Inactivation of Kv3.3 Potassium Channels in Heterologous Expression Systems*

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Kv3.3 K⁺ channels are believed to incorporate an NH₂-terminal domain to produce an intermediate rate of inactivation relative to the fast inactivating K⁺ channels Kv3.4 and Kv1.4. The rate of Kv3.3 inactivation has, however, been difficult to establish given problems in obtaining consistent rates of inactivation in expression systems. This study characterized the properties of AptKv3.3, the teleost homologue of Kv3.3, when expressed in Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells. We show that the properties of AptKv3.3 differ significantly between CHO and HEK cells, with the largest difference occurring in the rate and voltage dependence of inactivation. While AptKv3.3 in CHO cells showed a fast and voltage-dependent rate of inactivation consistent with N-type inactivation, currents in HEK cells showed rates of inactivation that were voltage-independent and more consistent with a slower C-type inactivation. Examination of the mRNA sequence revealed that the first methionine start site had a weak Kozak consensus sequence, suggesting that the lack of inactivation in HEK cells could be due to translation at a second methionine start site downstream of the NH₂-terminal coding region. Mutating the nucleotide sequence surrounding the first methionine start site to one more closely resembling a Kozak consensus sequence produced currents that inactivated at a second methionine start site more closely resembling a Kozak consensus sequence, both CHO and HEK cells. These results indicate that under the appropriate conditions Kv3.3 channels can exhibit fast and reliable inactivation that approaches that more typically expected of “A”-type K⁺ currents.

Fast inactivating potassium channels play an important role in regulating neuronal output by setting the initial delay and frequency of spike discharge upon membrane depolarization (1). The expression pattern and kinetics of inactivating K⁺ channels can also be controlled over the soma-dendritic axis of central neurons, allowing these channels to contribute to synaptic plasticity and the properties of backpropagating dendritic spikes (2–5). K⁺ channel α-subunits can exhibit slow C- or fast N-type inactivation, the latter incorporating an NH₂-terminal “ball and chain” motif on the α-subunit to block the channel pore (6–8). Within the Kv1–4 families, fast inactivating K⁺ channels include Kv1.4, 3.4, and all three members of the Kv4 family, while an intermediate rate of inactivation has been reported for Kv3.3 (9–11). The voltage dependence and rate of inactivation of these channel subtypes are critical to determining their contribution to neuronal excitability.

The inactivation properties of Kv1.4, Kv3.4, and Kv4.1–4.3 have all been well characterized in mammalian heterologous systems (12–15). While inactivating Kv3.3 channels have been successfully expressed in Xenopus oocytes (9) and more recently in Chinese hamster ovary (CHO) cells (10), obtaining consistent inactivation of rat Kv3.3 has been difficult in the human embryonic kidney (HEK) cell expression system (16). Understanding the kinetic properties of Kv3.3 channels is important as this channel can be densely distributed over dendritic membranes of both principal cells and interneurons (17, 18). Moreover, a reduction in the contribution of these channels to dendritic spike repolarization has been shown to augment burst discharge in an electroreceptive neuron (18). The rate of inactivation of Kv3.3 channels may even contribute to a dynamic modulation of dendritic refractory period during repetitive activity that underlies burst discharge (19).

We previously reported on some of the properties of teleost Kv3.3 channels (AptKv3.3) when expressed in tSA201 HEK cells and recorded in the outside-out patch configuration (18). However, as reported by others (16), the inactivation rate of Kv3.3 whole-cell currents in HEK cells often showed little if no inactivation during 100-ms step commands. The present study examined the kinetic properties of whole-cell currents of AptKv3.3 channels expressed in CHO and HEK cells. We find that AptKv3.3 cDNA gives rise to a fast inactivating whole-cell current in CHO but not HEK cells. By mutating nucleotides upstream of the first start site to one more closely resembling the optimal Kozak consensus sequence, both CHO and HEK cells express fast inactivating AptKv3.3 current. Under these conditions AptKv3.3 currents are capable of inactivating at rates that are close to that reported for even the fast inactivating mammalian Kv3.4.

