapted eels. Northern blot analysis was performed using seawater- and freshwater-adapted eel mRNA. Poly(A)⁺RNA from freshwater and freshwater eel gills were electrophoresed on a 1.2% agarose gel, transferred to a nylon membrane, and hybridized with an eKir 3²P-labeled cDNA probe. A position of 28 S and 18 S ribosomal RNAs are as noted in figure. Hybridization to an eel β-actin probe (corresponding to nucleotides 206–343 in rat sequence) demonstrated equal loading of the lanes. FW, freshwater eels; SW, seawater eels.

**FIG. 2.** Nucleotide and deduced amino acid sequences of eKir cDNA. The nucleotide sequence of the longest clone and its amino acid sequence are shown. Numbers to the left refer to the first amino acids on the lines, and the numbers to the right refer to the last nucleotides on the lines. Two putative membrane-spanning regions (M1 and M2) and pore-forming region (H5) are underlined. Potential phosphorylation sites for protein kinase C are indicated by #, and putative polyadenylation signals are boxed. The DDBJ/EMBL/GenBank™ accession number is AB009669.
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The expression pattern of eKir observed in the differential mRNA display was confirmed by Northern blot analysis (Fig. 1). A single eKir mRNA species of 2 kilobase pairs was detected, which was strongly induced in the gill of seawater eels.

Cloning and Sequence Analysis of Full-length eKir—On Northern blot analysis, the SW300-derived eKir probe hybridized to a 2-kilobase pair transcript. To isolate eKir cDNA clones, eel homologs of the Kir family members and their mammalian counterparts were used as probes. The eel homologs of Kir1.3, Kir2.1, Kir3.4, and Kir4.1 and rat ROMK1 (Kir1.1a) (7). Gaps are inserted to achieve maximum similarity. Amino acid identities are indicated by background shading.

Fig. 3. Sequence identity between the eKir amino acid sequence and four members of the inward rectifier K⁺ channel family. The aligned sequences are eKir, rat channel subunit BIR9 (Kir1.1a) (17), mouse IRK1 (Kir2.1) (8), mouse GIRK1 (Kir3.1) (33), and rat ROMK1 (Kir1.1a) (7). Gaps are inserted to achieve maximum similarity. Amino acid identities are indicated by background shading.

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Cloning and Sequence Analysis of Full-length eKir—On Northern blot analysis, the SW300-derived eKir probe hybridized to a 2-kilobase pair transcript. To isolate eKir cDNA clones of this size, we constructed a seawater eel gill cDNA library in the ZAP II vector and obtained 3 positive clones by screening a new member of the Kir5.0 subfamily. The 42-kDa band may therefore represent eKir whose calculated molecular mass is 41 kDa. The identity of the band was further confirmed using eKir expressed in mammalian cells.

Immunochemical Characterization of eKir—An antiserum was raised against eKir produced in E. coli as a fusion protein (MBP-eKir). On Western blot analysis of seawater eel gill membranes, the antiserum stained a band of 42 kDa (Fig. 5, lane 1). No bands were detected in freshwater eel gill (lane 2). The 42-kDa band may therefore represent eKir whose calculated molecular mass is 41 kDa. The identity of the band was further confirmed using eKir expressed in mammalian cells.

Characterization of eKir by Functional Expression in COS-7 Cells—The electrophysiological properties of eKir were determined by expressing the cloned channel protein in COS-7 cells using pcDL-SR0296 as a vector and cationic liposome as a transfection reagent. pEGFP-N2 (CLONTECH) was co-trans-
fected to select cells for recordings. The expression levels of eKir were assessed by Western blot analysis (Fig. 5). Fig. 6A shows the membrane currents from an eKir-transfected cell (left) and a cell transfected with the mock vector (right) under voltage clamp in the extracellular solution containing 140 mM K\(^+\). The eKir-transfected cells produced large inward currents \((n = 10)\) that were not seen in mock-transfected cells \((n = 10)\). Almost identical currents were seen in eKir-transfected GH\(_3\) cells (data not shown). The current-voltage (I-V) relationship of an eKir current is plotted in Fig. 6B (closed circles). This current had sensitivity to Ba\(^{2+}\) (1 mM, closed squares) and Cs\(^+\) (1 mM, closed triangles), as described previously for inward rectifier K\(^+\) channel (Kir) family members (7, 8). Fig. 6C shows the extracellular K\(^+\) dependence of the reversal potentials. These reversal potentials were close to the equilibrium potentials of K\(^+\) predicted by the Nernst’s equation which suggests a high selectivity for K\(^+\). These data indicate that the eKir constitutes a K\(^+\) channel that belongs to the Kir family.

Tissue Distribution and Time Course of Induction—To determine the tissue distribution of eKir mRNA and to compare its expression levels in freshwater and seawater eels, we performed RNase protection analysis using total RNA preparations from various tissues of freshwater and seawater eels including the gill, brain, heart, liver, stomach, posterior intestine, anterior intestine, kidney, and head kidney. A strong signal was detected in the gill, posterior intestine, and kidney but not in the other tissues examined (Fig. 7). Comparison between the freshwater and seawater eel samples indicated that there was a 3–5-fold increase in the eKir mRNA expression during adaptation to seawater.

Fig. 8 shows the time course of the induction of eKir mRNA expression in the gill following transfer of eels from freshwater to seawater. The changes occurred over a time course of hours to days.

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**FIG. 5.** Western blot analysis of the eKir protein. Eel gill membrane proteins were electrophoresed and blotted to polyvinylidene difluoride membrane and stained with antiserum against recombinant eKir. A 42-kDa band was observed in seawater eel (lane 2) but not in freshwater (lane 1). This antiserum recognized a 42-kDa protein in COS-7 cells transfected with the eKir expression construct (lane 3) but not in vector-transfected cells (lane 4). Positions of molecular mass markers are shown on the left.

