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Inwardly Rectifying K^+ (Kir) Channels in *Drosophila*

A CRUCIAL ROLE OF CELLULAR MILIEU FACTORS FOR Kir CHANNEL FUNCTION*

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Three cDNAs encoding inwardly rectifying potassium (Kir) channels were isolated from *Drosophila melanogaster*. The protein sequences of *Drosophila* KirI (dKirI) and dKirII are moderately (<44%) and dKirIII sequence is weakly (<27%) identical to human Kir channel subunits. During fly development, five dKir channel transcripts derived from three genes are differentially expressed. Whole mount *in situ* hybridizations revealed dKirI transcripts absent from embryos, but dKirII and dKirIII are expressed in the embryonic hind gut and in Malpighian tubules, respectively, thus covering the entire osmoregulatory system of the developing fly. In the head of adult flies, predominantly dKirII transcripts were detected. When expressed in *Xenopus* oocytes, dKir channel activity was only observed after amino acid substitutions in their cytosolic tails (*e.g.* exchange of a unique valine in the NH_2 terminus). In contrast, heterologous expression of wild type dKirI and dKirII in *Drosophila* S2 cells readily evoked typical inwardly rectifying K^+ currents, which were weakly sensitive to Ba^{2+}. Thus, the specific milieu of insect cells provides a crucial cellular environment for proper function of dKir channels.

Neurobiological studies in *Drosophila* greatly contributed in identifying novel ion channels and elucidating their biological function. *Drosophila* is a particularly attractive model system for such investigations, because chemical and molecular genetic approaches allow the generation of mutants that can be screened for new gene-function relationships. *Drosophila* mutants defective in K^+ channel genes (*e.g. shaker* (1–4) or *ether à go-go* (5)) enabled the isolation of homologues from other species, including humans. Recently, sequencing of the *Drosophila* genome was completed (6), and subsequent data analysis revealed ion channels that were previously unrecognized (7). Thus, the fruit fly's entire set of ion channels is now available in the *Drosophila* genome data base.

In mammals, inwardly rectifying K^+ (Kir)[1] channels are involved in important cellular functions such as control of the resting membrane potential, maintenance of K^+ homeostasis, and transduction of cellular metabolism into excitability. Kir channels are formed by the assembly of four subunits with a common structure: a pore-forming domain (H5) surrounded by two transmembrane segments (M1 and M2) and NH_2- and COOH-terminal tails located in the cytosol. 15 mammalian Kir channel subunits have been characterized and identified in various cell types (*e.g.* cardiomyocytes, endothelial cells, epithelial cells, myoblasts, pancreatic β-cells, glial cells, and neurons) (8–14). Kir subunits are grouped into six subfamilies according to sequence similarity as well as structural and functional features (reviewed in Ref. 15). Although for many cells the physiological role remains to be elucidated, there are several functional properties of Kir channels that are very well understood. (i) For instance, homomeric assembly of Kir2 subfamily members forms constitutively active K^+ channels. Their steep rectification results from intracellular blockage of the channel pore by positively charged polyamines and Mg^{2+} ions in a voltage- and $[K^+]_o$-dependent manner (*e.g.* Refs. 16 and 17). (ii) Heteromeric assembly of Kir3 subunits gives rise to G-protein-gated Kir (GIRK) channels. Their function depends on the activation of G-protein-coupled receptors, which release $G_{\beta\gamma}$ subunits, leading to GIRK channel opening. In the heart, release of acetycholine after vagal stimulation activates I_{KACh} (Kir3.1 + Kir3.4), which culminates in slowing of the heart rate (18). (iii) In pancreatic β-cells, secretion of insulin is regulated by the activity of K_{ATP} channels that are formed by coassembly of four Kir6 subunits with four sulfonylurea receptor polypeptides. An increase in the cellular ADP/ATP ratio opens K_{ATP} channels, which reduces insulin secretion (19).

Drosophila Kir channel homologues have not been described so far. In the present study, three genes (dKirI–III) were identified from the *Drosophila* genome data base, and their gene products were characterized. The expression profiles of all dKir channel transcripts are highly dynamic during fly development. Functional expression of inwardly rectifying dKirI and dKirII channels was only achieved in *Drosophila* S2 cells and not in *Xenopus* oocytes, pointing to an important role of the insect cellular environment in protein integrity.

EXPERIMENTAL PROCEDURES

Molecular Cloning—Protein sequences of mammalian Kir channels were used to search for homologues in the Celera genome data base of *Drosophila melanogaster* (6) by using the BLAST software (20). Three proteins (dKirI-III) with structural Kir channel features were identified. Corresponding cDNAs were amplified from total RNA of adult fruit flies by reverse transcriptase-PCR using *Taq* polymerase (Qiagen, Hilden, Germany). Specific primers covering the entire ORF were deduced from predicted transcripts of the *Drosophila* genome data base: dKirI (5′-CGGAATTCGCCACCATGTACATCTTGTTGTTCCGTTTC-3′; 5′-CCGCTCGAGTCAACAAATGGAATCGATG-3′), dKirII (5′-CGGAATTCGCCACCATGAGACGTGTTCGCCGAC-3′; 5′-CCGCTCGAGTTACAAGGGCACCGAGAGCTG-3′), dKirIII (5′-CGGAATTCGCCAC-

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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)AJ344344 (for dKirI cDNA), AJ344345 and AJ344346 (for cDNAs of dKirII splice variants), and AJ344347 (for dKirIII cDNA).

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[1] The abbreviations used are: Kir, inwardly rectifying K^+; dKir, *Drosophila* Kir; rKir, rat Kir; EGFP, enhanced green fluorescent protein; ORF, open reading frame.

