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Identification and Characterization of a Novel Bacterial ATP-Sensitive K^+ Channel

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Five bacterial species that are most likely to have putative prokaryotic inward rectifier K^+ (Kir) channels were selected by *in silico* sequence homology and membrane topology analyses with respect to the number of transmembrane domains (TMs) and the presence of K^+ selectivity filter and/or ATP binding sites in reference to rabbit heart inward rectifier K^+ channel (Kir6.2). A dot blot assay with genomic DNAs when probed with whole rabbit Kir6.2 cDNA further supported the *in silico* analysis by exhibiting a stronger hybridization in species with putative Kir's compared to one without a Kir. Among them, *Chromobacterium violaceum* gave rise to a putative Kir channel gene, which was PCR-cloned into the bacterial expression vector pET30b(+), and its expression was induced in *Escherichia coli* and confirmed by gel purification and immunoblotting. On the other hand, this putative bacterial Kir channel was functionally expressed in *Xenopus* oocytes and its channel activity was measured electrophysiologically by using two electrode voltage clamping (TEVC). Results revealed a K^+ current with characteristics similar to those of the ATP-sensitive K^+ (K-ATP) channel. Collectively, cloning and functional characterization of bacterial ion channels could be greatly facilitated by combining the *in silico* analysis and heterologous expression in *Xenopus* oocytes.

***Keywords*:** bacterial ATP-sensitive K^+ channel, *C. violaceum*, *in silico* homology analysis, *Xenopus* oocyte expression, TEVC

K^+ channels that are sensitive to the cellular level of adenosine 5′-triphosphate (ATP) or K-ATP channels are found in a wide variety of tissues, where their primary role is to couple cell metabolism to electrical activity and transmembrane K^+ flux. As such, they are involved in many cellular functions, including the response to cerebral and cardiac ischemia, and the regulation of vascular smooth muscle tone, epithelial K^+ transport, and electrical activity of neurons (Ashcroft, 1988; Ashcroft and Ashcroft, 1990; Edwards and Weston, 1993; Quayle *et al*., 1997). Their physiological role is best understood in the pancreatic β-cell, where they link changes in blood glucose concentration to insulin secretion. Under normal conditions, the K-ATP channel is open and sets the β-cell resting membrane potential. Elevation of blood glucose concentration results in increased glucose uptake and metabolism by the β-cell. This then closes the K-ATP channel, producing a depolarization that activates voltage-gated Ca^{2+} channels and thereby induces a rise in intracellular Ca^{2+} which stimulates the insulin release (Ashcroft and Rorsman, 1989).

The mammalian K-ATP channel is a heterooctameric protein complex composed of two subunit types, namely, a pore forming subunit or Kir6.2, a member of the inwardly rectifying family of K^+ channel, and the sulfonylurea receptor subunit (SUR), a member of the ATP binding cassette family of proteins (Clement *et al*., 1997; Aguilar-Bryan and Bryan, 1999; Fujita and Kurachi, 2000). The fact that channel properties are intrinsic to Kir6.2, which is conferred by association with SUR, has been complicated by the inability to obtain functional expression of Kir6.2 independently of the sulphonylurea receptor. This issue has now been resolved using deletion approaches. It was found that isoforms of Kir6.2, in which either the last 26 (Kir6.2△C26) or 36 (Kir6.2△C36) amino acids had been deleted, produced functional channels in the absence of SUR. This study further demonstrates that the primary site for ATP inhibition does not reside on SUR but on Kir6.2 (Tucker *et al*., 1997, 1998; Giblin *et al*., 2002; Trapp *et al*., 2003).

As more studies on molecular biology and electrophysiology of the K-ATP channel accumulate, knowledge of its structure and molecular diversity is increasingly being understood (Bichet *et al*., 2003). But it has long been assumed that the x-ray structure of a mammalian ion channel is impossible to obtain mainly due to its close association with the lipid membrane. Recently, however, many laboratories began to succeed to reveal the crystal structure of bacterial K^+ channel as a substitute for mammalian counterparts, which open a whole new concept of how ion channels work at atomic level (Doyle *et al*., 1998; Kuo *et al*., 2003).

