The weaver mutation changes the ion selectivity of the affected inwardly rectifying potassium channel GIRK2

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Abstract The weaver mutation in mice has recently been identified as a single base-pair mutation in the *Girk2* gene, which encodes a G-protein-activated inwardly rectifying potassium channel, GIRK2. The mutation results in a Gly to Ser substitution at residue 156, in the putative pore-forming region of the potassium channel. In the present study, we used *Xenopus* oocytes to express mutant GIRK2, and to characterize the effects of the mutation on the channel. The mutation results in a loss of the normal high selectivity for K^+ over Na^+, with little effect on other channel properties such as activation by the mu opioid receptor. The resulting increase in basal Na^+ permeability causes a marked depolarization of oocytes expressing the mutant GIRK2 protein. This result was observed even when the mutant GIRK2 was coexpressed with GIRK1, a situation more analogous to that seen in vivo. Thus, the increased Na^+ permeability and resulting depolarization may contribute to the pathology of cerebellar granule cells and substantia nigra dopaminergic neurons observed in the weaver mice.

Key words: Potassium channel; G-protein-coupled K^+ channel; Oocyte; Ion permeability

1. Introduction

The weaver mutation in mice results in motor deficits, and from an early age on homozygous weaver mice display severe ataxia – a weaving gait, hence the name. At the cellular level, the mutant mouse brain exhibits depletion of both granule cells in the cerebellum and dopaminergic neurons in the substantia nigra [1,2]. Although weaver mice have been identified and studied for many years, the molecular defect was identified only recently in the *Girk2* gene [3]. This gene encodes a potassium (K^+) channel, GIRK2. GIRK2 and other members of this gene family (e.g. GIRK1, GIRK3 and GIRK4) form inwardly rectifying K^+ channels whose basal activity can be enhanced in a membrane-delimited fashion by G-protein-coupled receptors such as the serotonin 1A, $GABA_B$, and the mu and delta opioid receptors [4–7]. The weaver mutation consists of a Gly to Ser substitution at residue 156 in the GIRK2 protein. This destroys the Gly-Tyr-Gly motif in the putative pore-forming region (H5 domain), a region that is highly conserved in several potassium channel families. It has been reported that mutations of conserved residues in the H5 domain of another potassium channel (ShB) either eliminate channel conductance or alter ion selectivity [8].

Xenopus oocytes have been used in many functional studies for membrane receptors and ion channels [9,10]. In particular, GIRK channels have been shown to be coupled to opioid receptors in the oocytes, via pertussis toxin-sensitive G-proteins [11–14]. In the present study, we used an oocyte expression system to characterize the effects of the weaver mutation on the K^+ channel.

2. Materials and methods

2.1. Materials

Opioid ligands were from Peninsula Laboratories Inc., Research Biochemicals International, and National Institute on Drug Abuse. *X. laevis* frogs were from Xenopus I and African Fish Farm. In vitro transcription kit mMessage mMachine was from Ambion. L15 medium was from Gibco BRL. All other chemicals were from Sigma.

2.2. Complementary DNA clones

The mutant and wild-type GIRK2 cDNA clones were isolated by RT-PCR from normal and homozygous weaver mice, respectively, and then subcloned into a PCRII expression vector (Invitrogen). GIRK1 and mu opioid receptor cDNA clones were previously described [12,13]. The mRNAs were synthesized by in vitro transcription with a mMessage mMachine kit.

2.3. Oocyte injection and electrophysiology

Xenopus oocytes were prepared as described [15], and 1 ng of synthetic mRNA for each K^+ channel (wild-type and mutant GIRK2, GIRK1, or their combinations) were injected into stage V–VI *Xenopus* oocytes with an automatic Drummond nanoinjector. To examine the enhancement of K^+ currents by G-protein-coupled receptors, the mu opioid receptor was used, and 1 ng of the mu receptor mRNA was coinjected in all cases. Oocytes were incubated in 50% L-15 medium supplemented with 0.8 mM glutamine and 10 μg/ml of gentamycin at 18–21°C, unless indicated otherwise. Electrophysiological recordings were made at room temperature. Microelectrodes filled with 3 M KCl and having a tip resistance of 0.5–3 MΩ were used for recording membrane potential and for two-electrode voltage clamp with an Axoclamp-2A current and voltage clamp (Axon Instruments). Recording was made with the aid of pCLAMP software (Axon Instruments) and on a Gould chart recorder. During the recording, cells were bathed in normal Na^+-containing solution (ND96: 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM HEPES, pH 7.4) except where noted. The high K^+, Na^+-free solution con-

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tained 98 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM HEPES, pH 7.4. The high Na^+, K^+-free solution was the same as the high K^+ solution except that the K^+ was replaced with 98 mM Na^+.