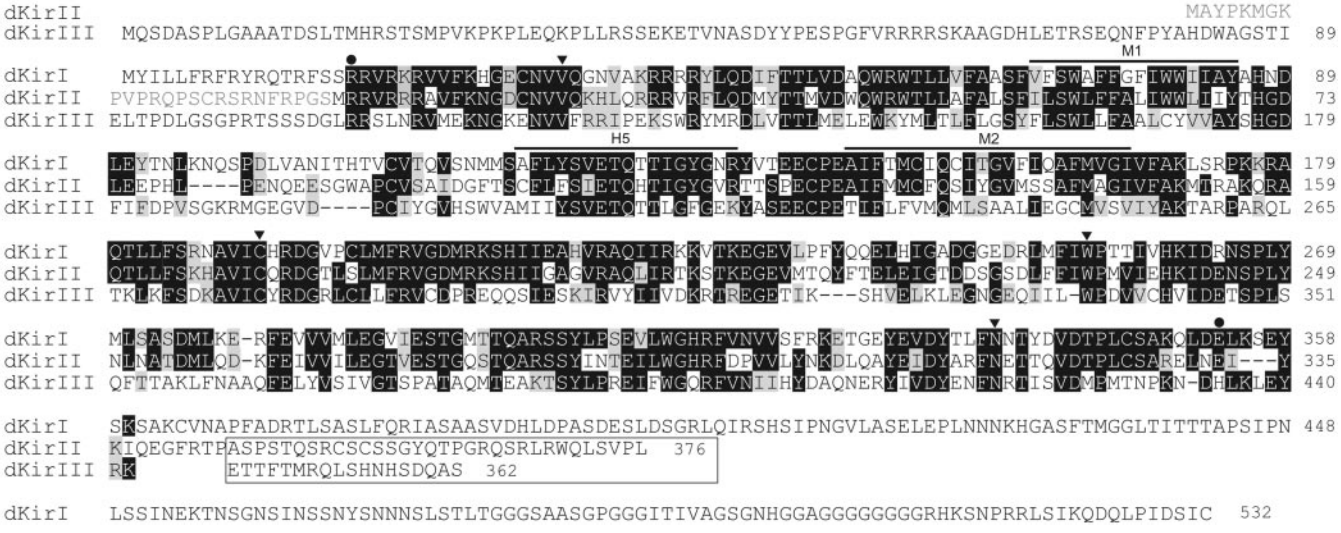


FIG. 1. **Sequence analysis of *Drosophila* Kir channels.** Protein sequences of dKirI–III (GenBank™ accession numbers AJ344344–AJ344347) are compared in the alignment. Transmembrane segments (M1 and M2) and pore-forming domain (H5) are indicated by *horizontal bars*. Identical amino acids are *boxed* in *black*, whereas conserved substitutions are *boxed* in *gray*. The *arrowheads* indicate amino acids that are unique for *Drosophila* Kir channels. The core region of Kir channels used for calculations in Fig. 2 is marked with *filled circles*. Two carboxyl-terminal splice variants of dKirII are highlighted. In dKirII, alternative translational start sites may result in an extended NH_2 terminus (*gray residues*).

CATGCAATCGGACGCATCTC-3′; 5′-CCGCTCGAGTCACTTTCTG-TACTCTAGC-3′). An expressed sequence tag clone of dKirII (GH11459) coding the entire ORF was identified and supplied by the Berkeley *Drosophila* Genome Project (21). PCR products were cloned into pCR-TOPO (Invitrogen, Groningen, The Netherlands), pSGEM (supplied by M. Hollmann (Bochum)), and pAc5.1 (Invitrogen) for functional expression in *Xenopus* oocytes and *Drosophila* S2 cells. Chimeric constructs of dKirI and rat Kir2.1 (rKir2.1) (Table I) were engineered with the "geneSOEing" method (22). All PCR fragments for chimeras and deletion constructs were amplified with *Pfu* polymerase (Stratagene, La Jolla, CA). *Drosophila* Kir channels were fused to enhanced green fluorescent protein (EGFP) by cloning into EGFP-N1 (CLONTECH, Palo Alto, CA), and subsequently the entire GFP constructs were cloned into pSGEM and pAc5.1. For introduction of point mutations, we used the QuikChange mutagenesis kit from Stratagene according to the manufacturer's instructions. All PCR products, chimeras, and mutants were sequenced on both strands on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). DNA analysis and sequence alignments were performed with Lasergene software (DNAstar, Madison, WI).

Northern Blots, in Situ Hybridization, and Immunohistochemistry—D. melanogaster strain Oregon R was reared on standard cornmeal-molasses-agar medium at 25 °C, and individuals of various developmental stages were collected and stored at −70 °C . Adult flies frozen in liquid nitrogen were vortexed and passed through a sieve to separate head and body fractions of the animal. Total RNA was isolated from 400–700 mg of frozen *Drosophila* stages either by Qiagen RNeasy (embryos, larvae, pupae) or WAK-Chemie TRI Reagent (adult flies). For Northern blots, 1 μg of poly(A)$^+$ RNA purified by Oligotex (Qiagen) was separated on a denaturating agarose gel and transferred to BrightStar Plus nylon membranes (Ambion, Austin, TX). Northern blots were probed with [^{32}P]UTP-labeled antisense RNA specific for dKirI (ORF positions Met^1–Gly^{166}), dKirII (Met^1–Leu^{376}), and dKirIII (Met^1–Lys^{442}). As control, blots were probed with antisense RNA of the ribosomal protein RpL9 (23). Labeling, hybridization, and probe removal were performed with "NorthernMax" and "Strip-EZ" reagents (Ambion) according to the manufacturer's instructions.

In situ hybridizations of whole mount *Drosophila* embryos (Oregon R) were performed as described by Lehmann and Tautz (24). Fixed embryos were probed with digoxygenin-labeled antisense RNAs that were specific for dKirI, dKirII, and dKirIII. Whole mount embryo immunostaining following *in situ* hybridization was performed as described (25). Primary mouse monoclonal antibodies 2B10 (final dilution 1:20) and Cq4 (final dilution 1:10) directed against the *Drosophila* Cut and Crumbs proteins, respectively, were obtained from the Developmental Studies Hybridoma Bank. The Vectastain Elite ABC-peroxidase system (Vector Laboratories, Burlingame, CA) with a biotinylated anti-mouse secondary antibody and diaminobenzidine as substrate was used for signal detection according to the manufacturer's instructions.