This study was undertaken to obtain crystals of bacterial K-ATP channel proteins, which was prerequisite to the x-ray crystallographic studies to reveal the molecular structure of the K-ATP channel at atomic level. As a preliminary step, we have cloned a putative bacterial K-ATP channel based on prediction with the *in silico* analysis and biologically validated

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its ATP-sensitive K^+ conducting properties in *Xenopus* oocyte expression.

Materials and Methods

In silico analysis

Available bacterial genomic DNA sequences were downloaded from the National Center for Biotechnology Information (NCBI) GenBank at http://ncbi.nlm.nih.gov/genbanl/genomes, and sequence homology searches were performed with the web-based program Psi-Blast available at http://www.ncbi.nlm.nih.gov/MicrobJ3blast/unfinishedgenome.html. Prediction of the topology of transmembrane domains (TMs) was carried out by using two web-based programs: TMHMM from http://www.ncbi.nlm.nih.gov/MicrobJ3blast/unfinishedgenome.html; TMPRED from http://www.ch.embnet.org/software/TMPRED_form.html. To generate a 3-D structure of a channel protein, Geno3D from http://geno3d-pbil.ibcp.fr was employed. The 3-D structure of putative bacterial inward rectifier K^+ channels was constructed in reference to the model constructed based on KirBac1.1 (PBD id: 1P7B), for which x-ray structures have been available. CLUSTALW2.0 was used for the sequence alignment of K^+ selectivity filters and ATP-binding sites.

Bacterial strains and molecular cloning

Five bacterial species, *Chromobacterium violaceum*, *Ralstonia solanacearum* GMI1000, *Burkholderia fungorum* LB400, *Trichodesmium erythraeum* IMS101, and *Nostoc punctiforme* were purchased from the American Type Culture Collection (ATCC, USA) and maintained according to the vender's instruction. A putative bacterial Kir channel gene was cloned from the *C. violaceum* genomic DNA by polymerase chain reaction (PCR) with following primers: forward primer (5′-GGA ATT CCA TAT GGC TCC TCG-3′) (*Nde*I site, underlined); backward primer (5′-GGG GTA CCC TCC TCT TTT GGC-3′) (*Kpn*I site, underlined). The PCR-cloned genes were double-digested with *Nde*I and *Kpn*I, subcloned into a bacterial expression vector pET30b(+) (Novagen, USA) and propagated in *E. coli* strain DH5α (Invitrogen, USA) until use.

Dot blot hybridization assay

^{32}P-labeled cDNA probes were generated by using the random primer DNA labeling kit (TaKaRa, Japan) with rabbit heart Kir6.2 cDNA, which was kindly provided by Dr. Han at Inje University, South Korea. Unincorporated dNTPs were removed by Sephadex G-50 spin-column. Each bacterial strain was inoculated on a LB agar plate and incubated at 37°C overnight. A nitrocellulose filter (PROTRAN BA 85 nitrocellulose membrane, Schleicher & Schuell, Germany) was laid on the surface of the agar plate until it became thoroughly wetted. The filter was carefully peeled from the agar surface with its colony on, placed onto a puddle of the lysis buffer containing 2% SDS for 3 min, denatured in 0.5 M NaOH and 1.5 M NaCl for 3 min, and then transferred to the neutralization buffer consisting of 0.5 M Tris-Cl, 1.5 M NaCl, pH 7.4 for 3 min. DNA was cross-linked to the filter under a UV light (254 nm, Spectrolinker XL-1000 UV Cross linker, Krackeler Scietific, USA), and the filter was kept dry until hybridization. The filter was pre-hybridized in 1× SSC solution supplemented with 5× Denhardt's reagent, 0.5% SDS, and 100 μg/ml salmon sperm DNA by gently rotating for 4 h at 65°C. The ^{32}p-labeled probe was denatured by heating at 100°C for 3 min before added to the hybridization buffer. The filter was hybridized in the buffers/probe mixture by gently rotating for at least 16 h at 65°C. After hybridization, the filters were washed by gently rotation in 1× SSC that was pre-warmed to 65°C, followed by the addition of 0.05% SDS at 65°C. The filters were further washed three more times for 30 min each before being dried. For image enhancement and analysis, Bio-Rad GS-525 Molecular Imager System (Bio-Rad, USA) was used.