3. Results and discussion

3.1. Effect of K^+ channel expression on resting membrane potential in Xenopus oocytes

To study the K^+ channel properties of the mutant weaver GIRK2 (*wv* channel), wild-type and mutant GIRK2 cDNA clones were used to synthesize mRNAs by in vitro transcription, and these mRNAs were injected into *Xenopus* oocytes for functional expression of the K^+ channels. Because it has been proposed [16–18] that some combinations of GIRK proteins form heteromultimers with 5–40-fold increase in K^+ currents, and that these heteromultimers may exist in vivo, GIRK1, another G-protein-activated inwardly rectifying K^+ channel [11,19] and a GIRK2 homologue, were also used for coexpression. To investigate the regulation of the GIRK channels by G-protein coupled receptors, all the oocytes in this study were also coinjected with the mRNA for the mu opioid receptor [13,20], since activation of this receptor has been shown to increase the GIRK1 channel conductance [12,14].

After oocytes started to express the K^+ channels, we noticed that the resting membrane potential in the cells injected with weaver (*wv*) mRNA became less negative, a sign often seen during cell death, whereas the resting potential in the control cells (not injected with any RNA) or those expressing the wild-type GIRK2 maintained the normal negative potential. As shown in Fig. 1A, over the course of several days of mRNA expression in oocytes, the resting membrane potential in the cells with *wv* channel (open diamonds) depolarized toward 0 mV, and the resting potential in uninjected controls (filled circles) or in cells expressing the wild-type GIRK2 (open circles) remained unchanged. Wild-type GIRK1 (open squares) had an effect similar to the wild-type GIRK2, whereas cells with both *wv* and GIRK1 (filled diamonds) also became depolarized. Interestingly, cells with *wv* and GIRK2 combinations, whether or not GIRK1 was present, showed a lesser extent of membrane depolarization (open and filled triangles in Fig. 1A), in between those values from cells with control and *wv* channels. Different members of the inwardly rectifying K^+ channels are known to form heteromultimers with large conductances [16–18], and we observed that cells with both GIRK2 and GIRK1 (filled squares) had a more hyperpolarized resting potential (Fig. 1A). The resting membrane potential in all the oocytes returned toward the normal negative value after about 5–6 days (Fig. 1A), when protein turnover started to deplete the K^+ channels.

The loss of a negative resting potential in cells with the *wv* channel suggested that the mutation might be altering resting membrane permeability. To determine the ionic basis of the depolarization observed in oocytes expressing the *wv* channels, the Na^+ in the normal physiological recording solution was replaced by the large, inert Na^+ substitutes *N*-methyl-D-glucamine (NMG) or choline. Surprisingly, this substitution rapidly restored the normal negative membrane potential in cells with *wv* channels (*wv*, *wv*+GIRK1, *wv*+GIRK2, *wv*+GIRK2+GIRK1) (see Fig. 1B), whereas it had little effect on the normal negative resting membrane potentials in control oocytes or cells with only wild-type K^+ channels (GIRK1, GIRK2, GIRK2+GIRK1). Removing Mg^{2+} or Ca^{2+} from, as well as adding EDTA or EGTA to, the extracellular solution did not change resting potential (data not shown). These results suggest that the mutant GIRK2 channels (*wv*) have lost their selectivity for K^+ over Na^+, and that the resulting increase in resting Na^+ permeability in the *wv* channels caused the loss of a negative resting potential in the cells.

