Inverse Modulation of Neuronal Kv12.1 and Kv11.1 Channels by 4-Aminopyridine and NS1643

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The three members of the ether-à-go-go-gene-like (Elk; Kv12.1-Kv12.3) family of voltage-gated K+ channels are predominantly expressed in neurons, but only little information is available on their physiological relevance. It was shown that Kv12.2 channels modulate excitability of hippocampal neurons, but no native current could be attributed to Kv12.1 and Kv12.3 subunits yet. This may appear somewhat surprising, given high expression of their mRNA transcripts in several brain areas. Native Kv12 currents may have been overlooked so far due to limited knowledge on their biophysical properties and lack of specific pharmacology. Except for Kv12.2, appropriate genetically modified mouse models have not been described; therefore, identification of Kv12-mediated currents in native cell types must rely on characterization of unique properties of the channels. We focused on recombinant human Kv12.1 to identify distinct properties of these channels. We found that Kv12.1 channels exhibited significant mode shift of activation, i.e., stabilization of the voltage sensor domain in a “relaxed” open state after prolonged channel activation. This mode shift manifested by a slowing of deactivation and, most prominently, a significant shift of voltage dependence to hyperpolarized potentials. In contrast to related Kv11.1, mode shift was not sensitive to extracellular Na+, which allowed for discrimination between these isoforms. Sensitivity of Kv12.1 and Kv11.1 to the broad-spectrum K+ antagonist 4-aminopyridine was similar. However, 4-AP strongly activated Kv12.1 channels, but it was an inhibitor of Kv11 channels. Interestingly, the agonist of Kv11 channels NS1643 also differentially modulated the activity of these channels, i.e., NS1643 activated Kv11.1, but strongly inhibited Kv12.1 channels. Thus, these closely related channels are distinguished by inverse pharmacological profiles. In summary, we identified unique biophysical and pharmacological properties of Kv12.1 channels and established straightforward experimental protocols to characterize Kv12.1-mediated currents. Identification of currents in native cell types with mode shift that are activated through 4-AP and inhibited by NS1643 can provide strong evidence for contribution of Kv12.1 to whole cell currents.

Keywords: Kv10, Kv11, Kv12, HERG, mode shift, voltage-dependent potentiation, 4-aminopyridine, NS1643
INTRODUCTION

The ether-à-go-go (Eag) superfamily of voltage-gated K⁺ channels comprises three evolutionary conserved families that share high sequence homology: Ether-à-go-go (Eag; Kv10), ether-à-go-go-related-gene (Erg; Kv11) and ether-à-go-go-gene-like (Elk; Kv12) channels (Bauer and Schwarz, 2001). The best-studied member, Kv11.1 (the human isof orm is referred to as HERG channel) mediates rapidly activating K⁺ current Ikᵣ in cardiac myocytes determining heart action potential duration (Sanguinetti et al., 1995). Accordingly, loss of Kv11.1 channel function through mutations or drug treatment causes cardiac arrhythmia and sudden death in humans (Curran et al., 1995; Sanguinetti et al., 1995; Trudeau et al., 1995). Kv11 channels also mediate important K⁺ currents in neurons of the auditory brainstem (Hardman and Forsythe, 2009), the olfactory bulb (Hirdes et al., 2009) and the midbrain (Ji et al., 2012). Kv10.1 channels regulate cell cycle progression and proliferation (Sanchez et al., 2016; Urrego et al., 2016), and are frequently overexpressed in human cancers with poor prognosis (Pardo and Sanchez et al., 2016; Urrego et al., 2016), and are frequently overexpressed in human cancers with poor prognosis (Pardo and Stuhmer, 2014). Kv10.1 channel mutations cause developmental disorders and epilepsy (Kortum et al., 2015; Simons et al., 2015).

In contrast to Kv10 and Kv11 channels, only little information on physiological relevance is available for the three members of the Kv12 family that are expressed predominantly in neurons (Engeland et al., 1998; Shi et al., 1998; Miyake et al., 1999; Trudeau et al., 1999; Saganich et al., 2001; Zou et al., 2003). Kv12.2 channels regulate excitability in pyramidal neurons of hippocampus in mice (Zhang et al., 2010), but no native current component could be attributed to Kv12.1 and Kv12.3 subunits despite expression of their mRNA transcripts in several brain areas (Shi et al., 1998; Miyake et al., 1999; Saganich et al., 2001; Zou et al., 2003). We consider that Kv12.1/Kv12.3-mediated currents in neurons were overlooked so far due to insufficient knowledge on biophysical properties and lack of specific pharmacological tools.

Recently, it was shown that Kv12.1 channels exhibit mode shift of activation (also termed pre-pulse facilitation or voltage-dependent potentiation) (Li et al., 2015; Dai and Zagotta, 2017). Mode shift denotes time-dependent stabilization of the voltage sensor domain in a “relaxed” open state after prolonged channel activation through depolarized (conditioning) membrane potentials (Bezanilla et al., 1982; Villalba-Galea et al., 2008). It manifests by slowing of deactivation and a shift of voltage dependence to hyperpolarized potentials (Li et al., 2015; Dai and Zagotta, 2017). Accordingly, when measured with routine voltage clamp protocols (e.g., holding potentials of −60 mV) human Kv12.1 channels mediate “conventional” K⁺ currents that activate similar to many other Kv channels with voltages at half-maximal activation of around −30 mV (c.f. Figure 1) (Li et al., 2015). These currents could easily go unnoticed in cell types expressing different endogenous K⁺ currents. Taking into account their mode shift, appropriate voltage protocols (e.g., depolarized holding potentials) may uncover Kv12.1-mediated currents. However, it remains to be explored whether mode shift can be detected in cells expressing various K⁺ currents and whether it may be employed to identify native Kv12.1 channels. Mode shift is not an exclusive feature of Kv12.1 channels, but has been demonstrated also for voltage-gated Na⁺ channels (Bezanilla et al., 1982), HCN (Mannikko et al., 2005; Bruening-Wright and Larsson, 2007), Shaker (Olcese et al., 1997; Tilegenova et al., 2017), and Kv11.1 (Erg1) channels (Piper et al., 2003; Tan et al., 2012; Goodchild et al., 2015). Nevertheless, it may constitute a prominent hallmark to distinguish Kv12 channels from other K⁺ current components in native tissue.