EXPERIMENTAL PROCEDURES

Molecular Biology—Primers used to amplify the full coding region of AptKv3.3, including 10 base pairs of 5’-untranslated region (5’-UTR), have been described previously (18). In this study, the same AptKv3.3 cDNA was cloned into the expression vector pCDNA3.1 (Invitrogen).

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‡ The abbreviations used are: CHO, Chinese hamster ovary; HEK, human embryonic kidney; UTR, untranslated region; GFP, green fluorescent protein; TEA, tetraethylammonium; S, siemens; IRES, internal ribosome entry site.
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using XhoI and XhoI restriction sites present in the forward and reverse primers, respectively. To generate an AptKv3.3 expression construct in which the Kozak sequence around the initiation methionine was altered (AptKv3.3Koz), a fragment of cDNA encompassing nucleotides –6 to 628 of AptKv3.3 was PCR amplified from an AptKv3.3 expression construct. The following primers were used: forward, 5’-CAC TGC AGC CGC CAT GCT CAG TTC CGT GTG TGA-3’; reverse, 5’-TAG CGG GCA TXT TTA GAG GAG TAG-3’. The forward primer contained the altered sequence GCC GCC preceding the methionine codon and an XhoI at the 5’ end for subcloning. The PCR fragment encompassed an ApoI restriction site normally present in AptKv3.3 cDNA at position 590. After amplification, the PCR fragment was digested with XhoI and ApoI restriction enzymes and substituted for the XhoI-ApoI fragment present in the original AptKv3.3 expression vector. The presence of the altered Kozak sequence was confirmed by DNA sequencing. The amino-terminal truncated AptKv3.3 cDNA was prepared by replacement of the cDNA encoding amino acids 1–27 with the coding sequence of enhanced GFP in the expression vector pEGFP-C1 (Clontech).

Sequences for mouse, rat, and human Kv3.3 and Kv3.4 sequences were obtained from the GenBank™ data base. Accession numbers are as follows: AptKv3.3 (AF308934), rKv3.3a, rKv3.3b, rKv3.3c, and rKv3.3d (rKv3.3a, M84210; rKv3.3b, M84211; rKv3.3c, AY179603; rKv3.3d, AY179604.1), mKv3.3b (NM_008422), hKv3.3a (AF055889), rKv3.4a (X62941), hKv3.4a and hKv3.4b (NM_004978; hKv3.4b, NM_152783).

Heterologous Expression of AptKv3.3—HEK cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented by 10% fetal bovine serum and 1% penicillin-streptomycin. CHO (CHO-K1; ATCC CCL-61) were maintained in F-12K medium (Kaighn’s modification) (Invitrogen) supplemented by 10% fetal bovine serum and 1% penicillin-streptomycin. All cultures were kept at 37 °C. Cells were plated onto glass coverslips and co-transfected with 5 μg of AptKv3.3 vector cDNA and 1.0 μg of eGFP-C1 (Invitrogen) using 50 μl of 2.5 M CaCl₂ per 35-mm dish at 70% confluence. CHO cells were plated onto glass coverslips and co-transfected with 1.25 μg of AptKv3.3 vector cDNA and 0.7 μg of eGFP-C1 using 7.5 μl of Polyfect (Qiagen, Valencia, CA) at 70% confluence. All chemicals were obtained from BDH (Darmstadt, Germany) or Sigma unless otherwise indicated.

Electrophysiology—Patch electrodes were constructed from borosilicate glass (2.0 mm outer diameter or fiber-filled 1.5 mm outer diameter) (A-M Systems, Carlsborg, WA) using a Sutter P-87 microelectrode puller. Recordings were obtained using an Axopatch 200-A or Axopatch 1-D amplifier and PC clamp 8 software (Axon Instruments, Foster City, CA) and data stored on a PC for later analysis. Seals were typically 1.5 GΩ or greater, and any leak current in whole-cell or outside-out mode was subtracted off-line. Electrode resistance was 2–5 MΩ with series resistance of 5–15 MΩ corrected by series resistance compensation at 80–90%. The electrolyte consisted of (in mm): KCl, 144; Hepes, 5; MgCl₂, 1.5; EGTA, 5; MgATP, 1.5; 0.1 CaCl₂, pH 7.2. Whole-cell recordings were acquired at 1–2 kHz and single channel recordings in the on-cell or outside-out configuration were acquired at 2–5 kHz and digitally filtered during analysis at 1–2 kHz.