During the preparation of this manuscript, a report of a similar study appeared where deletion variants (termed Δ9-GIRK2 and Δ9-GIRK2 *wv* in the report) were used for the GIRK2 wild-type and weaver mutant [21]. It was reported that Δ9-GIRK2 *wv* channels, when expressed in *Xenopus* oocytes, caused similar Na^+ leakage to that observed in our study with the full-length WV channels, but that the expression of the Δ9-GIRK2 *wv* channels resulted in cell death when incubated in the ND 96 solution containing normal Na^+ concentration [21]. This latter phenomenon differed from our observations, where oocytes stayed viable for 6–7 days in the ND 96 solution without noticeable cell death (data not

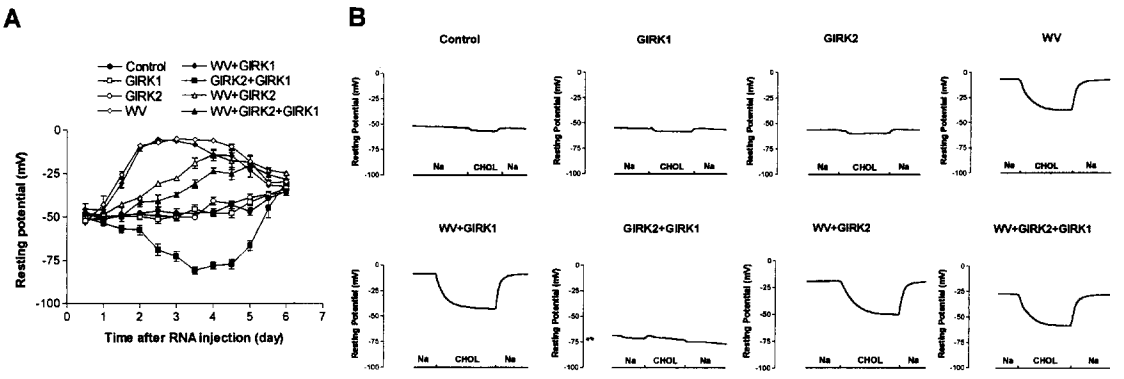

Fig. 1. Effect of K^+ channel expression on resting membrane potential in *Xenopus* oocytes. Resting membrane potentials were recorded from oocytes injected with mRNA for GIRK1 (GIRK1), wild-type GIRK2 (GIRK2), mutant GIRK2 from weaver mice (*wv*), and combinations thereof. Control cells were not injected with any mRNA. (A) Changes in oocyte resting potential over the course of an expression experiment. Cells were injected with mRNA on day 0. Membrane potentials were recorded in normal physiological Na^+-containing solution (ND96). Each point represents the mean ± S.E.M., from 3–5 oocytes. (B) Effect of substituting Na^+ on resting membrane potential. Representative membrane potential traces are shown from cells 4 days after mRNA injection. Cells were initially bathed in normal ND96 frog oocyte solution ('Na'). The bath solution was then switched to a solution in which the Na^+ was replaced with 96 mM choline chloride ('CHOL'), and then switched back to the normal Na^+ solution. Substitution with *N*-methyl-D-glucamine gave similar results (data not shown).

