The Extracellular K\(^+\) Concentration Dependence of Outward Currents through Kir2.1 Channels Is Regulated by Extracellular Na\(^+\) and Ca\(^{2+}\)\(^*\)

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It has been known for more than three decades that outward Kir currents (I\(_{K1}\)) increase with increasing extracellular K\(^+\) concentration ([K\(^+\)]\(_{o}\)). Although this increase in I\(_{K1}\) can have significant impacts under pathophysiological cardiac conditions, where [K\(^+\)]\(_{o}\) can be as high as 18 mM and thus predispose the heart to re-entrant ventricular arrhythmias, the underlying mechanism has remained unclear. Here, we show that the steep [K\(^+\)]\(_{o}\)-dependence of Kir2.1-mediated outward I\(_{K1}\) was due to [K\(^+\)]\(_{o}\)-dependent inhibition of outward I\(_{K1}\) by extracellular Na\(^+\) and Ca\(^{2+}\). This could be accounted for by Na\(^+\)/Ca\(^{2+}\)-inhibition of I\(_{K1}\) through screening of local negative surface charges. Consistent with this, extracellular Na\(^+\) and Ca\(^{2+}\) reduced the outward single-channel current and did not increase open-state noise or decrease the mean open time. In addition, neutralizing negative surface charges with a carboxylate esterifying agent inhibited outward I\(_{K1}\) in a similar [K\(^+\)]\(_{o}\)-dependent manner as Na\(^+\)/Ca\(^{2+}\). Site-directed mutagenesis studies identified Asp114 and Glu153 as the source of surface charges. Reducing K\(^+\) activation and surface electrostatic effects in an R148Y mutant mimicked the action of extracellular Na\(^+\) and Ca\(^{2+}\), suggesting that in addition to exerting a surface electrostatic effect, Na\(^+\) and Ca\(^{2+}\) might inhibit outward I\(_{K1}\) by inhibiting K\(^+\) activation. This study identified interactions of K\(^+\) with Na\(^+\) and Ca\(^{2+}\) that are important for the [K\(^+\)]\(_{o}\)-dependence of Kir2.1-mediated outward I\(_{K1}\).

Inward rectifier K\(^+\) channels (Kir)\(^3\) channels are important in maintaining stable resting membrane potentials, controlling excitability, and shaping the initial depolarization, as well as the final repolarization of action potentials in many cell types, including heart cells (1–6). The physiological functions of Kir channels are closely related to their unique inward rectification mechanism, which allows inward currents to pass through the channel more easily than outward currents (6). Although small, the outward I\(_{K1}\) plays a crucial role in controlling membrane excitability and action potential duration. The gain or loss of outward I\(_{K1}\) in the heart can lead to re-entry or arrhythmia, respectively (7). In contrast to normal conditions, in which the extracellular K\(^+\) concentration ([K\(^+\)]\(_{o}\)) is ~5 mM, under pathophysiological cardiac conditions, such as ischemia, tachycardia, and fibrillation, [K\(^+\)]\(_{o}\) can be as high as 18 mM (8, 9). Of particular relevance, extracellular K\(^+\) ions accumulate in intercellular clefts and t-tubules of cardiac myocytes, where Kir2.1 channels are expressed (10). When [K\(^+\)]\(_{o}\) is increased, the outward I\(_{K1}\) increases, despite a reduction in the electrochemical gradient (1, 11). Accordingly, increases in I\(_{K1}\) result in reduced excitability, slow conductance, and abbreviation of the refractory period, and thereby predispose the heart to re-entrant ventricular arrhythmias, the leading cause of death from coronary artery disease (12). The [K\(^+\)]\(_{o}\)-dependence of the outward I\(_{K1}\) thus might be important for regulating the physiological and pathological functions of the heart. Despite the importance of this long known [K\(^+\)]\(_{o}\)-dependence of the outward I\(_{K1}\), the underlying mechanism has remained unclear.

Previous studies have shown that the dependence of Kir channel activity on membrane potential (V\(_{m}\)) shifts in parallel with V\(_{m}\}E_K (1–6), where E\(_{K}\) is the equilibrium potential for K\(^+\). This property has been previously attributed to the driving force-dependent block of Kir2.1 channels by intracellular blockers (13, 14). According to this hypothesis, known as the blocking-particle model, cytoplasmic blockers are dragged into or pushed out of the channel pore by the outward or inward flux of K\(^+\), respectively. However, the results of experiments performed at various [K\(^+\)]\(_{o}\) and intracellular K\(^+\) concentrations indicate that Kir channels detect a combination of V\(_{m}\) and [K\(^+\)]\(_{o}\) rather than just V\(_{m}\}E_K (2–6, 15). Moreover, the Kir channel cannot conduct outward current in the absence of extracellular K\(^+\) (16). To account for the regulation of Kir activity by [K\(^+\)]\(_{o}\), an alternative K\(^+\)-activated K\(^+\) channel model has been proposed (3, 13, 17). Thus, although previous studies have provided insights into the regulation of Kir channel functions by [K\(^+\)]\(_{o}\), exactly how outward I\(_{K1}\) is increased by increases in [K\(^+\)]\(_{o}\) has remained unclear.