**FIG. 6.** Characterization of eKir. A, membrane currents from an eKir-transfected cell (left) and mock-transfected cell (right) under voltage clamp in the extracellular solution containing 140 mM K\(^+\). The membrane currents were evoked by pulse steps to +20, 0, -20, -40, -60, -80, -100, -120 mV from the holding potential of 0 mV. B, I-V relationships of the membrane current from the eKir-transfected cell in 140 mM K\(^+\) (closed circles) and in the presence of 1 mM Ba\(^{2+}\) (closed squares) and 1 mM Cs\(^+\) (closed triangles). C, the extracellular K\(^+\) dependence of the reversal potentials. Potassium was replaced by equimolar sodium, such that \([K^+]_o + [Na^+]_o = 140\) mM.

**FIG. 7.** eKir mRNA levels in various eel tissues under different salinity conditions. Eels were adapted to seawater or freshwater for 2 weeks, and total RNA were isolated from the eels, and then RNase protection analysis was performed as described under “Experimental Procedures”. FW, freshwater-adapted eels; SW, seawater-adapted eels.
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Fig. 8. Time course of eKir expression in eel gills under the conditions of hypertonic stress. Freshwater eels were adapted to seawater and their RNA were isolated at the time indicated in figure. RNase protection assay was carried out as described under "Experimental Procedures." Two eels were processed separately at each time point; each lane, therefore, represents mRNA from one eel.

Fig. 9. Immunohistochemical localization of eKir in seawater adapted eel gill. Sections of gill were stained with anti-eKir antiserum (A) or preimmune serum (B) at a 1:1000 dilution. Chloride cells were stained with antisemur but not with preimmune serum. CC, chloride cells; SL, secondary lamella; Ca, cartilage. Scale bars represent 50 $\mu$m.

Cellular and Subcellular Localization of eKir in the Gill—On immunohistochemistry, the anti-eKir antiserum specifically stained chloride cells that are located near the basal regions of the secondary lamella (Fig. 9). Chloride cells have a complex microtubular system formed by extensive invaginations of the basolateral plasma membranes that are heavily laden with channels and transporters such as Na⁺,K⁺-ATPase (30). To examine further the subcellular localization of eKir in the chloride cells, we performed immunoelectron microscopy on seawater eel gill sections using colloidal gold. Immunogold labeling was found in the microtubular system (Fig. 10), indicating that eKir is mainly present in the microtubular system of the chloride cells.

Discussion

Identification of the genes that are specifically expressed in seawater eels but not in freshwater eels, or vice versa, is important for understanding the molecular basis of the osmoregulation of euryhaline fishes. In the present study, we applied the RAP-PCR technique to compare mRNA species from freshwater and seawater eel gills, and we identified several differentially expressed cDNA bands, one of which was fully characterized and shown to be an inward rectifier K⁺ channel (eKir) highly expressed in the osmoregulatory chloride cells in the gill. The amino acid sequence of the channel protein is quite different from those of the currently known Kir family members; the sequence identity is less than 51% when compared with any of the members. Phylogenetic analysis suggested early divergence of the eKir channel from the other members (Fig. 4). The sequence divergence appears not to be due to the species difference since other members of the eel, salmon, and chick Kir families (eel Kir1.3, eel Kir2.1, eel Kir3.4, eel and salmon WIRK (16), and chick cIRK1 (10)) share more than 70% sequence identity with the corresponding mammalian homologs (Fig. 4). Based on these facts, therefore, we proposed that eKir is a new member of the subfamily Kir5.0. This conclusion is supported by its unique tissue distribution and inducibility; the eKir channel is confined to the gill, kidney, and posterior intestine, and its message levels are increased dramatically in response to osmotic challenge. Such distributions and inductions are not displayed by its predecessors.

The three locations of eKir (the gill, kidney, and intestine) are the major organs involved in osmoregulation. These locations, particularly the microtubular localization of eKir in the chloride cell (Fig. 10), may provide an important clue regarding its physiological roles. Since chloride cells are rich in Na⁺,K⁺-ATPase reflecting their extraordinary power of ion transport, eKir cloned here may be an osmoregulatory component working cooperatively with Na⁺,K⁺-ATPase, for example, by recycling K⁺. The localization in the posterior intestine but not in the anterior intestine is also particularly interesting since, to date, no significant difference in osmoregulatory roles has been found between the anterior and posterior parts of the intestine (31) that are divided by the presence of a constriction due to a sphincter muscle. Our finding is therefore expected to provide new avenues for further research on the intestinal fluid and electrolyte transport.

Osmoregulatory mechanisms of euryhaline fishes are known to be under endocrine control; for example, prolactin is the predominant osmoregulatory hormone in freshwater adaptation, and cortisol serves this function in seawater adaptation. In euryhaline fishes, these hormones seem to play a preparatory role and trigger the operation of the osmoregulatory system to the appropriate direction since their surge in serum levels precedes freshwater or seawater entry (32). In contrast, the inward rectifier potassium channel eKir appears to be downstream of these hormonal factors and plays an essential role for the subsequent survival in seawater as suggested by its time course of induction (Fig. 8). Our identification of an inducible channel protein, eKir, on which euryhaline fishes are supposed to depend in the seawater milieu may serve as a molecular clue for clarifying the molecular basis for seawater adaptation. Future studies should reveal how hypertonicity is recognized and how the recognition is converted to the activation of the eKir gene as well as the physiological function of the channel in association with other channels and transporters.

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