Here we describe biophysical and pharmacological properties of Kv12.1 channels and demonstrate straightforward experimental protocols that may be employed to identify Kv12 currents in neurons. We show, that these properties allowed detection of Kv12.1-mediated currents in cells expressing a variety of different K⁺ channels. Our findings may be utilized to identify physiological roles of Kv12.1 channels.

RESULTS

Voltage-Dependent Mode Shift of Human Kv12.1 Channels

Mode shift of human Kv12.1 channels was recently demonstrated (Li et al., 2015), but a detailed characterization is currently not available. In order to identify exclusive properties of human Kv12.1 channels, we thus set out with detailed biophysical analysis of these channels in an overexpression system. In CHO cells, activation of Kv12.1 channels through depolarizing voltage steps produced robust outwardly rectifying currents (Figure 1A) (Zou et al., 2003; Li et al., 2015; Dai and Zagotta, 2017). Channel deactivation at hyperpolarized potentials was best described by double exponential kinetics, and deactivation slowed down with more depolarized pre-potentials (Figures 1B,C). When we activated Kv12.1 channels at 0 mV for different time intervals, tail current amplitudes increased (Figures 1D,E) and deactivation slowed down with the duration of the pre-pulse (Figure 1F). Tail current increase and slowing of deactivation saturated at a pulse duration of about 200 ms and 400–500 ms, respectively.

We then analyzed voltage dependence of human Kv12.1 channels with voltage protocols established previously to study mode shift of related Kv11.1 (Tan et al., 2012). We applied depolarizing holding potentials (conditioning potentials; 200 ms) before a series of activating voltage steps (pulse potentials from −140 mV to +10 mV; 600 ms) (Figure 1G and Supplementary Figures 1A–C). To minimize time intervals at hyperpolarized potentials that may counteract mode shift (c.f. Villalba-Galea, 2017), we at start recorded tail currents at correspondingly depolarized potentials (Figures 1G–I). In these experiments, amplitudes of Kv12.1-mediated outward currents were similar irrespective of conditioning potentials. This indicated that comparable steady-state channel activation was reached with all voltage protocols (Supplementary Figures 1A–C). After a conditioning potential of −60 mV, voltage dependence of Kv12.1 channels also did not change relevantly upon extension of the activating steps to 4000 ms (Figures 1J,K). This additionally demonstrated that steady-state activation of Kv12.1 channels was already reached by activating pulses as short as 600 ms.
FIGURE 1 | Voltage-dependent mode shift of human Kv12.1 channels. (A) Representative patch clamp recording of a CHO cell transiently transfected with human Kv12.1 channels measured with the indicated voltage protocol. (B) Representative tail currents elicited at –120 mV after activating pulses of –60 mV (black) and +10 mV (red). Currents were normalized to maximum amplitude for visualization of deactivation kinetics (currents from recording shown in A). (C) Tail current deactivation was best described by double exponential kinetics: Tau1 and tau2 were derived from double-exponential fits to the decaying phase of tail currents (voltage protocols as in A). Deactivation slowed down with channel activation at more depolarized potentials. (D–F) When Kv12.1 channels were activated at 0 mV for different time intervals, (E) tail current amplitudes increased and (F) deactivation slowed-down with pre-pulse duration. Tail current increase and slowing of deactivation saturated at a pulse duration of about 200 ms and 400–500 ms, respectively (D shows a representative recording). (G–I) Analysis of voltage dependence of recombinant Kv12.1 channels. (G) Voltage protocols consisted of a 200 ms conditioning potential step to –120 mV (blue), –60 mV (black), 0 mV (red), or +40 mV (orange), followed by 600 ms activating pulse potentials from –140 to +10 mV (10 mV increments). In these experiments, tail currents were elicited either at –120 mV or at 0 mV (for representative recordings see Supplementary Figures 1A,C). (H) Summary of voltage dependence of human Kv12.1 channels derived from Boltzmann fits to individual recordings as shown in Supplementary Figures 1A,C (solid line represents a Boltzmann fit to averaged data). Depolarized conditioning potentials of +40 and 0 mV induced a large shift of voltage dependence to hyperpolarized potentials. (I) shows mean $V_h$ of channel activation in dependence of conditioning potentials (derived from fits shown in H). (J,K) Upon extension of the activating pulse to 4000 ms, significantly shifted voltage dependence of Kv12.1 channels to depolarized potentials after conditioning at 0 mV ($P \leq 0.01$; open red squares). For data shown in (J) eight independent recordings were averaged (each cell measured with all four voltage protocols). There was no significant difference between corresponding data in (J,K) and (H,I) (conditioning potential –60 mV, 600 ms).
For 600 ms activating pulses, half-maximal voltages of activation ($V_h$) were $-29.3 \pm 2.3$ mV and $-24.1 \pm 1.6$ mV for negative conditioning potentials of $-120$ mV or $-60$ mV, respectively ($n = 7$; 600 ms activating pulses; Figures 1H,I). When cells were held at depolarized conditioning pulses of 0 mV or $+40$ mV, $V_h$ was $-85.3 \pm 0.9$ mV and $-87.6 \pm 0.8$ mV, respectively (Figures 1H, I; 600 ms activating pulses). In these experiments, slope factors derived from Boltzmann fits to the recordings changed from experiments, slope factors derived from Boltzmann fits to the recordings changed from $-16.4 \pm 0.9$ mV (−60 mV) to $-8.6 \pm 0.1$ mV (0 mV) and $-8.9 \pm 0.6$ (+40 mV) ($n = 7$; Supplementary Figure 1D; 600 ms activating pulses). Accordingly, depolarizing conditioning potentials induced a large shift of voltage dependence by about $-60$ mV, and the full shift occurred across a potential range of 60 mV (between holding potentials of $-60$ and 0 mV). In analogous experiments, the same conditioning voltages shifted $V_h$ of related Kv11.1 channels from $-6.8 \pm 4.4$ mV (conditioning voltage $-60$ mV) to $-62.4 \pm 1.5$ mV (conditioning voltage $+40$ mV; $n = 7$; Figures 2A, B), consistent with a previous report (Tan et al., 2012).