Expression and purification of putative bacterial Kir channels in *E. coli*

E. coli strain BL21 (DE3) (Novagen, Germany) was transformed with pET30b(+) plasmids that bear a putative bacterial Kir channel gene from *C. violaceum* and grown in 2× YT medium supplemented with 50 μg/ml kanamycin. Expression of the channel proteins was induced overnight by the addition of isopropyl-1-thio-β-galactopyranoside (IPTG) at the final concentration of 0.5 mM. Bacterial cell lysates were obtained by a standard procedure, and induced putative bacterial Kir channel proteins were purified by using QI*Express* Ni-NTA Fast Start kit (QIAGEN, USA) and subjected to SDS-PAGE and immunoblotting with primary polyclonal antibodies recognizing hexa-histidine tag inherent in the plasmid and secondary polyclonal antibodies (anti-rabbit IgG) conjugated with horseradish peroxidase (Santa Cruz Biotech, USA). After color development, data were analyzed by using LAS1000 image analyzer (Fuji Photo Film, Japan).

Electrophysiology

Female *Xenopus laevis* were purchased from Xenopus One (Ann Harbor, MI) and maintained according to the vender's instruction. Frogs were anesthetized by immersion in cold 0.2% MS-222 (Sigma, USA) for 30 min, and stage V or VI oocytes were surgically removed. For oocyte expression, the DNA in pET30b(+) was linearized by digestion with *Bgl*II and synthesis of capped mRNAs or cRNAs was carried out by using the Ribomax Large Scale *in vitro* Transcription kit with T7 RNA polymerase (Promega, USA). Unincorporated cap and DNA template were removed by an appropriate clean-up procedure according to the manufacturer's manual. An oocyte was micro-injected with 2-5 ng of cRNA and maintained at 18°C in an incubation medium of ND96^{++} (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 1.8 mM $CaCl_2$, 2.5 mM Na-pyruvate, 50 μg/ml gentamicin, pH 7.4). The final injection volume was ~50 nl per oocyte. Oocytes were maintained in a tissue culture dish at 18°C with gentle shaking. Electrophysiological recordings were performed 2-3 days post-injection using a standard 2-electrode voltage clamp configuration as described before (Smith *et al*., 1980) with Geneclamp 500 amplifier (Axon Instruments, USA) interfaced to an IBM PC via Digidata 1200 (Axon Instruments). The bath solution was, in mM, 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.4 with KOH). Currents were measured 290-300 ms after currents were elicited by the voltage pulse from the holding potential of -10 mV using pCLAMP software (Axon Instruments).

Results

In silico identification of putative bacterial inward rectifier K^+ channel genes

In silico search for bacterial genes that are homologous to the rabbit cardiac Kir6.2 was performed with two web-based programs Psi-Blast and CLUSTALW2.0. The sequence homology search was based on two common structural features of mammalian Kir, i.e., TXGXG K^+ selectivity filter motif and two transmembrane domains interrupted by a, so called, pore-loop, collectively referred to as TM-P-TM topology, as depicted in Fig. 1. After vigorous homology searches against

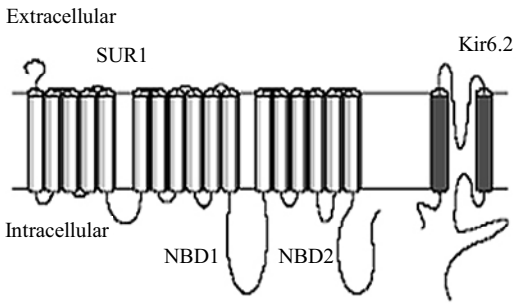

Fig. 1. A schematic topological transmembrane illustration of the mammalian K-ATP channel. Note the number of transmembrane domains (TMs) of SUR1 and Kir6.2 subunit, and the pore-loop (p-loop) domain seen in Kir6.2, both of which are the signature landmarks of the inwardly rectifying K^+ channel (Kir) families [adopted from Clement *et al*. (1997)].

