Interaction of the K Channel β Subunit, Hyperkinetic, with eag Family Members

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Assembly of K channel α subunits of the Shaker (Sh) family occurs in a subfamily specific manner. It has been suggested that subfamily specificity also applies in the association of β subunits with Sh channels (Rhodes, K. J., Keilbaugh, S. A., Barrezeuta, N. X., Lopez, K. L., and Trimmer, J. S. (1995) J. Neurosci. 15, 5360–5371; Sewing, S., Roeper, J. and Pongs, O. (1996) Neuron 16, 455–463; Yu, W., Xu, J., and Li, M. (1996) Neuron 16, 441–453). Here we show that the Drosophila β subunit homologue Hyperkinetic (Hk) associates with members of the ether α-go-go (eag), as well as Sh, families. Anti-EAG antibody coprecipitates EAG and HK indicating a physical association between proteins. Heterologously expressed Hk dramatically increases the amplitudes of eag currents and also affects gating and modulation by progesterone. Through their ability to interact with a range of α subunits, the β subunits of voltage-gated K channels are likely to have a much broader impact on the signaling properties of neurons and muscle fibers than previously suggested.

Voltage-gated ion channels can be profoundly affected by the presence of auxiliary subunits (1). For voltage-gated K (Kv) channels, affinity purification and immunoprecipitation of mammalian α-dentotoxin-sensitive complexes has identified two β subunits, Kβ1 and Kβ2, that associate with α subunits of the Sh family (2). Subsequent studies of α-β interactions have suggested that coassembly with this class of β subunits is restricted to the Kβ1 subtype of α subunits (3–5). Several Kβ subunits have been identified including Drosophila, rat, ferret, bovine, and human subunits (6–10).

The Drosophila Kβ subunit Hk shares ~42% amino acid identity with rat Kβ1 and ~48% identity with rat and bovine Kβ2 (6). Mutations of the Hk locus result in ether-sensitive K channel shaking and hypereexcitability in nerve and muscle, characteristics that are shared by Sh and eag mutants (11). Sh:HK heteromultimers in vivo and expressed in oocytes exhibit currents that are increased in amplitude with a voltage dependence that is shifted to more negative values and more rapid kinetics when compared with the currents of SH alone (6, 12). It is not clear, however, whether these changes are sufficient to account for the full range and severity of the phenotypic defects observed in Hk mutants. For example, the leg shaking of Hk mutants occurs in a cyclical pattern; this cyclical pattern is superimposed on the leg shaking of Sh in Sh: HK double mutants (13). We therefore sought to determine whether Hk can interact with other α subunits in addition to SH. In immunoprecipitation experiments, we found that HK can associate with EAG α subunits. The functional consequences of this association included an increase in eag current amplitude, an acceleration of activation kinetics, and “protection” from a down-modulation in eag current amplitude that was otherwise observed in response to treatment with progesterone. We also found that the ability to interact with Hk was conserved in mouse eag (meag) and the human eag-related gene, Herg. Some of the data have been presented in abstract form (14).