Cell Culture and Transfection—The Schneider 2 (S2) cell line derived from late stage (20–24 h old) *D. melanogaster* embryos (26) was purchased from Invitrogen. S2 cells were grown in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptavidin at 25 °C without CO_2. Modified calcium phosphate precipitation was used for transfection of S2 cells. Briefly, cells were plated (35-mm dish) in 3 ml of complete medium 1 h before transfection. 19 μg of plasmid DNA dissolved in 300 μl of $CaCl_2$ (240 mM) were slowly mixed with 300 μl 2× HBS buffer (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.05), the mixture was incubated for 30 min at room temperature and subsequently added to the cells. After 16 h, the cells were washed with medium and were analyzed 40 h after transfection.

Electrophysiology—For expression of *Drosophila* Kir channels in *Xenopus laevis* oocytes, capped run-off poly(A$^+$) cRNA transcripts from linearized cDNA were synthesized, and ~6 ng were injected into defolliculated oocytes. Oocytes were prepared as described (27) and were incubated at 20 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4) supplemented with 100 μg/ml gentamicin and 2.5 mM sodium pyruvate. 48 h after injection, two electrode voltage clamp measurements were performed with a Turbo Tec-10 C amplifier (npi, Tamm, Germany). Oocytes were placed in a small volume perfusion chamber with a constant flow of ND96 or high K$^+$ solution (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4).

Whole cell recordings of S2 cells were performed at room temperature 40 h after transfection in a bath solution consisting of 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES, pH 6.8. Patch pipettes were pulled from borosilicate glass capillaries (Kimble Products, Sussex, UK) and heat-polished to give input resistances of 3–5 megaohms. The pipette recording solution contained 140 mM KCl, 0.01 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_2ATP, 0.1 mM cAMP, 0.1 mM GTP, and 5 mM HEPES, pH 7.3.

Currents were recorded from S2 cells with an EPC9 patch clamp amplifier (Heka Electronics, Lambrecht, Germany). Stimulation and data acquisition were controlled by Pulse/Pulsefit software (Heka) on a Macintosh computer, and data analysis was performed with Igor software (WaveMetrics, Lake Oswego, OR). Data were presented as means ± S.D. (number of cells).

RESULTS

Cloning of Drosophila Kir Channels—Three putative Kir channel genes (dKirI–III) were identified in the *Drosophila* genome data base by homology screening with conserved mammalian Kir sequences. Deduced protein sequences revealed structural motifs typical of inwardly rectifying K$^+$ channels (*i.e.* a conserved pore-forming region (H5) flanked by two trans-

A

	dKirI		dKirII		dKirIII	
dKirI			54.0	60.2	25.7	33.9
dKirII	54.0	60.2			27.9	32.7
dKirIII	25.7	33.9	27.9	32.7		
hKir2.1	40.0	50.5	42.8	47.7	23.7	29.4
hKir2.2	40.0	51.2	42.8	48.5	25.9	33.0
hKir2.3	40.4	49.6	42.3	47.3	24.0	32.1
hKir2.4	38.5	49.7	38.6	44.4	22.9	29.5
hKir3.1	33.5	47.2	39.6	44.8	21.0	26.9
hKir3.2	39.2	49.2	40.2	45.2	22.7	27.2
hKir3.3	43.8	50.5	43.4	50.5	24.7	29.3
hKir3.4	40.8	50.8	40.2	50.8	22.9	27.9
hKir6.1	37.7	45.6	39.9	45.6	23.3	29.4
hKir6.2	39.5	46.1	40.4	46.1	26.4	31.3

B

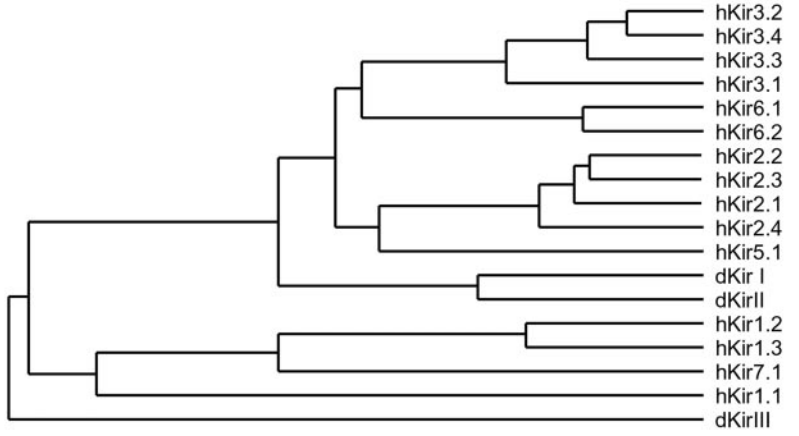


FIG. 2. **Comparison of *Drosophila* Kir channels with their human homologues.** *A*, sequence identities (in percentages) among *Drosophila* Kir channels and members of human Kir subfamilies 2, 3, and 6. For each *Drosophila* Kir channel, values of the entire sequence (*right columns*) and the core sequence (*left columns*) were calculated by the Clustal algorithm of the Lasergene software. *B*, phylogenic tree of all known *Drosophila* and human Kir channels deduced from the alignment of their core regions (indicated by *filled circles* in Fig. 1). dKirI and dKirII are related to human Kir subfamilies 2, 3, and 6, whereas dKirIII is located on a separate branch.

membrane segments M1 and M2) (Fig. 1). Primers covering the entire ORF were chosen to amplify native cDNAs from total RNA of adult fruit flies by reverse transcriptase-PCR. For each gene, a single transcript coding for a complete ORF was amplified: dKirI, 1599 bp; dKirII, 1131 bp; and dKirIII, 1329 bp in length. By screening the *Drosophila* expressed sequence tag data base, we identified a likely dKirII splice variant with an additional exon (21). This expressed sequence tag clone (GH11459; 1828 bp) gives rise to an ORF of 1089 bp. Two in frame ATG codons were found upstream of the putative start codon (Kozak sequence: $^{-6}$GGCAGC^{-1}) that may function as alternative translational start sites (*gray letters* in Fig. 1). Moreover, an exon (171 bp between Val^{144} and Gly^{145}) in the NH_2 terminus of dKirIII predicted by the Celera genome analysis was not confirmed in our dKirIII transcript. The entire protein sequences of dKirI (532 amino acids) and dKirII (376 amino acids) are 54% identical to each other, whereas dKirIII (362 amino acids) is <28% identical to dKir subunits I and II (Fig. 2*A*, *left columns*). A sequence alignment of the core regions (region between *filled circles* in Fig. 1) with all 15 known human Kir channel subunits indicated that dKirI and dKirII were phylogenetically most closely related to the human Kir2 channel subfamily (Fig. 2*B*, sequence similarity between 45 and 51%). In contrast, the dKirIII subunit is only between 27 and 33% identical to the human Kir channel polypeptides (Fig. 2*A*, *right columns*).