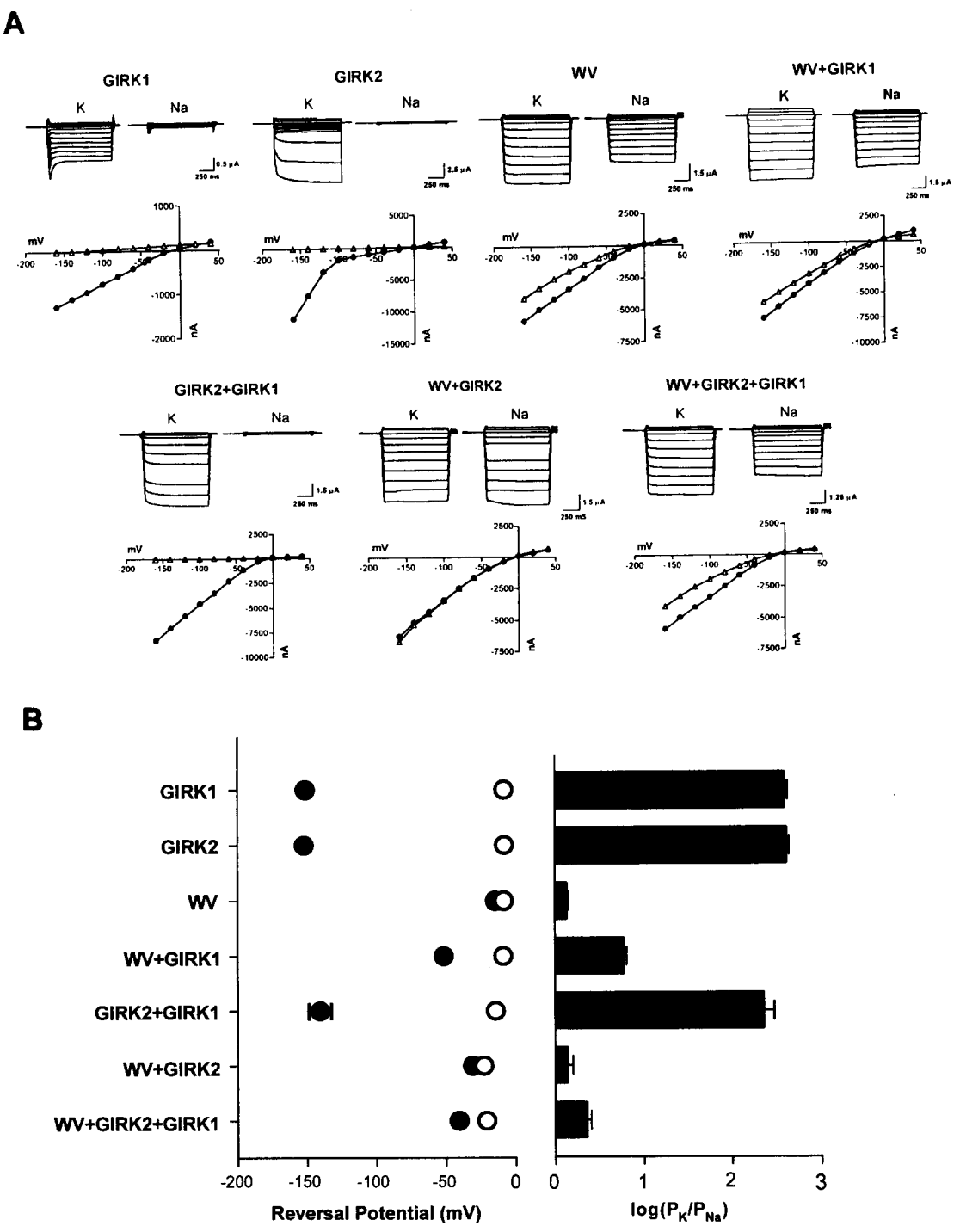

Fig. 2. Effect of the weaver mutation on K^+ and Na^+ permeability. (A) Typical current traces from mRNA-injected oocytes. Membrane currents were measured in a high K^+, Na^+-free solution (left traces in each pair) and in a high Na^+, K^+-free solution (right traces in each pair). The current-voltage relationship is shown below each current trace pair. (B) Average reversal potentials of the GIRK channels calculated from the current-voltage measurements (left panel), and the logarithm of the ratio of K^+ to Na^+ permeability (right panel), estimated from the reversal potential shifts with the Goldman-Hodgkin-Katz equation (see text). Open circles, reversal potentials in high K^+ solution; filled circles, reversal potentials in high Na^+ solution. The data are mean ± S.E.M., from 5–7 oocytes.

shown). It is not clear whether the N-terminal deletions used by Slesinger et al. contributed to the difference in cell survival.

3.2. *Effect of the weaver mutation on K^+ and Na^+ permeability*

To characterize further the apparent loss of ion selectivity of the *wv* channels, voltage clamp experiments were used to quantitate the ratio of K^+ permeability to Na^+ permeability in cells expressing the wild-type GIRK1 and GIRK2, the *wv* channel, and combinations thereof. As shown in Fig. 2A, membrane currents over the voltage range of −160 to +40 mV were recorded either in 98 mM K^+, Na^+-free solution ('K') or in 98 mM Na^+, K^+-free solution ('Na'). Reversal