We next tested whether mode shift was sensitive to the employed voltage protocol. Therefore, we again applied 200 ms depolarized conditioning pulses, but this time we extended the activating pulses to 4000 ms (Figures 1J, K). When in these experiments cells were held at conditioning pulses of $-60$ and 0 mV, $V_h$ was $-43.3 \pm 2.5$ mV and $-66.8 \pm 1.5$ mV, respectively (Figures 1J, K). Thus, mode shift of Kv12.1 channels was readily induced also when activating the channels for 4000 ms. However, the extent of mode shift was significantly reduced in these experiments compared to experiments with 600 ms activating pulses (Figure 1K; $P \geq 0.01$), i.e., increased time intervals at hyperpolarized holding potentials during these protocols presumably counteracted development of mode shift. Similarly, the extent of mode shift was reduced when we activated channels for 600 ms after conditioning pulses of $-60$ and 0 mV, but recorded tail currents at hyperpolarized holding potentials ($-120$ mV; Supplementary Figures 1E, F), i.e., we introduced additional hyperpolarizing potentials after every activating pulse. Thus, also in these experiments hyperpolarizing holding potentials between the conditioning pulses reduced the expression of mode shift.

Taken together, human Kv12.1 channels exhibited significant voltage-dependent mode shift that in response to depolarized holding potentials manifested by slowed channel deactivation and by a large hyperpolarizing shift of voltage dependence. This mode shift of Kv12.1 channels can be induced robustly employing different voltage protocols, but the extent of mode shift significantly varies with duration of hyperpolarized holding potentials.

In Contrast to Kv11.1, Kv12.1 Channels Are Insensitive to Extracellular Na$^+$

Inhibition of Kv11 channels by extracellular Na$^+$ is well established (Numaguchi et al., 2000; Sturm et al., 2005) and a hallmark used to identify Kv11-mediated currents in neurons (e.g., Hardman and Forsythe, 2009). In control experiments, replacement of extracellular Na$^+$ with NMDG without altering extracellular K$^+$ concentration slightly increased Kv11.1-mediated outward currents (Figure 2A), as previously reported (Numaguchi et al., 2000). Upon removal of extracellular Na$^+$, activation voltage range of Kv11.1 channels conditioned at $-60$ and $+40$ mV shifted to hyperpolarized potentials by $-24.4 \pm 1.5$ mV and $-13.9 \pm 0.7$ mV, respectively ($n = 7$; Figures 2B, C). Accordingly, overall mode shift of voltage dependence was attenuated from $-55.7 \pm 4.4$ mV under control conditions to $-45.1 \pm 3.5$ mV in absence of extracellular Na$^+$ ($n = 7$; $P \leq 0.05$). We then tested whether related Kv12.1 channels also exhibited such Na$^+$ sensitivity. We found that neither current amplitudes nor voltage dependence of Kv12.1 channels were affected by removal of extracellular Na$^+$ (Figures 2D–F). Consequently, the extent of mode shift was also insensitive to changes of the Na$^+$ concentration. To conclude, despite the high similarity in mode shift behavior, the absence of Na$^+$ sensitivity in Kv12.1 channels distinguishes Kv11.1-mediated currents from Kv12.1.

Kv12.1 Channels Are Not Sensitive to Kv Channel Blockers E-4031, XE991, and TEA

We then evaluated whether Kv12.1 channels were sensitive to channel inhibitors that are widely used to attribute neuronal K$^+$ currents to particular channel families. At concentrations generally applied to inhibit established target channels, human Kv12.1 channels were insensitive to both E-4031 (20 µM), a specific inhibitor of Kv11 channels (Supplementary Figures 2A, B) (Trudeau et al., 1995), and XE991 (10 µM), a specific antagonist of neuronal Kv7 (KCNQ) channels (Supplementary Figures 2D, E) (Wang et al., 1998). Kv12.1-mediated currents were also insensitive to E-4031 and XE991 concentrations up to 100 µM (Supplementary Figures 2C, F). Kv12.1 channels also were not affected by the broad-spectrum K$^+$ channel inhibitor tetraethylammonium (TEA) at a concentration that completely inhibited Kv7.2 channels (5 mM; Supplementary Figures 2G, H) (Hadley et al., 2000). However, Kv12.1 channels were slightly inhibited by 50 and 100 mM TEA ($I_{50\text{mMTEA}}/I_{\text{Start}} = 0.90 \pm 0.01$; $I_{100\text{mMTEA}}/I_{\text{Start}} = 0.83 \pm 0.01$; $n = 9$; Supplementary Figure 2I). Of note, low sensitivity of Kv12.1 channels to E-4031 and TEA has been shown in an early report (Shi et al., 1998). In summary, E-4031, XE991 and TEA cannot be used to inhibit Kv12.1 channels in native tissue, but may be used to isolate Kv12.1 channel activity through inhibition of other K$^+$ channels.