EXPERIMENTAL PROCEDURES

Generation of Antibodies—For production of anti-EAG antibody, fusion proteins containing the NH2-terminal portion of EAG (residues 44–210) along with a six-histidine tag (His-EAG-N) or glutathione S-transferase (GST-EAG-N) were generated. Proteins were expressed in Escherichia coli and purified using standard protocols (15). 250 µg of GST-EAG-N was injected into rabbits. Antibody was purified by passing the serum through a nickle column to which His-EAG-N was bound and eluted according to established procedures (Ref. 15, metal-chelate affinity chromatography, 10.11B). For anti-Hk antibody, an Hk cDNA, HC208 (6), was modified by polymerase chain reaction (Taq polymerase, Promega) to add an EcoRI site and a bacterial ribosomal binding site upstream of the start AUG (GAAGAGAATTCAGGAAGAGAAGCA-CATG, underlining indicates changes from the original cDNA; anti-sense primer ATGGGATCCGCCTTGTGGATGATG). The polymerase chain reaction product was digested with EcoRI and EcoRV (New England Biolabs) and subcloned into HC208 which was then inserted into the bacterial expression vector pPROK-1 (CLONTECH Laboratories, Inc.). After 4 h growth in 0.5 mM isoprropyl-1-thio-β-D-galactopyranoside, bacteria were lysed and treated with Benzonase (400 units; EM Science). The soluble fraction was precipitated with 75% NH4SO4 (w/v). Protein was resuspended in column buffer (100 mM NaCl, 20 mM Tris, pH 7.0, 5% ethylene glycol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and run over a Macro-Prep 50 CM column (Bio-Rad). The fraction enriched for HK (monitored by SDS-PAGE)1 was reapplied to the hydroxylapatite column, washed with 10–100 mM NaPO4 (pH 6.8), 1 mM β-mercaptoethanol, and then applied to an hydroxyapatite column (Bio-Rad) and eluted with a 10–500 mM NaPO4 gradient. The fraction enriched for HK (monitored by SDS-PAGE) was reapplied to the hydroxyapatite column, washed with 10–100 mM NaPO4, and re-eluted using steps from 125–500 mM NaPO4. Flow-through from the 150 mM elution contained essentially homogenous HK. Rabbits were inoculated with homogenized gel slices containing purified HK. An HK affinity column was prepared by incubating enriched HK fractions with Affi-Gel 10 (Bio-Rad). Crude rabbit sera were applied to the affinity column, washed with 1 M NaCl, eluted with 100 mM glycine (pH 2.5), 10% ethylene glycol, and collected in phosphate-buffered saline.

Immunoprecipitation—For expression in tsA201 cells, the vector contained the simian cytomegalovirus IE94 promoter/enhancer sequence upstream from the coding regions. A tag of 6 copies of the MYC-epitope was fused to the NH2-terminal of Hk. Hk and EAG subunits were coexpressed in tsA201 cells. After a shift from 25°C to 33°C, cells were lysed and the lysate was incubated with anti-Hk antibody, purified antibodies were added and incubated for 2 h at 4°C. Aliquots were then incubated with 200 µg of Protein G-Sepharose, pelleted, and washed three times with column buffer.

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§ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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modified Egel’s medium (Life Technologies, Inc.) with 10% cosin calf serum (HyClone Laboratories, Inc.) and transfected by the calcium phosphate method. 15 μg of DNA per 10-cm plate was used for each construct. Transfected cells were lysed and solubilized in buffer containing (in mM): 20 Tris-HCl, pH 7.5, 150 NaCl, 5 EDTA, 5 EDTA, 1% CHAPS, 1 dithiothreitol, 1 phenylmethylsulfonyl fluoride, and leupeptin, aprotinin, and pepstatin A at 1 μg/ml. 700 μg of total protein from each supernatant was incubated overnight at 4 °C with anti-EAG antibody. The immunocomplex was precipitated with 50% rProtein A Avidigel F (BioProbe International, Inc.) and extensively washed. 

**Electrophysiology**—For expression in Xenopus oocytes, constructs were subcloned into the pGEMHE vector (provided by E. Goulding) (6, 16), a version of pGEMHE containing the 5’- and 3’-untranslated regions of the Xenopus β-globin gene. Hk- and α-subunit-containing plasmids were linearized using Not1 and Nol1, respectively, and capped RNA transcribed in vitro using T7 RNA polymerase (Promega). Stage V-VI oocytes, obtained from adult females (Nasco, Ft. Atkinson, WI) as described previously (6), were maintained in L-15 media (containing 50% L-15 (Life Technologies, Inc.), 15 mM Hepes, 1 mM L-glutamine, 50 mg/ml gentamycin, and 5 mg/ml bovine serum albumin, pH 7.4) at 18 °C. Oocytes were injected with ~30 nl of solution containing mRNA for the α subunit and Hk or RNA for the α subunit and an equal amount of diethyl pyrocarbonate-treated water (~1–6 ng of RNA/oocyte). A 2-fold excess of Hk RNA was used to maximize coassembly. Two to three-fold increases in the amount of a subunit RNA produced linear increases in current amplitudes. Recordings were performed using an OC-725B amplifier (Warner Instrument Corp.) and pCLAMP 6 (Axon Instruments). Currents were filtered at 1–2 kHz (~3 dB, 8 pole Bessel) and sampled at 5–10 kHz. Linear leak and capacitative currents were subtracted using P/n methods. The extracellular recording solution typically contained, in mM: 140 NaCl, 2 KCl, 1 MgCl2, 10 Hepes (pH 7.1, NaOH). An extracellular solution high in K+ was used to enhance tail currents during measurements of the voltage dependence of activation and to record Herg currents; this solution contained, in mM: 100 KCl, 1.8 CaCl2, 1 mM MgCl2, 5 Hepes (pH 7.4, KOH). Pipettes (2–3 m) had resistances of 0.5–2 MΩ. Experiments were performed at room temperature (19–22 °C). Stauronosporine, H7, phorbol 12-myristate 13-acetate, and caffeine (Alexis Corp.), D609 (Calbiochem), and progesterone were prepared as stock solutions using dimethyl sulfoxide or extracellular recording solution. The concentration of dimethyl sulfoxide in the bath did not exceed 0.1%. 