In addition to the conspicuous length of the cytoplasmic NH_2 terminus in dKirIII and the COOH terminus in dKirI, we identified four positions in dKir channels (Val^{34}, Cys^{181}, Trp^{254}, and Asn^{336} in dKirI) that are unique in *Drosophila* (Fig. 1).

Localization of Drosophila Kir Channels—The expression profile of all dKir channels was monitored at the transcript level during different metamorphic stages (Fig. 3, *A–C*, *lanes 1–5*). For the dKirI gene, a major transcript of ~3.4 kb was identified at all stages with increasing amounts during development. In addition, a second dKirI transcript of ~3.1 kb was found in larvae, pupae, and adult flies (Fig. 3*A*, *lanes 3–5*). High levels of dKirII transcripts (~1.8 kb) were observed in embryos and larvae (Fig. 3*B*, *lanes 1–3*) but are less abundant in adult flies. Instead, a second population of transcripts, 2.0 kb in length, is strongly expressed in adults (Fig. 3*B*, *lane 5*). For dKirIII, a single transcript is present at all stages with highest expression levels in the adult (Fig. 3*C*).

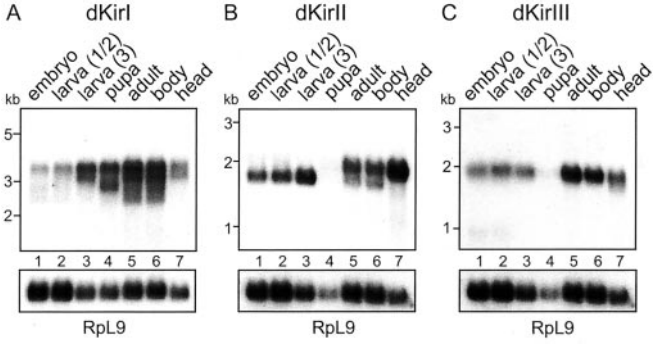


FIG. 3. **Northern blot analysis of Kir channel expression in *Drosophila*.** Blots containing 1 μg of poly(A)$^+$ RNA/lane were probed with ^{32}P-labeled antisense cRNA of dKirI (*A*), dKirII (*B*), and dKirIII (*C*). Samples in *lanes 1* and *4* represent all embryonic and pupal stages, respectively, whereas larval developmental stages are further subdivided in first plus second (*lane 2*) and third instar larvae (*lane 3*). In adult flies, Kir channel expression was verified either in entire animals (*lane 5*) or in fractions of body (*lane 6*) and head (*lane 7*). Probes detecting ribosomal protein RpL9 were used to normalize hybridization signals (*bottom panels*). The position of RNA size markers in kb is indicated on the *left* in *A–C*.

It should be noted that the apparent lack of dKirII and KirIII signals in pupae (Fig. 3, *B* and *C*, *lane 4*) may be partly due to lower amounts of sample RNA as revealed by control hybridizations with a probe against RpL9 (*lower panels*). Nevertheless, overexposure of x-ray films clearly detected dKirII and dKirIII transcripts also in *Drosophila* pupae (data not shown).

To test for a potential neuronal expression of *Drosophila* Kir channels, head and body fractions of adult flies were analyzed separately (Fig. 3, *lanes 6* and *7*). Our analysis revealed all Kir channels present in both compartments with dKirI more strongly expressed in the body and dKirII more prominently expressed in the head.

The tissue distribution of the dKirI–III channel transcripts was verified by whole mount *in situ* hybridizations of *Drosophila* embryos with digoxygenin-labeled antisense cRNA probes. At the end of embryogenesis, dKirII transcripts were detected in the hind gut (Fig. 4*A*), and the dKirIII subunit was found in anterior Malpighian tubules (stage 17; Fig. 4*B*). The latter are connected to the digestive tract before the hind gut and thereby form the continuous osmoregulatory system of the fly. To allow

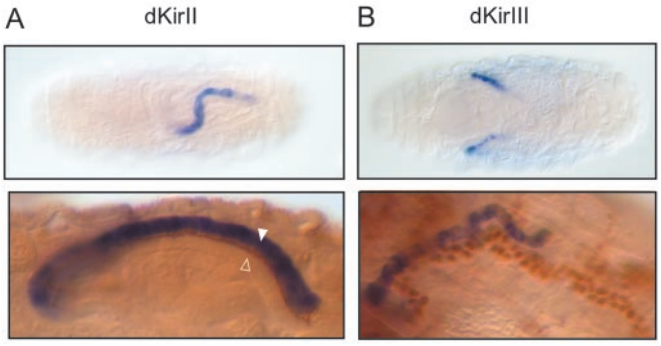


FIG. 4. **Localization of dKirII (*A*) and dKirIII (*B*) transcripts in *Drosophila* embryos.** The expression of *Drosophila* Kir channels in fly embryos was analyzed by whole mount *in situ* hybridization with digoxigenin-labeled antisense cRNA (*upper panels*). Transcripts of dKirII were detected in the hind gut (*A*), whereas dKirIII mRNA was found in the proximal part of the anterior Malpighian tubules (*B*). No embryonic transcripts of dKirI were found (data not shown). *Lower panels* (lateral views on stage 16 embryos; anterior is *left*), high power magnifications of embryos *in situ* hybridized with dKir-specific cRNA probes (*dark blue*) and counterstained with tissue-specific antibodies (*dark brown*). *A*, *lower panel*, dKirII is expressed in the dorsal (*filled arrowhead*) but not the ventral (*open arrowhead*) epithelium of the hind gut (lumen highlighted by α-Crumbs). *B*, *lower panel*, MT cells are marked by α-Cut. Partial colocalization of cytoplasmic dKirIII mRNA and nuclear Cut protein shows dKirIII expression restricted to the distal part of the anterior MT. Note that the proximal part of the anterior MT runs from posterior to anterior and bends back with the distal part heading in the posterior direction.

a high resolution analysis of dKir channel expression, *in situ* hybridized embryos were coimmunostained with tissue-specific antibodies. Counterstaining with antibody α-Crumbs (apical marker for hind gut epithelium) demonstrated that dKirII is present only in one-half of the hind gut (Fig. 4*A*, *lower panel*), whereas colabeling with α−Cut antibody (nuclear marker for Malpighian tubule cells) showed that dKirIII transcripts are present only in the distal part of the anterior Malpighian tubules (Fig. 4*B*, *lower panel*). At midembryogenesis (stage 11), dKirIII is expressed in the amnioserosa and in hemocytes (data not shown). Virtually no expression of dKirI was detected at all embryonic stages of the fly.