The Broad-Spectrum K$^+$ Channel Antagonist 4-Aminopyridine (4-AP) Activates Kv12.1

We then tested whether human Kv12.1 channels were sensitive to 4-AP, another established antagonist of several Kv families (Gutman et al., 2005). Surprisingly, 4-AP at a concentration well in the range often used to isolate neuronal K$^+$ currents (3 mM; e.g., Marcotti et al., 2003) increased Kv12.1-mediated steady-state and tail currents within seconds (Figures 3A–C). Current potentiation was the same for conditioning pulses of $-60$ and
Under these experimental conditions, 4-AP potentiated K$_{11.1}$ currents in a concentration-dependent manner with an EC$_{50}$ of about 2.1 and a Hill coefficient of about 0.8 (Figure 3F, red trace). In contrast, K$_{11.1}$ channels were inhibited by 4-AP with an IC$_{50}$ of approximately 2.6 mM and a Hill coefficient of about 0.7 (Supplementary Figure 3), consistent with previous reports (e.g., Ridley et al., 2003). Thus, despite inverse effects of 4-AP on K$_{11.1}$ and K$_{12.1}$, the sensitivity of both channels to 4-AP was quite similar. When we applied 5 mM or 10 mM 4-AP without adjusting pH, we found that these concentrations activated K$_{12.1}$ channel even stronger than at physiological pH (Figures 3E,G). Without adjusting pH at higher concentrations, the EC$_{50}$ was about 3.1 and the Hill coefficient was 0.9 (Figure 3F, black trace). As in line with a previous study (Kazmierczak et al., 2013) increasing pH of the extracellular solution from 7.4 to 8.1 or 9.0 without addition of 4-AP did not potentiate K$_{12.1}$-mediated steady-state currents (Figure 3G), these data suggested that 4-AP activated K$_{12.1}$ channels more efficiently at more alkaline pH. Indeed, increasing pH of the extracellular solution containing 3 mM 4-AP to 8.1 further increased K$_{12.1}$-mediated steady-state outward currents (Figure 3H).

**K$_{12.1}$ Channels Are Also Activated by Isomeric Aminopyridines**

Several other aminopyridines have been shown to inhibit voltage-gated K$^+$ channels, albeit antagonistic efficiency of these substances was lower than that of 4-AP (Robertson et al., 2003).
FIGURE 3 | 4-AP potentiates currents through human Kv12.1 channels. (A,B) Representative recordings from a CHO cell transiently transfected with Kv12.1 before (left) and after extracellular application of 3 mM 4-AP (right) after conditioning pulses of (A) –60 mV and (B) 0 mV. (C) 4-AP-dependent activation of Kv12.1-mediated steady-state outward currents was independent on employed voltage protocols (summary of recordings as presented in A,B). 4-AP potentiated currents at all holding potentials positive to –60 mV. (D) 4-AP-dependent potentiation of Kv12.1 currents was reversible, as current amplitudes returned to control levels within 2–3 min after removal of the substance [shown are representative recordings under control conditions (gray), after application of 3 mM 4-AP (green) and at different time points (30, 90, and 150 s) after washout of 4-AP]. (E) 3 mM 4-AP shifted the voltage dependence of human Kv12.1 channels to hyperpolarized potentials (open squares) [the panel shows Boltzmann fits to averaged data; data obtained from recordings as shown in (A,B)]. (F) 4-AP activates Kv12.1 channels in a dose-dependent manner. When pH of the extracellular solution was adjusted to 7.4 at higher 4-AP concentrations, the EC50 was about 2.1 mM and the Hill coefficient was about 0.8 (red trace). When pH of the solutions was not adjusted, the EC50 was about 3.1 and Hill coefficient was 0.9 (black trace). Parameters were derived from fits of averaged data to a Hill equation described in Section “Materials and Methods” (solid line represents fit; note that addition of 4-AP to 5 and 10 mM 4-AP increased the pH of the solution to 8.1 and 9.0, respectively). (G) Increasing the pH of the extracellular solution from 7.4 to 8.1 or 9.0 without addition of 4-AP did not increase Kv12.1-mediated steady-state currents. In contrast, application of 5 mM or 10 mM 4-AP in extracellular solution without adjusting pH activated Kv12.1 channels even more strongly than when pH of the solution was adjusted to pH 7.4. (H) Application of 3 mM 4-AP that does not change pH of the extracellular solution strongly potentiated Kv12.1-mediated currents. Current potentiation through 3 mM 4-AP was further increased when the pH of the extracellular solution was increased to pH 8.1.

and Nelson, 1994; Sedehizadeh et al., 2012; Strupp et al., 2017). We thus wondered whether we could identify isomeric aminopyridines that activated Kv12.1, at best without affecting other K+ channels. We found that 2-aminopyridine (2-AP) and 3-aminopyridine (3-AP) potentiated Kv12.1-mediated currents at a concentration close to the EC50 of 4-AP (3 mM) by about 20% \((P \leq 0.001)\) and 7% \((P \leq 0.001)\), respectively (Figure 4). At the same concentration, 4-diaminopyridine (4-DAP; 3 mM) was ineffective. Hence, 2-AP \((P \leq 0.01)\) and 3-AP \((P \leq 0.001)\) activated Kv12.1 channels significantly less than 4-AP mirroring efficacy of inhibition of other K+ channels by these substances (Robertson and Nelson, 1994; Sedehizadeh et al., 2012; Strupp et al., 2017).