bacterial genomes that had been completely or partially sequenced and thus available at http://ncbi.nlm.nih.gov/genbanl/genomes, we identified five prokaryotes *C. violaceum*, *R. solanacearum* GMI1000, *B. fungorum* LB400, *T. erythraeum* IMS101, and *Nostoc punctifome*, whose genomes contain a gene(s) coding for proteins whose closest relatives in current sequence databases were eukaryotic Kir's. The overall homology of putative bacterial Kir's to rabbit Kir6.2 is about 20-30% in amino acid sequences. Locations and sequences of the selectivity filter motif and TMs are well preserved along with ATP-binding sequences (Fig. 2A).

Topology of putative bacterial inward rectifier K^+ channels

To further validate our prediction of putative bacterial Kir's, the topology of two TM regions was examined by two transmembrane protein topology prediction methods, namely,

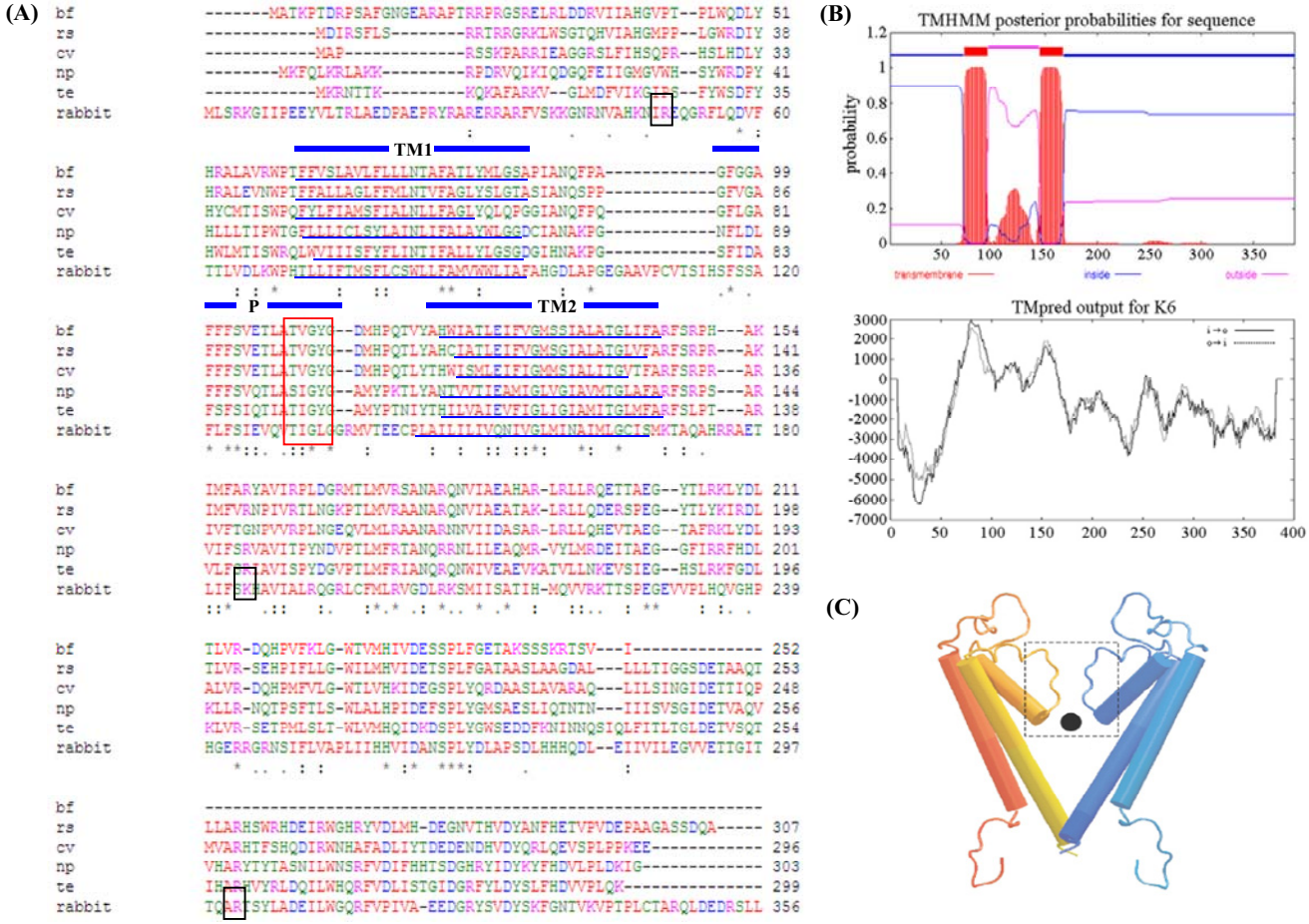

Fig. 2. Sequence alignments and transmembrane topologies of putative bacterial Kir channels. (A) *In silico* analyses of microbial genomes available in the NCBI databases by web-based programs, PsiBlast and CLUSTALW2.0, in search for DNA sequences that are homologous to rabbit heart inward rectifier K^+ channels (Kir6.2) have yielded five DNA sequences from *C. violaceum*, *R. solanacearum* GMI1000, *B. fungorum* LB400, *T. erythraeum* IMS101, and *N. punctifome*, which, with a high probability, may contain DNA sequences that encode protein(s) whose closet relatives in current sequence databases were eukaryotic Kir's. The TMs and pore loop (P) are indicated above the sequences, and the K^+ selectivity filter and the ATP-binding sites are indicated in a red box and in black boxes, respectively. (B) The topology of TMs of putative bacterial Kir channels from *C. violaceum* are predicted by two web-based programs TMHMM (upper) and TMPRED (lower panel). (C) Geno3D was used to construct a 3-D structure of a putative bacterial Kir channel of *C. violaceum*. The model was built based on the x-ray structure of KirBac1.1 (PBDID:1P7B) described by Doyle *et al*. (1998). For simplicity, only two Kir subunits are viewed, showing a conformation of transmembrane helix (outer and inner helix), pore loops, and selectivity filters. The black dot represents a K^+ ion.

TMHMM and TMPRED. All the TM prediction methods gave rise to two clearly distinct TMs and one pore loop in between (Fig. 2B). On the other hand, a 3-D structure of a putative bacterial Kir channel was generated by using the web-based program Geno3D based on the atomic structure of KirBac1.1 that was revealed by the x-ray crystallographic studies (Doyle *et al*., 1998) (Fig. 2C). The predicted 3-D structure of the bacterial Kir channel clearly indicated the location of two TMs and characteristic molecular structure of the K^+ selectivity filter and its nearby amino acid residues.

Dot blot hybridization

To biologically ascertain the *in silico* prediction of bacterial genome having a gene(s) encoding a member of Kir channel families, each bacterial chromosomal DNA was subjected to a dot blotting probed with ^{32}p-labelled rabbit whole Kir6.2 cDNA. Note that to compare the hybridization intensity, roughly equal numbers of bacteria were subjected to the assay. As expected, four bacterial species except *Trichodesmium erythraeum*, which we had difficulty to grow, exhibited a positive hybridization signal but to a varying extent, with the strongest signals manifested from *C. violaceum* (Fig. 3A), further supporting a biological relevance of our *in silico* prediction.