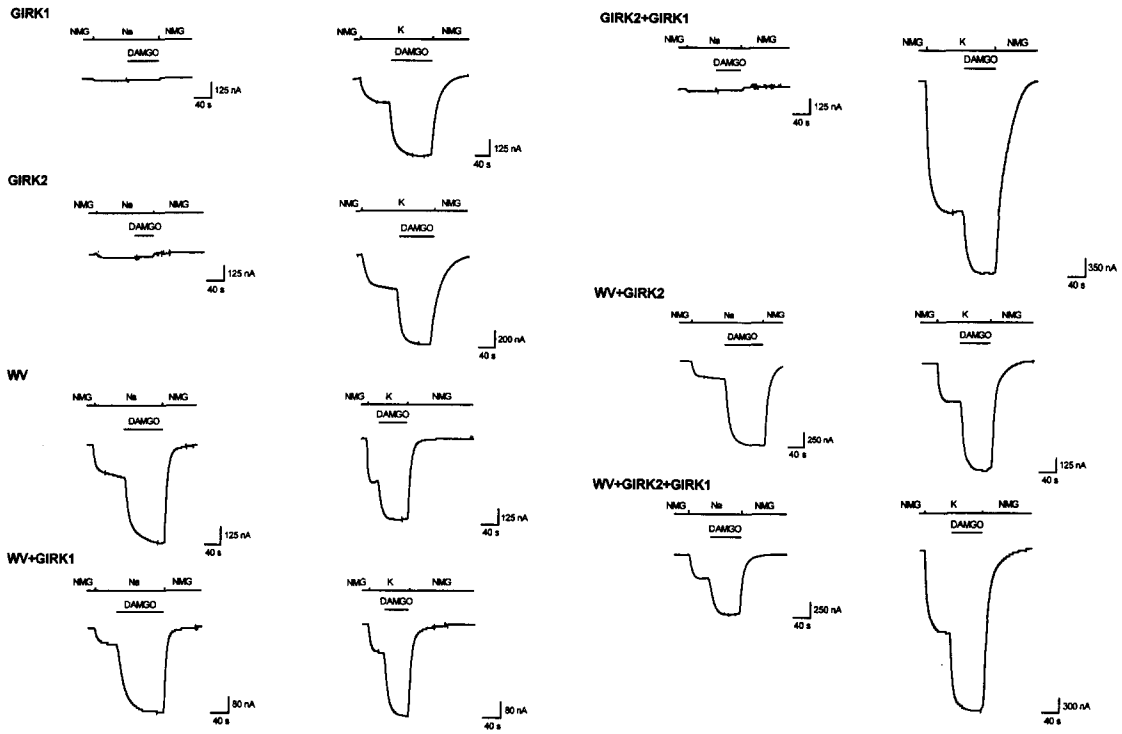

Fig. 3. Ability of the mu opioid receptor to increase GIRK channel currents in Na^+- or K^+-containing solutions. Inward Na^+ and K^+ currents were recorded with a two-microelectrode voltage clamp from oocytes expressing the mu opioid receptor and different GIRK channels or combinations of GIRK channels as indicated. Holding potential was −80 mV. Oocytes were first incubated in Na^+- and K^+-free ('NMG') solution for a few minutes until the negative resting potential was restored, and then the cells were voltage-clamped to record the membrane currents. During the recording, the bath solution was switched to the high Na ('Na') solution (left trace in each pair), and then to the high K^+ ('K') solution (right trace in each pair). The time course of the solution changes is shown on the horizontal line above the current traces. To measure the ability of the mu opioid receptor to increase the GIRK currents, the mu-selective agonist [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO) was added at the concentration of 50 nM as indicated by the bar.

potentials in both conditions ($E_{rev}(K^+)$ or $E_{rev}(Na^+)$) were obtained by measuring the current-voltage relationship. The ratio of K^+ permeability (P_K) to Na^+ permeability (P_{Na}) was calculated according to the Goldman-Hodgkin-Katz (GHK) equation:

$$\log(P_K/P_{Na}) = E_{rev}(K^+) - E_{rev}(Na^+)$$

As expected, *wv* channels had very low selectivity for K^+ over Na^+ (permeability ratio less than 2:1 in cells expressing *wv* channel alone) compared with the highly K^+-selective wild-type GIRK channels (permeability ratio about 400:1) (Fig. 2B). The current-voltage relationship of cells expressing the mutant GIRK2 showed strong inward rectification not only in high external K^+ solutions similar to that seen in the wild-type channel, but also in high external Na^+ solutions.