**NS1643, an Activator of Kv11 Channels, Inhibits Kv12.1 Channels**

We then turned to NS1643, a partial agonist of the Kv11 channel family (Casis et al., 2006). As shown earlier (c.f. Hansen et al., 2006), NS1643 (30 µM) slowed channel deactivation and
potentiated K<sub>11.1</sub>-mediated outward currents (Figures 5A–C). In contrast, the same concentration of NS1643 (30 µM) completely inhibited K<sub>12.1</sub> channels with a time constant of 16.8 ± 2.6 s (n = 7; Figures 5A–C). When we applied only 10 µM NS1643, a concentration close to the reported EC<sub>50</sub> of NS1643 for activation of K<sub>11.1</sub> channels (Casis et al., 2006), K<sub>12.1</sub>-mediated currents were reduced to 37.1 ± 4.1% of initial current amplitudes. Inhibition of currents by 10 µM was much slower than when 30 µM NS1643 was applied (n = 6; compare Figure 5B and Supplementary Figure 4). We then analyzed voltage dependence of residual K<sub>12.1</sub> currents in the presence of 10 µM NS1643 (Figures 5D–F): Application of NS1643 (10 µM) shifted V<sub>1/2</sub> by +8.8 ± 2.9 mV (n = 6; P ≤ 0.05) and by +21.7 ± 5.2 mV (n = 6; P ≤ 0.01) after condition voltage pulses of −60 and 0 mV, respectively (Figures 5D–F). At the same time, slope factors changed by +8.0 ± 1.5 mV (conditioning pulse of −60 mV; n = 6; P ≤ 0.05) and −1.8 ± 0.9 mV (conditioning pulse 0 mV; n = 6; P ≤ 0.01). Furthermore, NS1643 (10 µM) significantly accelerated deactivation of K<sub>12.1</sub> channels at hyperpolarized potentials (Figures 5G,H; P ≤ 0.05; n = 6).

### Identification of K<sub>12.1</sub> Currents in Cells Expressing Different K<sup>+</sup> Currents

Our results suggested that voltage-clamp protocols designed to detect mode shift in combination with pharmacology using 4-AP or NS1643 should provide a robust approach for isolation of K<sub>12.1</sub>-mediated currents in native cell types. As proof of principle, we sought to isolate K<sub>12.1</sub> channel activity in cells expressing different K<sup>+</sup> channels (Figure 6). To this end, we co-expressed K<sub>12.1</sub> channels together with K<sub>7.2</sub> and typical neuronal K<sup>+</sup> channels (K<sub>7.3</sub> and Kir2.1). In these experiments, CHO cells were transiently transfected with equal amounts of plasmid DNA encoding the channel subunits (see section “Materials and Methods” for details). We first analyzed whether we could isolate Kir2.1- and K<sub>7</sub>-mediated currents in those cells. In all cells tested, we found large inward currents at hyperpolarized potentials and XE991-sensitive outward currents at depolarized potentials demonstrating expression of functional Kir2.1 and K<sub>7</sub> channels, respectively (Supplementary Figure 5). As measure for abundance of K<sub>11.1</sub> and K<sub>12.1</sub> channels, we then tested whether we could detect mode shift of voltage dependence in the mix of K<sup>+</sup> currents. As determined from whole cell currents, V<sub>1/2</sub> = −6.7 ± 1.3 mV and −80.0 ± 2.1 mV after conditioning potentials of −60 and 0 mV, respectively (n = 7; Figures 6A–C). Thus, mode shift of channels expressed in these cells (K<sub>11.1</sub> and K<sub>12.1</sub> channels) was readily detectable even among a complement of different voltage-dependent K<sup>+</sup> channels. We next attempted to isolate K<sub>12.1</sub>-mediated currents among the mixture of K<sup>+</sup> channel by making use of the pharmacological profile established above. 4-AP (3 mM) shifted the voltage dependence of whole cell currents by −8.5 ± 0.9 mV (n = 7; P ≤ 0.001) and by −12.3 ± 2.3 mV (P < 0.05) to hyperpolarized potentials after conditioning voltages of −60 and 0 mV, respectively (Figures 6B,C). Thus, although being less pronounced than for K<sub>12.1</sub> channels alone (Figure 6C), the 4-AP-induced shift of voltage dependence was readily detectable in the mixed K<sup>+</sup> current situation. At the same time, 4-AP (3 mM) potentiated whole cell currents elicited at −60 and 0 mV to about 210% and 140% of baseline amplitudes, respectively (Figures 6A,D–F). Finally, we applied 30 µM NS1643 (on top of 4-AP) to the same cells and found that the substance inhibited outward currents and inward tail currents (Figures 6A,D,E). NS1643 also slowed deactivation kinetics of remaining currents (c.f. Figure 6A) indicating that NS1643-sensitive and functional K<sub>11.1</sub> channels were expressed in these cells.

Taken together, using abovementioned experimental protocols we could unequivocally identify properties of K<sub>11.1</sub> channels (mode shift, 4-AP and NS1643 sensitivity) among a mixture of K<sup>+</sup> currents. These results therefore suggest that the same protocols may be used to assign K<sup>+</sup> current components to K<sub>12.1</sub> channels in complex (native) cellular/neuronal settings.
DISCUSSION

The three members of the ether-à-go-go-like channel family of voltage-gated $K^+$ channels ($K_{v}12.1-K_{v}12.3$) are predominantly expressed in neurons, as shown by mRNA transcripts in several brain areas including cerebral cortex and hippocampus (Miyake et al., 1999; Trudeau et al., 1999; Saganich et al., 2001; Zou et al., 2003), as well as in sympathetic ganglia (Shi et al., 1998). However, neuronal current components and their physiological relevance have been resolved for $K_{v}12.2$ subunits exclusively: as demonstrated by application of a selective inhibitor, $K_{v}12.2$ activity controls resting membrane potential and spontaneous firing of pyramidal neurons in hippocampus (Zhang et al., 2010). Also, in $K_{v}12.2$ knock-out mice it was shown that $K_{v}12.2$ channels regulate hippocampal excitability (Zhang et al., 2010) and may be important for processing of spatial working memory (Miyake et al., 2009). However, it is not known whether $K_{v}12$ channels mediate relevant currents in any other physiological system.
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Mode shift of activation was recently demonstrated for K\textsubscript{v}12.1 channels in Xenopus oocytes (Li et al., 2015; Dai and Zagotta, 2017). However, mode shift of these channels has also been observed in mammalian cells in vitro. The 4-AP-induced shift of voltage dependence was smaller than in cells expressing only K\textsubscript{v}12.1 channels. Data were derived from experiments also shown in Figure 3E. (D) Representative current traces recorded in cells expressing a mix of K\textsubscript{v}12.1 alone (black) and the mix of K\textsubscript{v}12.1 and K\textsubscript{v}11.1 channels together with a mix of neuronal K\textsuperscript{+} currents. Data were derived from recordings as presented in (D), and currents elicited at –60 mV and at 0 mV were analyzed.