Amplitude measurements refer to the maximum current observed during the test pulse. Time constants were obtained by fitting traces in their entirety (excluding capacitative transients) with the number of exponentials noted in the text and a steady state. Estimates of the activation rate of Herg were obtained by varying the duration of a depolarizing prepulse to +10 mV. The amplitude of the inward current during the subsequent hyperpolarization to ~110 mV was corrected by extrapolating a single exponential fit to the deactivating phase of the inward current to the onset of the hyperpolarizing step. Conductance-voltage (G-V) relations were determined using the equation, $G - I$(V, V) = $G_s$ - $G_i$ = tail current observed during a pulse to ~120 mV following a test pulse to the indicated potential, $V$. For Herg, $I_v$ was corrected using a single exponential fit to the deactivating phase of the current and extrapolating to the onset of the pulse to ~120 mV. Reversal potentials, $V_r$, were determined for each oocyte. Each G-V curve was normalized by the maximum conductance and fit with a Boltzman distribution. 

**RESULTS AND DISCUSSION**

**Eag shares only 12% amino acid identity with Sh and distinct structural features define it as a member of a separate family (17). To examine whether there is a physiological interaction between HK and EAG, six copies of the MYC-epitope were fused in-frame to the amino-terminus of EAG in a mammalian expression vector. tsA201 cells were transfected with either eag, Hk, or both constructs and the resulting complexes were immunoprecipitated from cell extracts using an anti-MYC-tag. EAG protein was evident as an ~139-kDa band that was observed in the lanes corresponding to cells transfected with the eag construct (Fig. 1A, bottom). After stripping, the immunoblot was reprobed with antibody to HK. HK was observed only in the lane corresponding to cells transfected with both constructs (Fig. 1A, middle). There was no obvious cross-reactivity between the anti-EAG antibody and HK (Fig. 1B). A separate immunoblot of the same whole cell extracts revealed no difference in the level of HK expression in Hk and Hk+Eag+Hk transfected cells (not shown).**

The above results suggest that there is a physical interaction between EAG and HK. To determine the physiological effect of the interaction, we examined currents in oocytes injected with eag RNA, either alone or together with RNA encoding Hk. Fig. 2 shows the results obtained on day 3 postinjection for one batch of oocytes. Expression with Hk produced a 3.9-fold increase in the mean current amplitudes recorded in response to test pulses to +40 mV (Fig. 2A and B). Because the voltage dependence of activation did not change in the presence of Hk (Fig. 2E), the increase in amplitude is likely to be a result of an increase in the number of functional channels or an increase in the single channel conductance.

**Hk also accelerated the activation of eag currents. For the oocytes in Fig. 2, the time to 80% of the maximum current was decreased from the control value of 51.5 ± 3.5 ms (n = 5) to 37.8 ± 3.4 ms (n = 4; test pulse, 0 mV). The rising phase was best fit by a sum of two exponentials, $\tau_1$ and $\tau_2$; both were significantly decreased (Table I), an effect that was most dramatic at lower potentials (Fig. 2, C and D). The activation rate of eag also has been shown to vary with changes in the voltage preceding the test pulse. Activation occurs more rapidly with more positive prepulses, an effect that is reminiscent of the Cole-Moore shift observed for K currents in the crayfish axon (18, 19). As shown for $\tau_2$ (Fig. 2f), in the presence of Hk activation was faster at all prepulse potentials. Finally, eag currents exhibited an inactivating component at test pulse voltages above +20 mV. Inactivation was well described by a single exponential that was slowed from 45.5 ± 4.7 to 54.0 ± 2.9 ms in the presence of Hk (n = 5 and 4, respectively; test pulse, +20 mV, Fig. 2G). A detailed summary of results, averaged across the four batches of oocytes examined using the same recording solution, is given in Table I. The above changes in kinetics were observed in oocytes with currents ranging from 1 to 15 μA.**