Heterologous Expression of dKir Channels in X. laevis Oocytes—When cRNAs of dKirI–III were injected into *Xenopus* oocytes, none of the three wild type subunits initially revealed measurable membrane currents in the two-electrode voltage clamp condition (*e.g.* Fig. 5, *A* and *C*). This functional deficiency was observed despite the fact that the channel protein appeared to be properly targeted to the outer plasma membrane as revealed by fluorescence measurements of EGFP-tagged dKirI channels (Fig. 5*A*, *inset*). To get information on the putative structural determinants for the lack of membrane currents, we began to analyze various genetically altered channel constructs as outlined in Table I (see also measurements in S2 cells below). Since the pore region (M1-H5-M2) of mammalian and *Drosophila* inward rectifiers is highly conserved, we focused our analysis on both the NH_2- and COOH-terminal cytoplasmic domains. Thus, in a first series of experiments, both the NH_2 and COOH termini of dKirI were exchanged with the corresponding regions of rat Kir2.1 (rKir2.1) which is close in primary sequence and expresses well in heterologous systems. Briefly, these experiments demonstrated that prominent K^+ inward currents could be detected when both cytoplasmic domains were replaced (dKirI[N+C], 20.2 ± 10.9 μA; see Table I), but moderate currents were also elicited when either terminus was replaced (dKirI[N] and dKirI[C], 2.8 ± 0.74 and 29.3 ± 11.8 μA, respectively).

To outline our further experimental strategy, we describe the identification of functional domains in the NH_2 terminus of dKirI. First, when in dKirI[N] the outermost NH_2 terminus (amino acids 2–40) was deleted (dKirI[ΔN]), the amplitude and properties of the resulting membrane currents were indistinguishable from those of dKirI[N]. This suggested the presence of residues critical for channel function in the remaining NH_2-terminal region of rKir2.1. Next, three spots in dKirI with the highest sequence divergence were changed to the sequence in rKir2.1 (dKirI[FVK]$^{24-26}$, dKirI[HCNVQF]$^{30-35}$, and dKirI[EKGQ]$^{40-43}$). Whereas dKirI[FVK]$^{24-26}$ did not result in K^+ currents above background level (0.12±.04 μA), dKirI[HCNVQF]$^{30-35}$ and dKirI[EKGQ]$^{40-43}$ evoked typical K^+ currents with amplitudes of 1.56 ± 0.21 μA and 0.72 ± 0.08 μA, respectively (Fig. 5, Table I).

When expressed in *Xenopus* oocytes, dKirI[HCNVQF]$^{30-35}$ gave rise to rapidly activating inwardly rectifying K^+ currents (Fig. 5*A*). Elevating the outer potassium concentration from 2 to 96 mM K^+ resulted in a shift of the reversal potential E_K from −92 to −7 mV (Fig. 5*B*), which is in good agreement with the shift of E_K according to the Nernst equation. A 10-fold increase in $[K^+]_o$ led to a 52.3-mV shift in E_K, indicating that the channel is mainly permeant to K^+ ions. As typical for inwardly rectifying K^+ channels, dKirI[HCNVQF]$^{30-35}$ is reversibly blocked by external Ba^{2+} ions with a half-maximal concentration (K_i) of 50 μM.

The significance of the mutated region for the function of *Drosophila* Kir channels was confirmed by analyzing the homologous mutant of dKirII (dKirII[HCNVQQ]$^{14-19}$), which is also expressed in *Xenopus* oocytes (Fig. 5, *C* and *D*). Two-electrode voltage clamp recordings evoked inwardly rectifying K^+ currents (10-fold increase in $[K^+]_o$ shifted the reversal potential by 48 mV). dKirII[HCVNQQ]$^{14-19}$ exhibited a different time course of activation compared with dKirI[HCNVQF]$^{30-35}$. In addition to a fast component (<5 ms), dKirII[HCVNQQ]$^{14-19}$ displayed a slow component (37 ± 5 ms at V_h = −140 mV) in the activation time constant in response to hyperpolarizing potentials (Fig. 5, *A* and *C*). Inward currents were completely blocked by 1 mM extracellular Ba^{2+} (K_i = 200 μM, data not shown).

We noted that in the critical region described above, all three dKir channels harbor a valine residue, absent from all mammalian Kir sequences (Fig. 1), which may represent a key residue for channel function in the fly. Whereas dKirIIV14Q and dKirIII V124Q did not express in *Xenopus* oocytes, point mutant dKirIV34Q in fact evoked typical Kir currents with average amplitudes of 0.56 ± 0.09 μA (Table I).