Here, we focused on recombinant human K\textsubscript{v}12.1 to identify distinct biophysical and pharmacological properties allowing for attribution of native currents to these channels.

**Human K\textsubscript{v}12.1 Channels Exhibit Mode Shift of Activation**

Mode shift of activation was recently demonstrated for K\textsubscript{v}12.1 channel isoforms of humans and zebrafish heterologously expressed in Xenopus laevis oocytes (Li et al., 2015; Dai and Zagotta, 2017). However, mode shift of these channels has not been characterized in mammalian cells in detail yet. We found that mode shift of human K\textsubscript{v}12.1 was readily induced by depolarized holding potentials between −60 and 0 mV and that it manifested by slowed channel deactivation and a striking shift of voltage dependence to hyperpolarized potentials. Deceleration of deactivation is generally considered a biophysical hallmark of mode shift, as channels undergo additional (time-consuming) transitions from this “relaxed” (metastable) open state into deactivation (Bezanilla et al., 1982; Villalba-Galea et al., 2008; Corbin-Leftwich et al., 2016; Villalba-Galea, 2017). Similar to K\textsubscript{v}11.1 (Tan et al., 2012) and zebrafish K\textsubscript{v}12.1 (Dai and Zagotta, 2017), mode shift of human K\textsubscript{v}12.1 channels manifested by a striking −60 mV shift of voltage dependence. Thus, mode shift-dependent hyperpolarizing shifts of voltage dependence were qualitatively and quantitatively comparable for these three ion channels. Of note, mode shift of K\textsubscript{v}12.1 channels was readily induced using different voltage protocols, albeit the extent of the hyperpolarizing shift of activation potentials varied considerably with duration of hyperpolarized voltage steps. This demonstrated high sensitivity of K\textsubscript{v}12.1 channels to the holding potential, but also highlighted that voltage dependence of native K\textsubscript{v}12.1 currents might also vary with the used voltage protocols. In contrast to zebrafish K\textsubscript{v}12.1 (Dai and Zagotta, 2017), activation of human K\textsubscript{v}12.1 channels did not exhibit prominent double exponential kinetics that might indicate transition into a more stable open conformation. However, human K\textsubscript{v}11.1 channels that...
beyond doubt exhibit mode shift do not display such kinetics neither (Tan et al., 2012; Goodchild et al., 2015). This indicates that either kinetics were masked by channel inactivation, or transition was too fast in the human channel isoforms.

Time course of transition into the relaxed state varies considerably between channels requiring depolarization for minutes in Na+, (Bezanilla et al., 1982), seconds in Shaker (Olcese et al., 1997) and some hundreds of milliseconds in K\textsubscript{v11.1} channels (Piper et al., 2003). For human K\textsubscript{v12.1}, we found that also some hundreds of milliseconds of conditioning depolarization was sufficient for significant alterations of voltage dependence and kinetics. Thus, time course of development of K\textsubscript{v12.1} mode shift was well in the range of that published for K\textsubscript{v11.1} channels (Piper et al., 2003). As K\textsubscript{v12.1} channels are highly sensitive to changes of the holding potential, voltage dependence of the channels strongly depends on the employed voltage protocols. In fact, such protocol differences could account for considerable variations in voltage dependence of K\textsubscript{v12.1} channels reported in different studies (V\textsubscript{h} close to 0 mV in Shi et al., 1998; V\textsubscript{h} of about −60 mV in Zou et al., 2003).

K\textsubscript{v12.1} Channels Are Activated by 4-AP, an Established K\textsubscript{v} Channel Inhibitor

4-AP is a rather selective blocker of voltage-gated K\textsuperscript{+} channels: At micromolar concentrations, it reversibly inhibits activity of K\textsubscript{v1} and K\textsubscript{v3} family members, but at higher concentrations (in the millimolar range) 4-AP also blocks other K\textsubscript{v} channels (e.g., K\textsubscript{v11}) (Gutman et al., 2005; Alexander et al., 2015). Thus, it was somewhat surprising to find that 4-AP activates K\textsubscript{v12.1} channels. As an early study mentioned potentiation of K\textsubscript{v12.3} through 4-AP without, however, showing any recordings (named rELK1; Engelstad et al., 1998), such 4-AP sensitivity may constitute a general feature of the K\textsubscript{v12} family.

Earlier, it was shown that currents through K\textsubscript{v12.1} channels that are normally inhibited by 4-AP were potentiated by the substance, but only when K\textsubscript{v6.4} subunits were co-expressed (Stas et al., 2015). As K\textsubscript{v2.1} and K\textsubscript{v6.4} co-assemble into functional channels (reviewed in Bocksteins, 2016), this suggested that K\textsubscript{v6.4} largely determined altered 4-AP sensitivity of the resulting heteromeric channels. 4-AP suppressed closed state inactivation of K\textsubscript{v2.1}/K\textsubscript{v6.4} resulting in exclusive current potentiation of currents through those heteromers (Stas et al., 2015). In contrast, K\textsubscript{v12.1} channels did not inactivate in our experiments, and thus 4-AP probably does not potentiate K\textsubscript{v12.1}-mediated currents through a similar mechanism. As 4-AP, in contrast to K\textsubscript{v2.1}/K\textsubscript{v6.4}, heteromers, also modulated voltage dependence of K\textsubscript{v12.1}, actions of 4-AP are probably even more complex for these channels. Interestingly, a recent study demonstrated 4-AP-dependent activation of K\textsubscript{v7.4} channels (Khammy et al., 2017) indicating that 4-AP-dependent activation of voltage-gated K\textsuperscript{+} channels may constitute a more frequent phenomenon than expected. However, further work is needed to elucidate whether other members of the K\textsubscript{v} channel superfamily also exhibit this special 4-AP sensitivity.