Coverslips containing cells were mounted on the stage of an upright submersed lens (Zeiss Axioskop I) or inverted microscope (Olympus) and perfused at room temperature with a medium consisting of (in mm): NaCl, 148; Hepes, 10; K₂HPO₄, 1.25; KCl, 2; CaCl₂, 1.5; MgCl₂, 1.5; d-glucose, 20; pH 7.4. Recordings were typically obtained 48–72 h after transfection of cells and identified by GFP fluorescence. The rate of transfection as judged by GFP fluorescence was typically 1–2%, with the vast majority of fluorescent cells expressing AptKv3.3 current. Recordings were restricted to cells expressing Iₚ between 5–8 nA whole-cell current. Cells were typically 32–38 pS in CHO cells and 8.2 ± 0.8 nA in CHO cells (157). The rate of inactivation was calculated from the peak of the tail current to steady-state inactivation. Exponential fits were used and calculated using the following equation.

\[
I(t) = A_t(1 - e^{-q(t-t_0)/τ})
\]  

where \( q \) is a parameter characteristic of the voltage dependence of inactivation \( τ \) is fit using the following equation.

\[
I(t) = A_t(1 - e^{-\frac{t}{τ}})
\]  

Plots of the voltage dependence of activation \( τ \) fit using the following equation.

\[
\frac{I(V)}{I(V_0)} = \frac{A_1 - A_2}{1 + e^{(V - V_0)/V_1}} + A_2
\]  

where \( V \) is the voltage, and \( k \) is a slope factor. The data points of TEA effects were fit to calculate IC₅₀ using the following logistic equation function,

\[
\frac{[V]}{[V_0]} = \frac{A_1 - A_2}{1 + e^{-k(V - V_0)}} + A_2
\]  

where \( [V] \) is the TEA concentration and \( p \) is the equivalent Hill coefficient. Activation \( τ \) was calculated between the end of the compensated capacitive transient and the peak current using a single exponential fit raised to a power of 4 (see Equation 3).

\[
I(t) = A_t(1 - e^{-q(t-t_0)/τ})^4
\]  

Inactivation \( τ \) was calculated from the peak of the evoked current to a point corresponding to steady-state inactivation and deactivation \( τ \) from the peak of the tail current to steady-state. Exponential fits were used and calculated using the following equation.

\[
I(t) = A_t(1 - e^{-\frac{t}{τ}})
\]  

The time course of recovery from inactivation was fit using a single exponential function (see the following equation).

\[
I(V) = A_1 - A_2
\]  

Origin 7.1 (Microcal, Northampton, MA) and Clampfit 8.2 (Axon Instruments, Foster City, CA) were used for fitting. Statistical significance was determined using a two-sample Student’s t test. Average values are expressed as mean ± S.E.

RESULTS

Current Activation and Deactivation—The majority of recordings were made in the whole-cell mode from AptKv3.3 channels expressed in CHO or HEK cells. Step commands from a holding potential of −90 mV in 10 mV depolarizing steps revealed an outward rectifying current that first became apparent for steps between −30 to −10 mV (Fig. 1A). Activation of the current in both expression systems was fast in exhibiting a \( τ \) for activation of <1.2 ms for steps from −90 to 30 mV (Table 1). The \( τ \) for the rising phase of activation decreased with increasing voltage steps (Fig. 1B) but was significantly slower in CHO than HEK cells, with the greatest difference at low voltages. Once above activation voltage, plots of evoked current rose in a linear fashion with no reduction of current at higher voltages, as reported for some expressed Kv3 channels (9, 21). The voltage for activation plots for AptKv3.3 in CHO and HEK cells could be superimposed (Fig. 1C), with a \( V_0 = 7.6 ± 0.8 \) mV for CHO cells and 8.2 ± 0.8 mV for HEK cells (Table 1).