**Hk not only affected the properties, but also the modulation,
of eag currents. To examine the role of Hk in modulation, we used progesterone which alters the activity of a number of intracellular messengers to trigger the transition from the G2 to M phase of the cell cycle in a process referred to as maturation (20). When recording from oocytes injected with eag alone, progesterone (30 μM) decreased current amplitudes by 20–50% (mean fractional current remaining in the presence of progesterone = 0.68 ± 0.03, n = 14; e.g., Fig. 3A). The decrease was initiated within the first minute following application and current levels stabilized at the new lower level within 5–10 min (e.g. Fig. 3A, bottom). Currents were restored following washout of progesterone (n = 7). In contrast, when Hk was coexpressed with eag, progesterone (30 μM) failed to decrease amplitudes in 10 of the 12 oocytes examined even when currents were monitored for 30 min (mean fractional current remaining = 0.96 ± 0.02; n = 12; e.g. Fig. 3B). Thus, the association with Hk either reduced the sensitivity of eag channels to progesterone or inhibited the response. To further identify the intracellular messengers involved in the response to progesterone, oocytes were incubated in staurosporine (1–2 μM) or H7 (50 μM), two nonspecific serine-threonine kinase inhibitors, for 30–90 min prior to the application of progesterone. Both staurosporine and H7 failed to block the effect (n = 6 and 9, respectively, not shown). In addition, caffeine (10 mM), which increases calcium levels by releasing calcium from intracellular stores, decreased eag:Hk, as well as eag, currents and therefore did not mimic the action of progesterone (n = 3 and 11, respectively, not shown). Phorbol 12-myristate 13-acetate (20–50 nM), a phorbol ester that activates protein kinase C, failed to produce a consistent change in eag currents (n = 12, not shown). We therefore attempted to block the response to progesterone at an earlier point in the signaling cascade. D609 is a specific inhibitor of phosphatidylcholine-specific phospholipase C (21) and, in oocytes, breakdown of phosphatidylcholine is responsible for the majority of diacylglycerol released following progesterone treatment (22). Preincubation of eag-expressing oocytes in bath solution containing D609 (50 μg/ml, 1 h) resulted in a complete block of the response to progesterone in 10 of 12 oocytes examined (mean fractional current remaining in the presence of progesterone = 0.89 ± 0.06, n = 12; e.g., Fig. 3C).

To determine whether the interaction with Hk is specific to eag or whether the interaction with Hk transcends both species and eag subfamilies, we examined the effects of Hk on two other members of the eag family which have been isolated and physiologically characterized, meag and Herg (16, 17, 19, 23). With the exception of inactivation which is not observed for meag, the properties of meag currents affected by Hk were the properties affected in the case of eag, namely current amplitudes and activation kinetics (Fig. 4, A and B, Table I). Herg, in contrast to eag and meag, carries a predominantly inward K current and represents a distinct subtype within the eag family (16, 17). The inward rectification of Herg channels has been suggested to be the result of a “C-type” inactivation that is much more rapid than activation (24). In the comparison of Herg in the presence and absence of Hk, little difference in current was detected when recording at days 2 and 3 postinjection. By day 6, however, oocytes coexpressing Hk could be clearly distinguished from controls. Hk increased current amplitudes by 6.8-fold (test pulse, −110 mV), the largest increase observed for any of the α subunits tested (Fig. 4, C and D). An estimate of the activation rate was obtained for a subset of oocytes by varying the duration of a prepulse to +10 mV. The amplitude of the inward current during the subsequent hyperpolarization to −110 mV, corrected for deactivation and normalized to the maximum observed (see “Experimental Procedures”), was used to determine the fraction of channels that had passed from the closed to open state during the preceding depolarization. The resulting data were well fit by a single exponential with a time constant of 81.5 ± 2.3 ms in controls (n = 7, Fig. 4D). In the presence of Hk the time constant was...