Yet, we do not know whether 4-AP directly activates K\textsubscript{v12.1} channels or whether an auxiliary subunit endogenously expressed in CHO cells confers 4-AP activation to K\textsubscript{v12.1}. Unfortunately, at present nothing is known about physiologically relevant interaction partners of K\textsubscript{v12.1} channels. As K\textsubscript{v} channels (with K\textsubscript{v2.1} as exception) normally do not form functional channels with members of other K\textsubscript{v} families, heteromization of K\textsubscript{v12.1} channels with another pore forming or subunit apart from K\textsubscript{v12.2} or K\textsubscript{v12.3} is quite unlikely (Zou et al., 2003). Accordingly, a mechanism as shown for K\textsubscript{v2.1} is rather implausible, and it is hard to imagine how a non-pore-forming auxiliary subunit could reverse 4-AP sensitivity from inhibition to activation. Furthermore, any endogenously expressed auxiliary subunit would need to be expressed at high abundance to saturate overexpressed K\textsubscript{v12.1} channels. Hence, a straightforward model proposes direct activation of K\textsubscript{v12.1} channels through 4-AP. However, we want to point-out that we cannot exclude indirect actions on the channels at the moment.

**NS1643, a “Specific” Activator of K\textsubscript{v11} Channels, Inhibits K\textsubscript{v12.1}**

NS1643 is a well-characterized partial agonist of K\textsubscript{v11} channels that slows deactivation, increases tail-current amplitude, and shifts voltage dependence of activation to hyperpolarized potentials and voltage dependence of C-type inactivation to depolarized potentials (Casis et al., 2006; Hansen et al., 2006). At the same time, NS1643 exhibits weak antagonistic effects on K\textsubscript{v11} channels as evident by an attenuation of drug-induced current increase at higher concentrations (Casis et al., 2006; Schuster et al., 2011). K\textsubscript{v11.3} channels are even inhibited by higher concentrations of NS1643. For K\textsubscript{v12.1} channels, NS1643 accelerated deactivation, inhibited outward and tail currents and shifted voltage dependence of activation to depolarized potentials (c.f. Figure 5). Thus, NS1643 affected K\textsubscript{v11} and K\textsubscript{v12.1} channels exactly in opposite directions. Similar to K\textsubscript{v11.3} channels, NS1643 increased slope factor of voltage dependence of K\textsubscript{v12.1} channels, even though already at lower concentrations. This suggests that NS1643 similarly affects K\textsubscript{v11} and K\textsubscript{v12.1} channels, but antagonistic effects might dominate over activation for K\textsubscript{v12.1}. In line, the concentration range of NS1643 effects was similar for these channel isoforms (Casis et al., 2006). However, so far we cannot tell whether NS1643 also exhibits weak agonistic effects on K\textsubscript{v12.1} channels or whether NS1643 binds to homologous regions in K\textsubscript{v11} and K\textsubscript{v12.1} channels.

**Conclusion and Outlook**

As pharmacological tools and appropriate mouse models are currently missing, identification of native K\textsubscript{v12.1}-mediated currents critically depends on identification of unique biophysical and pharmacological properties. Indeed, native currents were successfully attributed to related K\textsubscript{v10} and K\textsubscript{v11} channels by exploiting their unique activation kinetics (c.f. Cole-Moore shift; e.g., Meyer and Heinemann, 1998) or exclusive Na\textsuperscript{+} sensitivity and pharmacology (Hirdes et al., 2005, 2009; Hardman and Forsythe, 2009), respectively. Here, we present distinctive pharmacological properties, and straightforward experimental protocols that may be employed to isolate K\textsubscript{v12.1} channel activity in native tissue. As mode shift readily manifested in
cells expressing various neuronal K\(^+\) currents, this experimental protocol may be used to demonstrate expression of channels with mode shift in native cell types. In neurons expressing various K\(^+\) current components, such changes of voltage dependence may be easier to detect than associated changes of deactivation kinetics. Such experiments may not provide definite proof for K\(_{12}\) channel expression. However, expression of mode shift may be employed together with expression analyses, Na\(^+\) sensitivity, activation through 4-AP and inhibition by NS1643 to narrow down (or exclude) contribution of K\(_{12}\) channels to whole cell currents. Identification of the combination of these properties would provide strong evidence for expression and thus potential physiological relevance of K\(_{12}\) channels.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**

Chinese hamster ovary (CHO) dhFR\(^-\) cells were maintained as previously reported (Leitner et al., 2016). Cells were kept in MEM Alpha Medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (pen/strep) (all Invitrogen GmbH, Darmstadt, Germany) in a humidified atmosphere at 5% CO\(_2\) and 37°C. Transient transfection of cells was done with jetPEI transfection reagent (Polyplus Transfection, Illkirch, France). All experiments were performed 24–48 h after transfection at room temperature (22–25°C). The expression vectors used were: K\(_{\text{v}}\)7.2-pBK-CMV (gene: human KCNQ2; UniProt accession number: O43526), K\(_{\text{v}}\)7.3-pBK-CMV (human KCNQ3; O43525), K\(_{\text{v}}\)7.4-pBK-CMV (human KCNQ4; P56696) K\(_{\text{v}}\)11.1 (Erg1)-pcDNA3.1 (rat KCNH2; O08962), K\(_{\text{v}}\)12.1(Elk1)-pcDNA3.1-IRESeGFP (human KCNH8; Q96L42), Kir2.1-pBK-CMV (human KCNJ2; P63252), and pEGFP-C1 (Addgene, Teddington, United Kingdom). For recordings presented in Figure 6 and Supplementary Figure 5, CHO cells were transiently transfected with identical amounts of plasmids encoding K\(_{\text{v}}\)7.2, K\(_{\text{v}}\)7.3, K\(_{\text{v}}\)11.1, K\(_{\text{v}}\)12.1, and Kir2.1 (0.6 µg of plasmid DNA for every subunit).