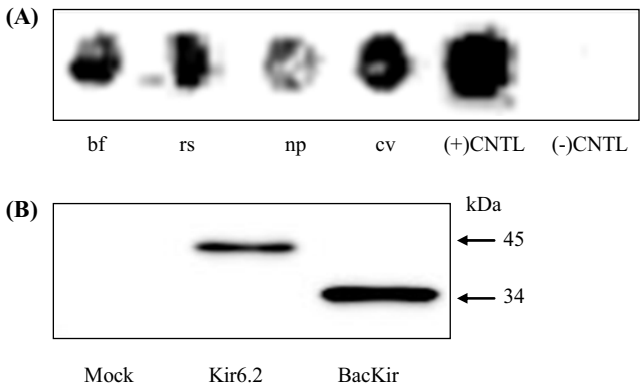

Fig. 3. Cloning and expression of putative bacterial Kir channels. (A) A dot blot analysis for the presence of putative bacterial Kir channel genes in *B. fungorum* LB400 (bf), *R. solanacearum* GMI1000 (rs), *Nostoc punctiforme* (np), and *C. violaceum* (cv). Roughly equal amount of bacterial genomic DNAs (based on the number of cells) were extracted and probed with ^{32}p-labelled rabbit whole Kir6.2 cDNA. All four bacterial genomes were labeled positively with the probe. The positive control or (+)CNTL is from *E. coli* transformed with rabbit Kir6.2 cDNA in pET30b(+) vector. The negative control or (-)CNTL is from *E. coli* without transformation. (B) Western blot analysis of putative Kir channel protein expression in *E. coli*. *E. coli* strain BL21 (DE3) was transformed with pET30b(+) vector harboring a putative bacterial Kir gene from *C. violaceum*, and its expression was induced by adding IPTG. The resulting bacterial Kir proteins were purified by virtue of histidine tagging present in pET30b(+) vector, separated on SDS-PAGE, and probed with antibodies raised against the hexa-histidine tag as described in 'Materials and Methods'. The molecular weights on the right indicate the size of eukaryotic Kir6.2 and BacKir, respectively. Mock, mock *E. coli* transformants; Kir6.2, eukaryotic Kir6.2 transformants; BacKir, transformants harboring putative Kir channels from *C. violaceum.*

Molecular cloning and expression of putative bacterial Kir channels in *E. coli*

A putative bacterial Kir channel gene was successfully isolated from the genome of *C. violaceum* by PCR and subcloned into a bacterial expression plasmid pET30b(+) vector. Following transformation of *E. coli* BL21 (DE3) strain with pET30b(+) vectors harboring a putative bacterial Kir gene, also referred to as pET30b(+)/BacKir vector, expression of the channel proteins was induced by IPTG. The resulting bacterial Kir channel proteins were purified by virtue of histidine tagging and analyzed by Western blotting with anti-hexa-histidine antibodies. Figure 3B showed the expression of both rabbit Kir6.2 protein and putative bacterial Kir channel protein in *E. coli* while no Kir type channel protein was detected with mock transformants. The molecular sizes of putative bacterial Kir proteins and Kir6.2 proteins estimated from the gel electrophoresis precisely matched those estimated from their amino acid sequences (34.0 vs 34.5 kDa for bacterial Kir; 45.0 vs 44.4 kDa for Kir6.2). The yield of the channel protein purification was estimated to be ≈110 μg/g bacteria.

Electrophysiological measurement of putative bacterial K-ATP channels

Most eukaryotic mRNAs contain a m7G(5′)ppp(5′)G cap at the 5′-end and a polyA tail at the 3′-end, which are known to be important for the binding of translation initiation factors and contribute to mRNA stability, respectively. The use of capped RNA is favored for programming certain translation systems (e.g., *Xenopus* oocytes) because uncapped RNA are rapidly degraded after microinjection into the cells (Quick and Lester, 1994; Kim *et al*., 1999). To achieve this, DNA templates were enzymatically linearized prior to *in vitro* transcription, and cRNA was produced *in vitro* in the presence of Ribo m7G cap analog (Promega). The *in vitro* transcription produced cRNAs for a putative bacterial Kir channel (data not shown), and these cRNAs were microinjected into *Xenopus* oocytes to reconstitute functional BacKir channels onto the surface membrane of oocytes. Figure 4 showed inwardly rectifying K^+ currents which are elicited in response to a series of voltage steps from -120 to +20 mV, and this putative bacterial Kir current was increased following azide treatment (3 mM for 15 min), implying that the putative bacterial Kir current is ATP-sensitive. Thus it can be envisioned that a putative bacterial K-ATP channel gene is cloned and functionally expressed in *Xenopus* oocytes.