| Properties of α subunits expressed in Xenopus oocytes with and without the Drosophila β subunit, Hk |
|-----------------|------------|--------|-----------------|--------|--------|-----------------|--------|-----------------|--------|--------|
|                 | eag        | eag + Hk | P    | meag           | meag + Hk | P    | Herg           | Herg + Hk | P    |
| N oocyte batches (oocytes) | 4 (17)    | 4 (22)  |       | 4 (18)         | 3 (14)    | 3 (17) |                 |                 |       |
| I_{peak} (μA at +40 mV; −110 mV) | 2.59 ± 0.42 | 5.07 ± 0.89 | <0.0001 | 2.72 ± 0.27 | 3.74 ± 0.54 | <0.0001 | −1.44 ± 0.29 | −5.78 ± 0.93 | <0.0001 |
| Fold increase in I |
| Activation |
| ms to 80% activation (0 mV) | 50.0 ± 3.4 | 42.8 ± 2.8 | <0.01 | 119.2 ± 4.8 | 103.5 ± 7.1 | <0.01 |
| τ1 (ms at 0 mV) | 18.2 ± 1.14 | 16.2 ± 1.09 | <0.01 | 15.53 ± 0.72 | 13.98 ± 0.85 | <0.01 |
| τ2 (ms at 0 mV; +40 mV) | 121.3 ± 10.4 | 96.9 ± 7.2 | <0.01 | 133.9 ± 4.6 | 122.8 ± 7.7 | <0.01 |
| Midpoint (mV) in 100 mM K+ | −5.21 ± 2.51 (11) | −6.19 ± 1.14 (10) | NS | −7.52 ± 1.94 (26) | −7.78 ± 1.55 (28) | NS |
| Slope (mV/e-fold) in 100 mM K+ | 11.29 ± 0.94 (11) | 11.25 ± 0.87 (10) | NS | 19.58 ± 1.42 (26) | 20.13 ± 0.99 (28) | NS |
| Inactivation |
| τ1 (ms at +40 mV) | 42.9 ± 2.7 | 55.0 ± 2.7 | <0.01 |
| Recovery from inactivation |
| ms to peak (−90 mV) |
| τ1 (ms at −90 mV) | 46.9 ± 2.3 | 58.0 ± 2.3 | <0.01 |
| Deactivation |
| τ1 (ms at +40 mV; −90 mV) | 5.58 ± 0.89 | 6.26 ± 0.66 | NS | 4.55 ± 0.51 | 4.84 ± 0.37 | NS |
| τ2 (ms at +40 mV; −90 mV) | 65.3 ± 5.0 | 63.0 ± 4.6 | NS | 76.2 ± 4.0 | 76.2 ± 2.5 | NS |

|                |
|-----------------|------------|--------|-----------------|--------|--------|-----------------|--------|-----------------|--------|--------|
|                |            |        |                 |        |        |                 |        |                 |        |        |
| aSignificance was determined using an unweighted means two-way analysis of variance with oocyte batch and the presence of Hk as variables. |
| bThe first voltage refers to the test pulse voltage used for eag and meag currents (holding potential = −80 mV), the second voltage refers to the test pulse voltage used for Herg currents (holding potential = +40 mV). |
| cFor each batch of oocytes the fold change was obtained by dividing the mean of +Hk currents by the mean of the control currents. The fold change was then averaged across the different oocyte batches. |
| dEstimates of Herg activation were obtained only for a subset of oocytes, the number of which is given in parentheses following the S.E. |
| eStudent's t test. |
| fThe N differ because G-V relations were determined separately in different oocytes. These oocytes, however, belonged to the same batches used in the measurement of the other features and exhibited increases in current amplitude similar to that observed in standard extracellular recording solution. |
| gNS, not significant. |
oocytes were incubated in bath solution containing 50 μM progesterone does not appear to be a consequence of other changes in the oocyte membrane. To demonstrate block of the progesterone effect, 

$$G \times V$$

to Fig. 2 and under “Experimental Procedures.” For those described for $eag$ and $meag$ conditions, respectively. Protocols differing from those described for $eag$ and $meag$ conditions, respectively.