Deactivation was rapid in both CHO and HEK cells (CHO \( τ = 0.55 ± 1.09 \) ms; HEK \( τ = 0.41 ± 0.81 \) ms) and voltage-dependent between −80 and −30 mV (Fig. 1, D and E). As found for activation, the \( τ \) of deactivation in CHO cells was significantly slower than that in HEK cells. The single channel slope conductance measured under presumed equimolar K⁺ in the on-cell configuration ranged between 32 and 38 pS and was not significantly different for channels expressed in CHO or HEK cells (Fig. 1F; see also Table 1).

As reported previously for mammalian Kv3.1 (22), initial activation of AptKv3.3 in HEK cells could be accompanied by an early transient peak of current but less so when expressed in CHO cells. This early peak in HEK cells has been attributed to a transient shift in extracellular K⁺ concentration (22). The
lower prevalence of this early peak in CHO cells may reflect the slower rate of activation, decreasing a putative increase in extracellular K⁺ ions. This was supported by preventing the early peak of current in HEK cells using a 5 ms voltage ramp command from −90 to 50 mV (28 mV/ms; data not shown).

**TEA Pharmacology**—As found for other Kv3 subtypes, AptKv3.3 channels were highly sensitive to external TEA application (Fig. 2A) (16, 23). In both cell types, substantial block was obtained for whole-cell currents in the range of 200 μM TEA or less (28 mV/ms; data not shown).

**Current Inactivation**—Expression of AptKv3.3 cDNA produced currents in CHO and HEK cells that differed dramatically in their inactivation properties. In CHO cells AptKv3.3 current reliably inactivated, with a rate of decay that could be fit well with a single exponential (Fig. 3, A and B). The τ of inactivation was voltage-dependent in decreasing at higher voltage steps from 251 ± 33.5 ms at 0 mV to 84 ± 14.0 ms at 50 mV (Fig. 3B). By comparison, the same cDNA expressed in HEK cells produced slower inactivating currents. Furthermore, there was a high degree of variability in the rate of inactivation, in that approximately half the transfected HEK cell population showed little or no inactivation within 10 s or required step commands to greater than 100 mV from a holding potential of −90 mV before any inactivation could be observed. Our analysis in HEK cells was restricted to those recordings reaching steady-state inactivation within 10 s for steps from −90 to −50 mV. This group presented a slow and voltage-independent inactivation that could only be fit with a single exponential (Fig. 3, D and E). An initial τ for inactivation in HEK cells ranged from 96.8 ± 17.7 to 132.8 ± 35 ms and a second τ from 1037.5 ± 215.7 ms to 1682.2 ± 526.2 ms (n = 9). The V_{1/2} for inactivation in HEK cells was significantly left-shifted compared with CHO cells, with a value of −30.0 ± 0.86 mV compared with −22.5 ± 0.7 mV in CHO cells (Fig. 3, C and F) (Table I). The rate of recovery of AptKv3.3 also differed between CHO and HEK cells (Fig. 3G), with a τ of recovery after steady-state inactivation of 255 ± 27 ms in CHO cells and 174 ± 21 ms in HEK cells (Table I).

The rate of inactivation of other mammalian K⁺ channels can vary depending on such factors as the cytosolic content of...
second messengers or redox environment (13, 24–28). These factors might then explain the different kinetics of AptKv3.3 observed in the two cell lines. To test this hypothesis we recorded AptKv3.3 currents in CHO and HEK cells in the outside-out configuration to maximize intracellular washout and reduce differences in cytosolic factors. We found in outside-out recordings that single channels of AptKv3.3 expressed in HEK cells showed a high steady-state open probability over 5 s, while those expressed in CHO cells showed clear inactivation and an extremely low steady-state open probability within 1 s (Fig. 3H; n = 10). In addition, macropatch recordings showed that large differences in the rate of inactivation persisted between CHO and HEK cells (Fig. 3I). These results indicate that the primary difference in the rate of inactivation between CHO and HEK cells does not likely arise from cytosolic factors.