The GHK equation assumes that the contribution of endogenous leakage currents in the oocyte is small, as verified by the observation that the resting conductance of uninjected oocytes in the high K^+ solution is at least 5–10 times smaller than that of oocytes injected with any of the GIRK proteins; and that the Cl^- permeability of the GIRK and mutant GIRK channels is negligible, as verified by the observation that the current-voltage relation for different GIRK injected oocytes in the NMG solution, which contains a normal amount of Cl^-, was very similar to that for uninjected oocytes in the normal Na^+-containing solution. The equation also assumes that divalent cation permeability was small, as verified by the lack of effect of removing Ca^{2+} or Mg^{2+}. Expression of the mutant GIRK2 channels did not induce a large current from the electrogenic Na-K-ATPase, which might have otherwise confounded the permeability ratio measurements, because addition of 0.4 mM ouabain to oocytes expressing the mutant GIRK2 did not change the resting membrane potential or the ability of NMG substitution to restore the negative resting membrane potential (data not shown).

3.3. *Ability of the mu opioid receptor to increase GIRK channel currents in Na^+- or K^+-containing solutions*

The above results indicate that the primary effect of the weaver mutation is to alter the selectivity of the inwardly rectifying GIRK2 K^+ channel. In oocytes expressing wild-type GIRK channels, a basal, inwardly rectifying current can be readily observed in high external K^+ solutions, and this current can be further enhanced by various G-protein coupled receptors [12–14]. The right-hand panel in Fig. 3 shows that, in high K^+ solutions, activating the coexpressed mu opioid receptor with the mu-selective agonist [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO) further enhanced the inward K^+ current. This effect of DAMGO is essentially similar in oocytes expressing wild-type GIRK channels and in oocytes expressing the *wv* channel, either alone or in combination with wild-type GIRKs. These results are consistent with the idea that the weaver mutation alters channel selectivity with no obvious effects on G-protein regulation. If this is the case, then one should be able to observe DAMGO-activated currents carried by Na^+ in oocytes expressing the mu-

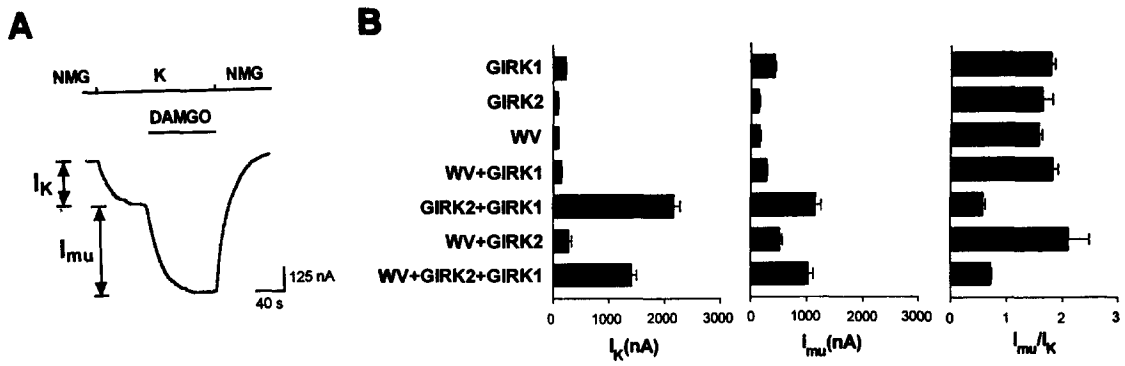

Fig. 4. Effect of the weaver mutation on the formation of high conductance channels. (A) An inwardly rectifying K^+ current recorded by the two-electrode voltage clamp at a holding potential of −80 mV. Measurements of the basal inward K^+ currents (I_K) in the high K^+ solution and the mu opioid receptor-enhanced currents (I_{mu}) were as marked. Solutions and DAMGO stimulation were as in Fig. 3. (B) Average inward basal K^+ currents (I_K) in the high K^+ solution and the additional current activated by DAMGO stimulation (I_{mu}), as well as the ratio of I_{mu}/I_K. Data are from 6–13 oocytes for each condition. The error bars show the S.E.M.

tant *wv* channel. As shown on the left panel of Fig. 3, this was indeed the case. After incubating the oocytes in the Na^+-free solution (NMG) for a few minutes, changing the solution to a high Na^+ solution revealed an inward Na^+ current. This reflects basal activity of inward rectifier 'Na^+ channels' in the oocytes expressing the *wv* channels. Notably, in these oocytes, additional currents carried by Na^+ were evoked by activating the coexpressed mu opioid receptor with DAMGO (Fig. 3, left panel).