$Hk$ and $erg$ currents in high K$^+$ recording solution in response to steps from $-120$ to $+20$ mV in 20 mV increments (holding potential, $+40$ mV). Scale bars, $0.5 \mu A$ and $100$ ms. $D$, properties of $Herg$ in the presence and absence of $Hk$. Data were obtained as described in the legend to Fig. 2 and under “Experimental Procedures.” For G-V curves, $n = 7$ and 6; otherwise, $n = 5$ and 4 for the $meag$ and $meag+Hk$ conditions, respectively. $C$, $Herg$ and $Herg+Hk$ currents in high K$^+$ recording solution in response to steps from $-120$ to $+20$ mV in 20 mV increments (holding potential, $+40$ mV). Scale bars, $0.5 \mu A$ and $100$ ms. $D$, properties of $Herg$ in the presence and absence of $Hk$. $n = 5$ and 4 for the $Herg$ and $Herg+Hk$ conditions, respectively. Protocols differing from those described for $eag$ are described in the text and under “Experimental Procedures.”

decreased to 65.9 ± 6.2 ms ($n = 4$), indicating that $Hk$ accelerates the activation of $Herg$, as well as $eag$ and $meag$ currents. In addition, $Hk$ slowed the time-to-peak of the inward current from 46.3 ± 2.3 to 58.8 ± 3.0 ms ($n = 8$ and 9, respectively; test pulse $-90$ mV). Exponential fits revealed that the slowing was largely a result of a slowing of deactivation (Fig. 4D). $Hk$ also produced a modest $-4$ mV shift in the midpoint of the Boltzmann curve describing the voltage dependence of activation with little change in slope (Fig. 4D). A summary of the results, averaged across oocyte batches, is given in Table I.

In conclusion, in a comparison with rat K1.1 whose primary effect on K1.1 appears to be an acceleration of inactivation (2), the primary role of $Hk$ is to produce a substantial increase in current amplitudes for each $\alpha$ subunit we have examined, including $Sh$ (6). In fact, an increase in amplitude is the most common consequence of the interaction between $\alpha$ and $\beta$ subunits for all voltage-gated channels (1). Our results also demonstrate that coexpression with $Hk$ can affect channel modulation and suggest that the role of $Hk$ and other $\beta$ subunits of this class cannot be fully understood by an examination of changes in the basal properties of $\alpha$ subunits. In this case, $Hk$ “protects” $eag$ currents from the down-regulation in amplitude.
that is otherwise observed in response to progesterone. Intrigu-
ingly, the pathways activated by progesterone in oocytes may be the same pathways whose activation at the Drosophila neuromuscular junction results in a prolonged alteration in K currents lasting many minutes (25).

Recent studies examining the interactions between the mammalian K\(_{\beta}\) subunits and the \(\alpha\) subunits of K channels have indicated that the \(\beta\) subunits associate specifically with the K\(_{1,1}\) subtype of \(\alpha\) subunits (3–5). Our results suggest that K\(_{\beta}\) subunits may be more promiscuous and that they associate with \(\alpha\) subunits from at least two families. Of interest is whether the interactions between K\(_{\beta}\) subunits and eag family members arise in vivo and whether the potential to interact with Herg is conserved in any of the mammalian K\(_{\beta}\) subunits. The Herg gene is the locus of one form of the inherited cardiac disorder known as long QT syndrome (LQT-2) (26) and, in our experiments, was the most profoundly affected by Hk. Recent evidence suggests overlap in the expression of the mammalian \(\alpha\) and \(\beta\) counterparts (2, 3, 6–9, 17, 18, 26, 27) and some alleles of the seizure (sei) locus, which encodes the Drosophila eag homologue (28, 29), exhibit an enhanced temperature sensitiv-
ity in Hk\(_{sei}\), but not Sh\(_{sei}\), double mutant combinations.\(^2\) Therefore, at least in Drosophila, there is an in vivo interaction between Hk and sei that is independent of the effects of Hk on Sh channels. The average increase in Herg current amplitude produced by Hk is substantially in excess of the recently re-
ported increase observed as a consequence of the association with minK (30). Thus, our results predict that this class of \(\beta\) subunits would make a large contribution to cardiac repolarization.

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