**Effects of Modifying the Kozak Consensus Sequence**—The efficiency of translation at a methionine start site has been shown to depend on the resemblance of the nucleotide sequence surrounding the AUG start site with respect to an optimal Kozak consensus sequence (29). A Kozak consensus sequence consists of a purine (A/G) at position −3 relative to adenine of the methionine mRNA codon (AUG) and/or a guanine at position +4. An ideal Kozak consensus sequence will incorporate both of these features, although only one is required to initiate translation at an AUG codon. mRNA sequences not corresponding closely to the Kozak consensus sequence can lead to leaky scanning, in which the ribosome does not begin translation at a given potential methionine start site (29, 30).

The mRNA sequence for mammalian Kv3.3 channels has been shown to contain two putative methionine start sites at the 5′ end (9, 10, 16). It has been suggested that translation from the different start sites could explain the differences in inactivation observed between Kv3.3 expressed in *Xenopus* oocytes and HEK cells (16). Similarly, in AptKv3.3 a second methionine in the open reading frame can be found 29 amino acids downstream from the first methionine, at the end of the putative inactivation domain (Fig. 4A). If translation started from the second methionine codon, the resulting currents encoded by the truncated isoform would presumably be lacking fast NH₂-terminal-mediated inactivation, potentially accounting for the slower rate of inactivation in HEK cells. This hypothesis is strengthened by the observation that the nucleotide sequence surrounding the first methionine codon in Kv3.3 does not match the Kozak consensus sequence for translation initiation (29). To examine whether preferential translation from the first methionine codon could increase the rate of inactivation of AptKv3.3, the six nucleotides preceding the first methionine codon were replaced with the sequence (gcc gcc) to provide a closer fit to the Kozak consensus sequence (AptKv3.3Koz) (Fig. 4B). The cytosine nucleotide at position +4 was not changed to the Kozak consensus of guanine, because it would change the amino acid at this position from leucine to valine. A second cDNA without the coding region for the first 29 amino acids was also produced (AptKv3.3Δ1–29) to test the result of removing the NH₂-terminal region on whole-cell currents.

**Inactivation of the Kozak-modified Constructs**—Whole-cell recordings from AptKv3.3Koz expressed in CHO cells generated currents that inactivated with an average τ that was 40% faster at 30 mV than those generated from the unmodified Kv3.3 expression vector (Fig. 5A). Expression from the AptKv3.3Koz vector in HEK cells now consistently produced a very fast inactivating current, exhibiting a τ that was 52% faster than even AptKv3.3Koz expressed in CHO cells (Fig. 5, A and B). By comparison, expression of AptKv3.3Δ1–29 resulted in a non-inactivating current with a similar profile to the original AptKv3.3 construct expressed in HEK cells, including a prominent transient peak of current often attributed to fast activation and extracellular K⁺ accumulation (Fig. 5A). The τ of AptKv3.3Koz inactivation was also voltage-dependent in both cell lines, ranging from 130 ± 12.0 ms at 0 mV to 52.9 ± 3.7 ms at 50 mV in CHO cells compared with 52.1 ± 5.0 ms at 0 mV to 25.4 ± 2.0 ms at 50 mV in HEK cells (Fig. 5B; see also Table 1).

AptKv3.3Koz in CHO cells showed a similar V½ of inactivation and k value to that of AptKv3.3 (cf. Figs. 5D and 3C). In HEK cells both the V½ of inactivation and k value for AptKv3.3Koz were significantly altered compared with the unmodified Kv3.3 expression vector (Fig. 5E and 3F) but to final values that were not significantly different from those in CHO cells. Recovery from inactivation was significantly lengthened when AptKv3.3Koz was expressed in HEK cells over that of AptKv3.3 (Fig. 5F). However, recovery from inactivation was not significantly different between those constructs predicted to express NH₂-terminal regions (AptKv3.3 in CHO and AptKv3.3Koz in CHO and HEK cells) (Fig. 5F).