Discussion

As a step toward obtaining K-ATP channel protein crystals, a prokaryotic K-ATP channel gene has been successfully cloned and expressed, and their ion channel activity has been measured. The *in silico* analysis has been instrumental in homology-searching DNA sequences and predicting 3-D structure of channel proteins. Specifically, it greatly facilitates to predict the number of transmembrane helices and the presence of certain sequence such as the K^+ selectivity filter. Based on TM-P-TM topology and presence of K^+ selectivity filter sequence, we have identified 5 putative bacterial Kir

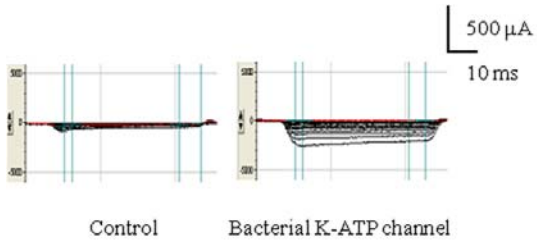

Fig. 4. Electrophysiological measurements of putative bacterial K-ATP currents. The putative bacterial K-ATP channel DNAs from *C. violaceum* was subcloned into pET30b(+) vector. cRNAs were synthesized by *in vitro* transcription as described in 'Materials and Methods', and microinjected into *Xenopus* oocytes. Isolated oocytes were recorded 2 days after the cRNA injection. Whole cell currents were recorded from an oocyte injected with cRNAs encoding a putative bacterial K-ATP channel in response to a series of voltage steps from -120 to +30 mV before or 15 min after exposure to 3 mM azide. Bath solution consisted of (mM): 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.4 with KOH; pipette solution consisted of 3 M KCl. Solutions were gravity-fed. Current traces represent inward rectifier currents that were produced by subtracting the currents obtained after exposure to 3 mM azide for 15 min from the currents before the azide treatment in either uninjected oocytes (control, left) or oocytes expressing putative bacterial K-ATP channels (right).

channel genes. Among them, *C. violaceum* has yielded putative bacterial Kir gene whose expression is confirmed in *E. coli* as evidenced by its purification and immunoreactivity. Furthermore, a heterologous expression in *Xenopus* oocyte reveals functional K-ATP channel activity. Next studies will be obtaining crystals of *C. violaceum* K-ATP channels for the x-ray crystalographic studies. The structural information obtained from the bacterial K-ATP channel is implemented to gain insight into the channel structure of cardiac K-ATP channel, which is the target channel of pathological significance, including the ischemic preconditioning.

Understanding of how ion channel works is a key to understanding of how the nervous system works, how muscles contract, and how cells secret among many others. The crystal structure of ion channels will open a new paradigm of the ion channel research. So far, the structure of ion channels has been deduced mainly based on the amino acid sequence and/or biophysical measurement of the altered channel (Loussouarn *et al*., 2000; Cartier *et al*., 2003). The x-ray structure of K^+ channels of prokaryotic origin, however, has been broadening the ways to gain insight into channel protein structure-function relation such as ion channel gating and voltage sensing (Morais-Cabral *et al*., 2001; Jiang *et al*., 2003). The primary reason why prokaryotic ion channels have been favored over eukaryotic counterparts for the x-ray studies would be: (*i*) the easiness to obtain crystals, possibly due to compatibility with the bacterial protein overexpression system, which has been unequivocally employed; (*ii*) structural and functional similarity between prokaryotic and eukaryotic ion channels (Heginbotham *et al*., 1998; Durell and Guy, 2001). A full elucidation of the x-ray structure of K-ATP channels would enable us to design or screen new drugs, whose primary target is the mammalian K-ATP channel (Dabrowski *et al*., 2004). In addition, structural information of ion channels will help to construct an, so called, ion channel proteome network in near future.

In conclusion, we have combined the *in silico* gene mining technique with the electrophysiological technique in *Xenopus* oocyte expression, which greatly facilitate a 'functional cloning' of ion channel genes of bacteria origin. In this study, a putative bacterial K-ATP channel has been cloned and functionally identified. This powerful technique will allow us to identify more bacterial ion channel proteins, which remains largely unknown until recently.

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