In the report by Slesinger et al. [21], it was noted that expression of Δ9-GIRK2 *wv* eliminated the ability of the channel to be activated by G-proteins. As shown in Fig. 3, the full-length WV channels can be activated by G-protein-coupled mu opioid receptor, in both high external Na^+ and K^+ solutions. Therefore, the full-length WV channels are capable of being activated by G-proteins, different from the Δ9-GIRK2 *wv* deletion variant used by Slesinger et al.

3.4. *Effect of the weaver mutation on the formation of high conductance channels*

It has been reported that GIRK channels are heteromultimers, and that while homomultimers can form functional channels when expressed from the clones, these channels have a lower conductance and somewhat altered properties from the channels found in vivo [16–18]. By measuring the basal (I_K) and the mu opioid receptor-mediated (I_{mu}) currents (Fig. 4A) in oocytes expressing various GIRK channels and their combinations, we showed that coexpression of GIRK1 and GIRK2 results in a much larger basal inward K^+ current than that expected from expressing either channel singly [16,17], and that these heteromultimers have a fractionally smaller further activation by DAMGO (Fig. 4B). This interaction of GIRK1 and GIRK2 is not blocked by coexpression of the mutant GIRK2 (*wv*). However, the mutant GIRK2 is unable to generate high conductance channels (in Na^+ or K^+) when coexpressed with GIRK1.

Our results indicate that in the weaver mutation, a single amino acid substitution in the H5 region of GIRK2 alters the ion selectivity of the channel and converts the G-protein-gated K^+ channel to a G-protein-gated K^+- and Na^+-permeable channel, yet has no effect on the G-protein-gating properties of the channel. The results are consistent with the report that the H5 region forms the pore of the channel [8]. Further study of this H5 region mutation may help elucidate the molecular mechanisms of ion selectivity.

Like the wild-type GIRK2, the *wv* channel displays basal activity that can be enhanced by receptor activation. The effect of the mutation is to increase the Na^+ permeability, both basal and receptor-activated. It has been suggested that the basal activity of the G-protein-gated K^+ channels contributes to the resting membrane potential in the absence of an activated receptor in physiological conditions [22–24]. However, in the case of the *wv* mutant, a large basal Na^+ permeability is seen as well. This causes depolarization of the oocyte's resting membrane potential. It is possible that this effect also occurs in vivo in the affected neurons and contributes to the death of these neurons in specific brain regions. Murtomäki et al. [25] reported that weaver cerebellar granule neurons had low resting membrane potential (−38 mV) in comparison with the resting membrane potential in wild-type neurons (−61 mV). In addition, further activation of the channel by appropriate G-protein coupled receptors might cause further depolarization of cells expressing the mutant channel, whereas in the wild-type cells these receptors are normally inhibitory, hyperpolarizing inputs.

The present study does not permit firm conclusions to be drawn about the ability of the mutant GIRK2 to form heteromultimers with wild-type GIRK proteins. However, in terms of the possible physiological implications of the Na^+ permeability observed in the mutant channel, it is relevant that we found that the mutant channel caused marked depolarization of resting potential and loss of K^+ selectivity even when coexpressed with wild-type GIRK1, a condition presumably closer to that found in the affected neurons. Also note that the oocytes coexpressing mutant and wild-type GIRK2 without, or especially with, GIRK1 did not depolarize as much as oocytes expressing *wv* channel alone. This intermediate effect on resting membrane potential may relate to the observation that the heterozygous mutant mouse has an intermediate cerebellar phenotype, between that of the homozygous mutant, and the normal, wild-type mouse. Taken together, these coexpression experiments support the idea that the permeability change observed in the oocyte expression system may be relevant to the effects of the weaver mutation observed in vivo.

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