The τ for activation of AptKv3.3Koz in HEK cells was significantly slower at all voltages compared with those expressed from the unmodified Kv3.3 construct (Fig. 5C) but more similar to that of AptKv3.3Koz in CHO cells (Fig. 5C). Removal of the NH₂-terminus in AptKv3.3Δ1–29 resulted in a significant increase in the rate of activation compared with the fast inactivating channels, with an activation profile very similar to the slow inactivating AptKv3.3 expressed in HEK cells. The V½ for activation was also negative-shifted for AptKv3.3Koz channels expressed in CHO or HEK cells compared with AptKv3.3, with a shift of −4.2 mV in CHO cells and −10.6 mV in HEK cells (cf.
Fig. 3. Inactivation kinetics of AptKv3.3 differ in CHO and HEK cells. Kinetics of inactivation in CHO (A–C) and HEK (D–F) cells. A and D, representative current traces of AptKv3.3 indicating fast inactivation in CHO and slow inactivation in HEK cells (note difference in time scales). For AptKv3.3 expressed in CHO cells, currents were held at −90 mV and stepped to 50 mV in 10-mV increments for 4 s, followed by a test pulse to 50 mV for 10 ms (inset). For AptKv3.3 expressed in HEK cells, the cell was held at −90 mV and stepped to 50 mV in 10-mV increments for 10–25 s, followed by a test pulse to 50 mV for 80 ms (inset). B and E, voltage dependence of inactivation time constants (τ). AptKv3.3 current activation was fit with a single exponential function in CHO cells and by a double exponential function in HEK cells. The τ of inactivation was voltage-dependent in CHO cells, and the relationship was fit with a single exponential decay function (B), while both the short (τ_short) and long (τ_long) time constants in HEK cells were voltage-independent (E). C and F, steady-state inactivation curves for AptKv3.3. The V_1/2 and k value of inactivation were significantly different for CHO and HEK cells (p < 0.05; n = 5 CHO, n = 9 HEK). Data points were fit with Boltzmann functions. G, time course of recovery from inactivation in CHO and HEK cells. The recovery of control current was significantly longer in CHO than in HEK cells (p < 0.05; n = 5 CHO, n = 7 HEK). The recovery of control current was significantly longer in CHO than in HEK cells (p < 0.05; n = 5 CHO, n = 7 HEK). The recovery of control current was significantly longer in CHO than in HEK cells (p < 0.05; n = 5 CHO, n = 7 HEK). The τ of recovery was determined by depolarizing the cells to 50 mV from a holding potential of −90 mV until steady-state inactivation was reached. A step to −90 mV of variable duration was followed with test pulses to 50 mV for 10 ms in increments of 100 ms. Data points were fit with a single exponential function. H, outside-out patches containing two to four AptKv3.3 channels expressed in CHO or HEK cells. Shown are individual traces obtained from a holding potential of −70 mV stepped to 10–30 mV. I, comparison of inactivation rates in CHO and HEK cells in the outside-out recording configuration with a step from −90 to 50 mV revealed similar differences as found for whole-cell currents. The same traces are shown but at a smaller time scale in the inset. Traces have been normalized in current amplitude for comparison, with the initial 100 ms of recording shown in the inset.

Fig. 4. Mutation of the AptKv3.3 start sequence to a Kozak consensus sequence. A, AptKv3.3 channels contain two putative methionine start sites for translation, M1 and a second (M2) 29 amino acids downstream. Sequence alignment with AptKv3.1 indicates that M2 of AptKv3.3 is at a position corresponding to the initiation methionine of AptKv3.1, past the putative inactivation domain of AptKv3.3 (amino acids 1–29). Boxed regions indicate identical amino acid sequences between AptKv3.3 and AptKv3.1. B, the sequence surrounding the M2 site represents a better Kozak consensus sequence for translation initiation than M1, possibly leading to leaky scanning by ribosomes to initiate translation at M2. Boxed regions indicate the six nucleotides preceding the ATG start sites at M1 (K1) and M2 (K2). To test whether an improved Kozak sequence at K1 would alter the rate of inactivation of whole-cell currents, we mutated the preceding six nucleotides to those shown in the K1 box.

Fig. 5. D and E, and Fig. 1C; see also Table I). The k values associated with activation did not differ significantly between Koz-modified and unmodified currents. The V_1/2 for activation of AptKv3.31–29 was 6.9 ± 1.3 mV with a k value of 14.0 ± 1.1 mV in CHO cells, which was not significantly different from the original AptKv3.3 expressed in CHO or HEK cells. These results suggest that the presence of the inactivation particle can interact with the activation gate to slow the rise time and slightly shift the voltage dependence of activation.