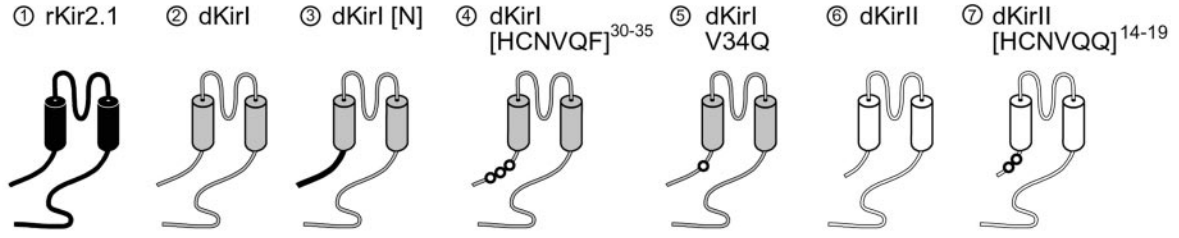
Drosophila S2 Cells Supply the Environment for Functional Expression of dKir Channels—Ion channel function is often dependent on milieu factors and the physiological conditions of their natural cellular environment. The S2 Schneider cell line derived from late stage *Drosophila* embryos was cultured at 25 °C in insect-specific medium at pH 6.8 and was chosen as an alternative expression system to test for the importance of cellular factors in the expression of dKir channels. When transfected with wild type dKirI and dKirII, whole-cell recordings from S2 cells readily revealed the presence of functional channels (Fig. 6). Transfected S2 cells were recognized by cotransfected EGFP (Fig. 6, *upper panels*) and showed prominent inward currents that averaged 0.52 ± 0.17 nA (n = 6) and 0.81 ± 0.63 nA (n = 6) in 50 mM $[K^+]_e$ for dKirI (Fig. 6*A*) and dKirII (Fig. 6*B*), respectively (V_h = −100 mV). The current–voltage relationship revealed strong rectification properties, similar to those displayed by mammalian Kir2 subfamily members. In response to hyperpolarizing voltage steps between +60 and −140 mV, the activation kinetics of dKirI and dKirII was similar to the corresponding mutants (dKirI[HCNVQF]$^{30-35}$

TABLE I

Qualitative expression analysis of Kir channel constructs in Xenopus oocytes

Terminology of mutant and chimeric dKir channels and description of the corresponding amino acid substitutions are shown. Sequences of rKir2.1 origin are indicated in parentheses. Single amino acid mutations are depicted by arrowheads. Note that circled numbers refer to structural diagrams at the bottom. Functional expression of the constructs in cRNA-injected oocytes was rated according to current amplitudes: +++, high; ++, moderate; +, low; −, no expression. r, rat; d, *Drosophila*; Δ, deletion. Amino acids are depicted in one-letter code.

Name	Mutation	Expression
rKir2.1 ①	wildtype	+++
dKirI ②	wildtype	−
dKirI[N+C]	dKirI [M^{1}-V^{86}]+[A^{178}-I^{427}]	+++
dKirI[C]	dKirI [A^{178}-I^{427}]	++
dKirI[N] ③	dKirI [M^{1}-V^{86}]	+
dKirI[ΔN]	dKirI [ΔY^{2}-R^{40} in N]	+
dKirI[FVK]$^{24\text{-}26}$	dKirI V^{24}►F, F^{26}►K	−
dKirI[HCNVQF]$^{30\text{-}35}$ ④	dKirI E^{30}►H, V^{34}►Q, Q^{35}►F	+
dKirI[EKGQ]$^{40\text{-}43}$	dKirI KRRR$^{40\text{-}43}$►EKGQ	+
dKirI V34Q ⑤	dKirI V^{34}►Q	+
dKirII ⑥	wildtype	−
dKirII[HCNVQQ]$^{14\text{-}19}$ ⑦	dKirII D^{14}►H, V^{18}►Q	+
dKirII V18Q	dKirII V^{18}►Q	−
dKirIII	wildtype	−
dKirIII V124Q	dKirIII V^{124}►Q	−
ΔdKirIII	ΔM^{1}- L^{106} dKirIII	−



and dKirII[HCVNQQ]$^{14-19}$) expressed in *Xenopus* oocytes: rapid activation in dKirI ($\tau_{ON} < 5$ ms) and an additional, slower activation time constant of 113 ± 12 ms ($n = 6$) in dKirII. The reversal potentials for different $[K^+]_o$ concentrations followed the Nernst potential with good agreement as predicted for K^+-selective channels (Fig. 6). dKirI and dKirII currents in S2 cells appeared to be less sensitive for Ba^{2+} and were blocked by 1 mM Ba^{2+} only by 53 and 14%, respectively.

In the cell-attached configuration, single channel recordings of S2 cells transfected with dKirI revealed channel openings (Fig. 7*A*, *left panel*) that were absent from nontransfected cells ($n = 15$). Transfection of S2 cells with dKirII also induced single channel activities with longer open and closed times and a significantly larger conductance compared with dKirI (Fig. 7*A*, *right panel*). The *I*-V profile displayed strong inward rectification for both channel populations with single channel conductances of 21 ± 3 pS (dKirI, $n = 6$) and 58 ± 5 pS (dKirII, $n = 6$), respectively (Fig. 7*B*). The open probabilities also differed between both channel types and were 0.36 ± 0.08 for dKirI and 0.54 ± 0.04 for dKirII.

DISCUSSION

The present report describes three Kir channel genes identified from the *Drosophila* genome and the functional characterization of their products in different heterologous expression systems. Sequence analysis demonstrated that typical structural features of mammalian inward rectifiers are also present in *Drosophila* Kir channels: (i) two conserved transmembrane segments, (ii) a pore-forming region with a G[YF]G motif as potassium selectivity filter, and (iii) conserved amino acids in the NH_2- and COOH-terminal domains. Two dKir channels are closely related (54% identity of dKirI and dKirII) and display profound similarity to sequences of human Kir subfamilies 2, 3, and 6. The third *Drosophila* Kir channel is located on a separate branch of the phylogenetic tree (Fig. 2*B*). Interestingly, the entire *Drosophila* genome revealed only three Kir channel genes (see also Ref. 7), giving rise to a handful of transcripts that may suffice to serve various physiological functions connected to K^+ homeostasis or regulation of the membrane potential. Three Kir channel genes are also present in *Caenorhabditis elegans* (28), but most mammals do carry at least 15 Kir channel genes. These numbers may be greatly different when inspecting other K^+ channel families, and thus the diversity of K^+ channel genes within a given family does not serve as an indicator for evolutionary progress within the animal kingdom. For instance, ~50 tandem pore domain potassium channel genes were found in *C. elegans*, whereas only 11 were predicted in *Drosophila* (7), and 14 genes were described in humans (29).