**Electrophysiological Recordings**

Electrophysiological recordings were performed in the whole cell configuration with an Axopatch 200B amplifier (Molecular Devices, Union City, CA, United States) or an HEKA EPC10 USB patch clamp amplifier HEKA (Elektronik, Lambrecht, Germany) in voltage-clamp mode (Leitner et al., 2011). All recordings were low-pass filtered at 2 kHz and sampled at 5 kHz. Currents were elicited by voltage protocols indicated in the figures. Dashed lines in representative recordings highlight zero current. Borosilicate glass patch pipettes (Sutter Instrument Company, Novato, CA, United States) were used with an open pipette resistance of 2–3 MΩ after back-filling with intracellular solution. Liquid junction potentials were not compensated (approximately −4 mV). Series resistance (R\(_s\)) typically was below 6 MΩ and compensated throughout the recordings (80–90%). Whole cell currents are normalized to the cell capacitance (current density; pA/pF) or as normalized to baseline current amplitude (I/I\(_{\text{Start}}\)). Extracellular solutions for most experiments contained (mM) 144 NaCl, 5.8 KCl, 1.3 CaCl\(_2\), 0.9 MgCl\(_2\), 0.7 NaH\(_2\)PO\(_4\), 10 HEPES and 5.6 D-glucose, pH 7.4 (with NaOH), 305–310 mOsm/kg. In some experiments, NaCl in the extracellular solution was substituted by N-methyl-D-glucamine (NMDG; Sigma–Aldrich) (c.f. Figure 2).

The standard intracellular solution contained (mM) 135 KCl, 2.41 CaCl\(_2\) (100 nM free Ca\(^{2+}\)), 3.5 MgCl\(_2\), 5 HEPES, 5 EGTA, 2.5 Na\(_2\)ATP, pH 7.3 (with KOH), 290–295 mOsm/kg (Wilke et al., 2014).

**Substances**

Tetraethylammonium (TEA, Sigma), NMDG (Sigma), 4-aminopyridine (≥99%; Sigma and Tocris Bioscience, Bristol, United Kingdom), 2-aminopyridine (2-AP; Sigma), 3-aminopyridine (3-AP; Sigma), 3,4-diaminopyridine (3,4-DAP; Sigma), 1,3-Bis-(2-hydroxy-5-trifuoromethyl-phenyl)-urea (NS 1643, Tocris), 10,10-bis-(4-Pyridinylmethyl)-9(10H)-anthracene dihydrochloride (XE991, Tocris), and N-[4-[1-[2-(6-Methyl-2-pyridinyl)ethyl]-4-piperidinyl][carbonyl]phenyl] methanesulphonamide dihydrochloride (E-4031, Tocris) were diluted in extracellular solution to concentrations indicated in “Results.” All substances were applied locally via a glass capillary through a custom-made application system.

**Note on 4-AP Solutions**

4-AP did not significantly change the pH of the extracellular solution at concentrations below 3 mM. After dilution of the substance, pH of the extracellular solution containing 5 mM or 10 mM 4-AP typically was about 8.1 or 9.0, respectively. As indicated in “Results,” in some experiments the pH of solutions containing 5 and 10 mM 4-AP was adjusted to 7.4 after dilution of 4-AP. At the concentration applied in the present study (3 mM), isomeric aminopyridines did not change pH of the extracellular solution (c.f. Figure 4).

**Data Analysis**

Patch clamp recordings were analyzed with PatchMaster (HEKA) and IgorPro (WaveMetrics, Lake Oswego, OR, United States). Voltage dependence of activation was derived from tail current amplitudes using voltage protocols indicated: Tail currents were fitted with a two-state Boltzmann function with I = I\(_{\text{min}}\) + (I\(_{\text{max}}\) − I\(_{\text{min}}\))/(1 + exp((V − V\(_h\))/s)), where I is current, V is the membrane voltage, V\(_h\) is the voltage at half maximal activation, and s describes the steepness of the curve. Results are shown as conductance-voltage curves, obtained from fits to data of individual experiments. Time constants of deactivation were derived from double-exponential fits to deactivating current components at indicated potentials. For dose-response relations, current potentiation at 0 mV (normalized to baseline) was fitted to a Hill equation with I/I\(_{\text{max}}\) = I\(_{\text{h}}\) + (I\(_{\text{max}}\)−I\(_{\text{h}}\))/(1 + (EC\(_{50}\)/[S])\(^n\)), where I is the (normalized) current, I\(_{\text{h}}\) and I\(_{\text{max}}\) denote minimal and maximal currents at low and high drug concentrations, EC\(_{50}\) is the concentration at the half maximal effect, [S] is the drug concentration and n\(_H\) is the Hill coefficient (Leitner et al., 2012).
Statistical Analysis
Isolated cells under investigation were randomly assigned to different treatment groups. Data recordings and analysis for experiments presented were not performed in a blinded manner. For some experiments, single recordings were normalized to base line values individually to account for baseline variations between cells. Statistical analysis was performed using two-tailed Student's t-test/Wilcoxon–Mann–Whitney test, and when appropriate comparisons between multiple groups were performed with ANOVA followed by Dunnett test. Significance was assigned at \( P < 0.05 \), \( * P < 0.01 \), \( ** P < 0.001 \). Data subjected to statistical analysis have \( n \) over 5 per group and data are presented as mean ± SEM. In electrophysiological experiments, \( n \) represents the number of individual cells and accordingly the number of independent experiments (no pseudo-replication).

AUTHOR CONTRIBUTIONS
MD, SE, BW, and ML planned and performed the experiments and analyzed the data. ML conceived study, designed the experiments, and wrote the paper together with MD. All authors revised and approved the final version of the manuscript. All experiments were conducted at the Institute of Physiology and Pathophysiology at the Philipps-University Marburg (Germany).

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SUPPLEMENTARY MATERIAL
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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