In summary, the net result of mutating the Kozak consensus
sequence preceding M1 was to produce currents that inactivated more rapidly with identical voltage dependence and at a rate that could be fit with a single exponential in both CHO and HEK cells.

**DISCUSSION**

This study establishes the kinetic properties of AptKv3.3 channels when expressed in two mammalian cell lines. Although several properties of activation, deactivation, and conductance were equivalent in the two systems, we encountered key differences in the properties of inactivation. The most significant difference was a fast inactivation when expressed in CHO cells, but only slow inactivation in HEK cells, as reported previously for mammalian Kv3.3 in HEK cells (16). These differences could be due to posttranslational modifications or even translational differences that affect channel structure. The potential influence of second messengers on channel inactivation was tested by recording AptKv3.3 currents in the outside-out configuration. Since large differences in the rate of inactivation persisted under conditions expected to promote substantial washout through dialysis by the electrode, cytosolic factors could not account for the major differences in inactivation rates. A more important role was identified in the translational process with respect to successful translation of an NH$_2$-terminal domain.

Others have identified two potential methionine start sites for translation in the NH$_2$-terminal region of mammalian Kv3.3 and Kv3.4 channels (16). Inspection of AptKv3.3 revealed that expression of the first of two potential start sites lacked key Kozak consensus sequence. We found that expression of the region preceding M1 was to produce currents that inactivated more rapidly with identical voltage dependence and at a rate that could be fit with a single exponential in both CHO and HEK cells.

The increased expression of inactivating currents with AptKv3.3 was consistent with an improved translation of NH$_2$-terminal domains. The slow rate of AptKv3.3 inactivation in HEK cells may then reflect a greater number of channels.
translated without the NH₂-terminal ball and chain motif. One can envision a population of channels that could range from homomeric combinations of subunits without any NH₂-terminal domains to heteromeric combinations with varying numbers of NH₂-terminus-containing subunits. In this regard, deletion-mutation analyses in Shaker B and Kv1.4 suggest that substantial inactivation can be achieved with even one N-ball in a tetramer and that the \( V_{1/2} \) of inactivation is independent of the number of N-balls (12, 31, 32). Similarly, co-expression of the inactivating Kv3.1 channel with a lower density of the inactivating Kv3.4 channel in oocytes produced a fast inactivating channel, leading again to the suggestion that only one or a small number of NH₂-terminus-containing subunits from Kv3.4 can induce fast inactivation in the presumed heteromeric channel (16). The findings from each of these channel types would thus suggest that even if a small number of AptKv3.3 channels expressed in HEK cells were translated with an NH₂-terminal domain, one would predict a much faster rate of inactivation than what we observed under whole-cell recording conditions. In support of this, outside-out recordings revealed that AptKv3.3 channels exhibited clear inactivation only when expressed in CHO cells (Fig. 3H). It is also interesting that the slow inactivating AptKv3.3 expressed in HEK cells exhibited a negatively shifted \( V_{1/2} \) of inactivation and a voltage-independent rate of inactivation, unlike all other expressed AptKv3.3 currents. Altogether these results imply an extremely low rate of NH₂-terminal domain translation of AptKv3.3 in HEK cells and a substantially different inactivation process that could include a greater contribution of slow pore-mediated C-type inactivation.

We also found that expressing fast inactivating currents lead to a significant decrease in the rate of activation. Conversely, deleting the NH₂ terminus of AptKv3.3 removed fast inactivation and increased the rate of activation. These results differ from a state-dependent model of inactivation, where the removal of inactivation reveals the slower and true steady state (33, 34). Removal of the NH₂ terminus in mammalian Kv3.4 produces currents that have a slower rate of rise compared with full-length sequences (13), a result that is compatible with a state-dependent model of inactivation. Our results suggest a more complex model for inactivation that may incorporate a greater voltage dependence in the inactivation process. We also found that the rate of inactivation was not the sole determinant of the activation rate, as AptKv3.3Koz in HEK cells had a faster rate of activation than AptKv3.3Koz in CHO despite having a faster rate of inactivation (Fig. 5, A–C). This suggests that the presence of the NH₂-terminal domain introduces additional modulatory sites that can differentially affect the rate of activation between expression systems.