In *Drosophila* embryos, dKirII and dKirIII were detected in the hind gut and in Malpighian tubules, respectively, whereas expression of dKirI was not detected (weak signal in the North-

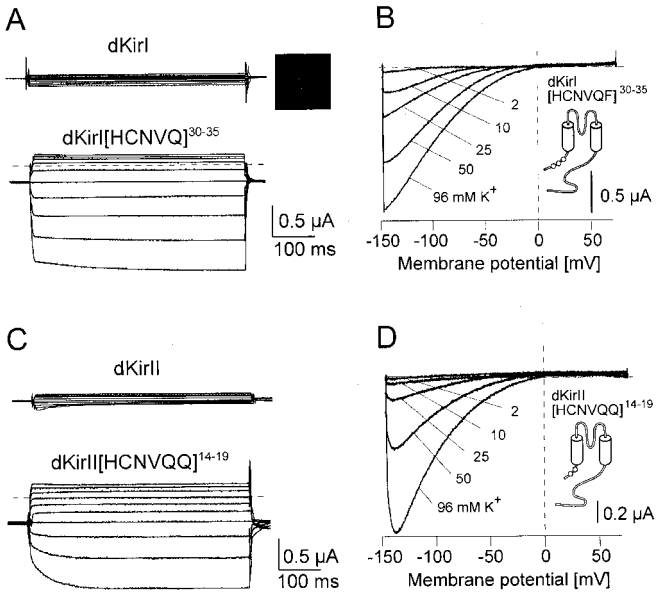


FIG. 5. **Heterologous expression of dKirI/dKirI[HCNVQF]$^{30-35}$ (*A* and *B*) and dKirII/dKirII[HCVQQ]$^{14-19}$ (*C* and *D*) channels in *Xenopus* oocytes.** *A* and *C*, two-electrode voltage clamp recordings depict current responses of cRNA injected oocytes to 500-ms voltage steps between +60 and −140 mV from a holding potential of −60 mV. External potassium concentration ($[K^+]_e$) = 96 mM. Recordings of dKirI and dKirII (*upper panels*) illustrate absence of currents in contrast to substantial currents evoked by the mutants (dKirI[HCNVQF]$^{30-35}$ and dKirII[HCVQQ]$^{14-19}$). The confocal image in *A* shows the fluorescence signal of EGFP-labeled dKirI in the oocyte membrane 48 h after cRNA injection. *B* and *D*, current recordings from oocytes expressing mutated dKir channels to voltage ramps from −150 to +60 mV elicit inward currents whose reversal potentials shift upon changes of the outer potassium concentration. The *insets* illustrate the position of mutated residues in cytosolic NH_2-terminal domains.

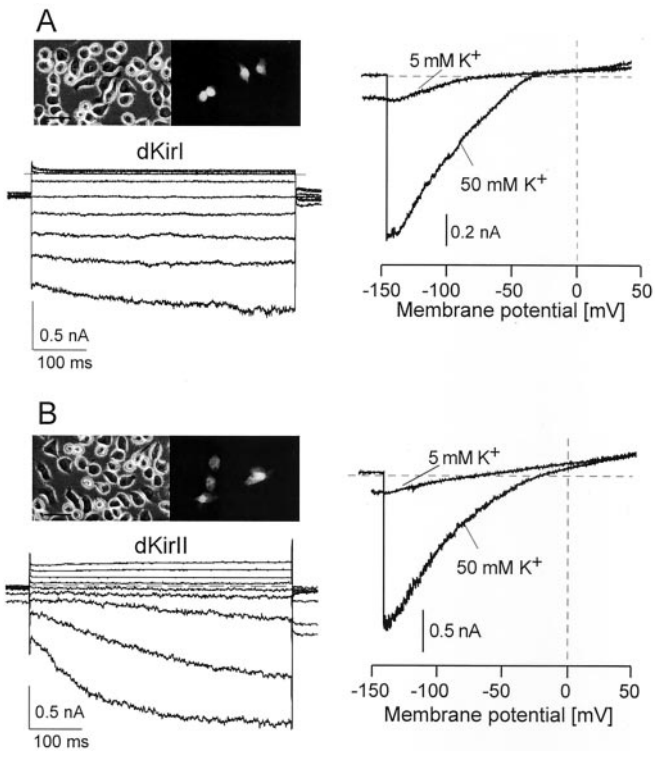


FIG. 6. **Expression of wild type dKir channels in Drosophila S2 cells.** Whole-cell current recordings from S2 cells transfected with cDNA of dKirI (*A*) and dKirII (*B*). *A* and *B*, as illustrated by phase contrast and fluorescent images (*upper panels*, *left* and *right*, respectively), transfected S2 cells were identified by coexpression of EGFP. Shown in the *lower left* are current recordings in response to 500-ms voltage steps between +80 mV and −140 mV from a holding potential of −60 mV ($[K^+]_e$ = 50 mM). Current-voltage relations recorded at 5 and 50 mM $[K^+]_e$ using voltage ramps between −150 mV and +60 mV are depicted on the *right*. *Scale bar*, 25 µm.

ern blot is probably of maternal origin). Thus, at the onset of fly development, Kir channels were found only in excretory tissues which are essential for potassium homeostasis. In mammals, Kir channels are also strongly expressed in kidney and intestine (30, 31), and their important functions in osmoregulation are well documented. Patients who are affected in the Bartter syndrome (32) suffer from defects in K^+ homeostasis that in many cases are caused by mutations in Kir1 channels (33).

The osmoregulatory system of insects is composed of Malpighian tubules and the hind gut. They are connected at the border of the midgut and hind gut, where the tubules enter the digestive tract. The hydrostatic pressure of hemolymph and the inside of the tubules is almost identical; thus, urine is not generated by filtration but is produced by secretion. The main driving force for the production of the primary urine is generated by active transport of potassium into the tubules, whereas most other substances follow passively as a consequence of the electrochemical gradient. The important function of K^+ in this process is evident from the high K^+ concentration of primary urine in all insects (34) as well as from enhanced production of primary urine with rising K^+ concentration in the lumen of Malpighian tubules. The reabsorbtion of ions (mainly KCl) and water is performed in the hind gut, where potassium is recycled into the hemolymph via potassium channels of unknown identity in the apical and basolateral membrane of epithelial cells. The interesting expression of dKirII in only one-half of the hind gut (Fig. 4*B*) is also known for other proteins (35), but a functional significance remains to be elucidated.

During rat development, the tissue distribution of Kir channel subunits has been shown to change in peripheral organs and also in the brain (36). Similarly, we show in the present report that the population and the amount of dKir channel transcripts change during fly development. Although dKir channels are not expressed in the embryonic nervous system, their presence in the central nervous system of adult flies is evident from Northern blots. From their RNA signals in the head, we conclude strong neuronal expression of dKirII (Fig. 3*B*, *lane 7*) and moderate expression of dKirI and -III (Fig. 3, *A* and *C*, *lane 7*) in the nervous system of *Drosophila*.

Functional expression of dKir channels in *Xenopus* oocytes was observed only after introducing mutations in the cytoplasmic domains. The exchange of a unique valine in the NH_2 terminus of dKirI (dKirIV34Q; Table I) was sufficient to evoke typical inwardly rectifying K^+ currents, indicating its key role for the function of dKir channels. Moreover, we found that current amplitudes of mutated dKir channels increased with extended alterations in both cytoplasmic termini. In summary, dKir channel activity in *Xenopus* oocytes appeared to be impaired by both cytoplasmic parts. Together, these parts may serve as a gate structure, which is disrupted by the exchange with homologous rKir2.1 amino acids. In potassium channels, various gating mechanisms have been evolved in general. (i) Intramolecular N- and C-type inactivation of Kv1 channels is mediated by the cytosolic termini of the α subunit. (ii) Intermolecular inactivation by various β subunits was described for Kv1 and Kv4 channels (37). (iii) Kir3 channels are gated by $G_{\beta\gamma}$ subunits released from various G-protein-coupled receptors (38). (iv) Voltage-dependent gating of Kir2 channels is mediated by intracellular block and release of positively charged polyamines and Mg^{2+} ions (17). (v)

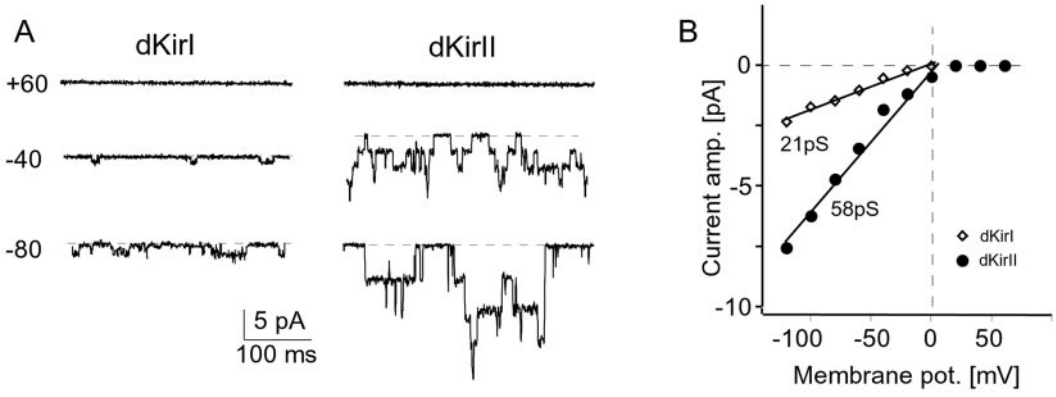


FIG. 7. **Single channel analysis of dKir channels expressed in S2 cells.** *A*, single channel events recorded in the cell-attached configuration from S2 cells transfected with dKirI (*left*) and dKirII (*right*). The currents were recorded with 50 mM K^+ in the pipette and were filtered at 1 kHz; sampling frequency was 2.5 kHz. *B*, unitary currents of dKirI and dKirII plotted against membrane potential reveal strong rectification and single channel amplitudes of 21 and 58 pS, respectively. Linear regression was fitted to data points.

Domains sensing the intracellular pH switch between open and closed state are described for Kir1 and tandem pore domain K^+ channels (29, 39). Thus, proper ion channel function often depends on the presence of cellular factors and other cell type-specific conditions.

As hypothesized, we found that *Drosophila* S2 cells transfected with dKirI and dKirII channels supplied the appropriate environment for functional Kir channel expression. Features of dKir currents in S2 cells resemble those evoked by mutated dKir channels in *Xenopus* oocytes. In both expression systems, dKirI channels are rapidly activated, and activation of dKirII currents is biphasic with a fast and a slow component. In both cell types, dKirII is less susceptible to block by external Ba^{2+} than dKirI with a generally lower sensitivity compared with mammalian Kir channels.

Using S2 cells as a heterologous expression system does not allow for functional expression of dKirIII, however. Since physiological functions of human Kir channels often depend on the interaction with other accessory proteins, this may result from the lack of such a component in S2 cells. Coassembly of Kir6 subunits and sulfonylurea receptors, for example, is obligatory for generating functional mammalian K_{ATP} channels, and a sulfonylurea receptor homologue has indeed been identified in the fruit fly (40). Similarly, mammalian Kir3 subunits aggregate as heterotetramers and accomplish their physiological role by interacting with $G_{\beta\gamma}$ subunits of trimeric G-proteins. Recently, three metabotropic $GABA_B$ receptor homologues were identified in *Drosophila* and were shown to couple to heteromeric Kir3 channels (41). However, in preliminary experiments, coexpression of heptahelical receptors with dKirIII failed to generate measurable K^+ currents, even in conjunction with dKirI and dKirII.[2]

Drosophila melanogaster serves as a model system for the investigation of many cellular and developmental processes common to higher eukaryotes including humans. Knowledge of the dKir gene loci (Celera genome data base: dKirI (CG6747), Chr 3R 18923011–80925051; dKirII (CG4370), Chr 3R 19255584–19254141; dKirIII (CG10369), Chr 2L 18545216–18543017) from the completed *Drosophila* genome sequence in combination with the data base of existing fly mutants (FlyBase; available on the World Wide Web at flybase.bio.indiana.edu) supplies attractive perspectives to uncover new physiological roles of Kir channels *in vivo*. In mutated or knockout animals, the limited number of dKir channels in the fly reduces compensatory effects by other channel isoforms.

[2] E. Wischmeyer, unpublished observations.

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MEMBRANE TRANSPORT STRUCTURE FUNCTION AND BIOGENESIS:

Inwardly Rectifying K^+ (Kir) Channels in *Drosophila* : A CRUCIAL ROLE OF CELLULAR MILIEU FACTORS FOR Kir CHANNEL FUNCTION

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