**Comparison with Mammalian Kv3 Currents**—Overall the voltage dependence and single channel conductance of AptKv3.3 was similar to mammalian Kv3.3. AptKv3.3 showed the first measurable whole-cell current in I–V plots for steps between −30 and −10 mV. In CHO and HEK cells the \( V_{1/2} \) for activation and deactivation of modified and unmodified AptKv3.3 constructs ranged between −2 and 8 mV with \( k \) values of 7–11 mV. This compares reasonably to a range for the mammalian Kv3.3 channel \( V_{1/2} \) of 7–12 mV and \( k \) values between 6 and 14 mV (9, 16, 35). Finally, our single channel conductance measured in on-cell mode fell between 32 and 38 pS, essentially identical to the 39 pS conductance reported for hKv3.3 under the same recording conditions (10).

AptKv3.3 channels differed, however, in exhibiting a much faster rate of activation and deactivation than mammalian Kv3.3 (9, 10). The rate of AptKv3.3 inactivation in CHO cells (\( r = 85 \) ms at 50 mV) was also faster than mammalian Kv3.3 (\( r = 120 \) ms at 50 mV) (9) and fell within the range reported for Kv1.4 (\( r = 43–160 \) ms) (12, 14, 32). AptKv3.3Koz inactivation in CHO and HEK cells was even faster (\( r = 25–53 \) ms at 50 mV) and thus close to the Kv3.4 channel (\( r = 10–20 \) ms at 50 mV) (13, 16, 21). These differences could relate to the temperature dependence of channel kinetics, as our recordings at room temperature (21 °C) are reasonably close to the native environment in which atheronotid electric fish live (26 °C). By comparison, all kinetic analyses of mammalian Kv3.3 channels have been carried out at room temperature. The faster kinetics observed in AptKv3.3 channels may then reflect channel activity closer to the physiological condition.

AptKv3.3 channels have been shown to play a role in spike repolarization (18, 19), but the exact contribution will depend critically on the rate of activation with respect to spike duration. In this regard, the fast rate of activation of AptKv3.3 channels should enable a significant role in spike repolarization, as shown for ELL pyramidal cells (18). The fast rate of inactivation shown here further makes this channel a possible candidate for the dendritic current proposed to promote burst output in ELL pyramidal cells through a cumulative inactivation (19, 36).

**Initiation of Translation**—Mammalian Kv3.3 and Kv3.4 channel subtypes have all been shown to have two potential methionine start sites: the first site enabling translation of the putative inactivation domain and the second corresponding to that shared with the non-inactivating Kv3.1 and 3.2 channel subtypes (16). Examination of mammalian mRNA sequences reveals that all Kv3.3 and Kv3.4 splice isoforms entirely lack a Kozak consensus sequence near the first methionine site but do have one at the second methionine site (Fig. 6). A poorly defined start site at the first methionine in AptKv3.3 is thus a ubiquitous feature among the fast inactivating Kv3 K⁺ channel subtypes.

The functional significance of this to translation of Kv3.3 channels and their inactivation rates in vivo is unknown. We
have previously recorded Kv3-like currents from ELL pyramidal cells (18). In the outside-out configuration, a single exponential fit procedure revealed an inactivation rate with a $\tau$ of $\sim 250–450$ ms at 0 mV (18), a rate of inactivation that is similar to that of Kv3.3 whole-cell currents in CHO cells at the same voltage ($\sim 250$ ms). Although we have shown that these currents do not correspond to dendrotoxin-sensitive Shaker-type K$^+$ channels or BK channels (19), we cannot entirely rule out an influence of a much lower expression level of Kv3.1 channels on the rate of inactivation. However, the observed rate of inactivation in pyramidal cells at least indicates that translation of AptKv3.3 in CHO cells, even if the Kozak sequence does not entirely match the optimal consensus site.

In summary, the present study identifies one explanation for the variability in the rate of inactivation of Kv3.3 observed in previous studies and indicates that under the appropriate conditions Kv3.3 channels are capable of fast inactivation at rates that approach those of more traditional A-type K$^+$ currents.

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