In this study, we examined the [K\(^+\)]\(_{o}\)-dependence of the Kir2.1-mediated outward I\(_{K1}\). Our results show that outward I\(_{K1}\) is steeply dependent on [K\(^+\)]\(_{o}\) in the pathophysiological range because of the [K\(^+\)]\(_{o}\)-dependent inhibition of outward I\(_{K1}\) by extracellular Na\(^+\) and Ca\(^{2+}\), which act by screening surface charges and, possibly, also by inhibiting the K\(^+\) activation...
**[K⁺]o Dependence of Outward I_{K₁}**

**TABLE 1**

Compositions of extracellular solutions

| Solutions with various [K⁺]o | KCl | KOH | EDTA | HEPES | K₂HPO₄ | KH₂PO₄ | NaCl₂ | CaCl₂ | XCl |
|---|---|---|---|---|---|---|---|---|---|
| 0 [Na⁺]₀/0 [Ca²⁺]₀ | 1 mm [K⁺]₀ | 1 | 0.1 | 1 | | | | | |
| 1 mm [K⁺]₀ | 2 | 1 | 0.1 | 1 |
| 5 mm [K⁺]₀ | 4 | 1 | 0.1 | 1 |
| 10 mm [K⁺]₀ | 1 | 0.5 | 4 | 1 |
| 20 mm [K⁺]₀ | 11 | 2 | 4 | 1 |

| 140 mm [Na⁺]₀ | 1 mm [K⁺]₀ | 1 | 0.1 | 1 | 140 |
| 3 mm [K⁺]₀ | 2 | 1 | 0.1 | 1 |
| 5 mm [K⁺]₀ | 4 | 1 | 0.1 | 1 |
| 10 mm [K⁺]₀ | 1 | 0.5 | 4 | 1 |
| 20 mm [K⁺]₀ | 11 | 2 | 4 | 1 |

| 140 mm [Na⁺]₀/1.8 mm[Ca²⁺]₀ | 1 mm [K⁺]₀ | 0.3 | 0.7 | 1 | 140 | 1.8 |
| 3 mm [K⁺]₀ | 2.3 | 0.7 | 1 |
| 5 mm [K⁺]₀ | 4.3 | 0.7 | 1 |
| 10 mm [K⁺]₀ | 9.3 | 0.7 | 1 |
| 20 mm [K⁺]₀ | 19.3 | 0.7 | 1 |

| Solutions used in Fig. 4 | K⁺ only | 4 | 1 | 0.1 | 1 |
| Na⁺ | 4 | 1 | 0.1 | 1 |
| Li⁺ | 4 | 1 | 0.1 | 1 |
| NMG⁺ | 4 | 1 | 0.1 | 1 |
| Ca²⁺ | 4.3 | 0.7 | 1 |
| Ca²⁺ | 4 | 1 | 0.1 | 1 |
| Sr²⁺ | 4 | 1 | 0.1 | 1 |

**Experimental Procedures**

Preparation of Xenopus Oocytes—Xenopus oocytes were isolated by partial ovariectomy from frogs anesthetized with 0.1% tricaine (3-aminobenzoic acid ethyl ester). After suturing the surgical incision, animals were monitored during the recovery period before being returned to their tank. All surgical and anesthesia procedures were reviewed and approved by the Academia Sinica Institutional Animal Care and Utilization Committee.

Molecular Biology—cRNAs for expression in Xenopus oocytes were obtained by in vitro transcription (mMessage mMach, Ambion, Dallas, TX). Site-directed mutations were generated using the PCR. Correct mutations were confirmed by sequencing cDNAs using the ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA).

Electrophysiological Recordings—Currents were recorded at room temperature (21–24 °C) using the patch clamp technique (18, 19) and an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). For experiments using giant outside-out patches, the 150 mm [K⁺]₀ solution (pH 7.4) contained (in mM) KCl, 121; KOH, 20; K₂HPO₄, 4; KH₂PO₄, 1; KF, 5; and EDTA, 5. The intracellular solution (pH 7.4) contained (in mM) KCl, 76; KOH, 20; K₂HPO₄, 4; KH₂PO₄, 1; KF, 5; EDTA, 5; Na₂VO₄, 0.1; and K₃PO₄, 10 (20). The results obtained with these solutions are considered control data. The compositions of other solutions are listed in Table 1. In some experiments (Fig. 1), 280 mm sucrose was added to solutions containing [K⁺]₀ ≤ 20 mm to maintain osmolarity. Single-channel recordings were carried out in inside-out patches at a sampling rate of 10 kHz and a filtering rate of 2 kHz. The [K⁺] of the intracellular solution was 150 mm, and the [K⁺] of the pipette solution was 5 or 20 mm (Table 1). For pretreatment of oocytes with trimethoxynonoxin (TMO), de-vitallized oocytes were placed in a solution containing 90 mm KCl and 100 mm HEPES (pH 8.0) (21). TMO was then added to the solution to yield a final TMO concentration of 50 μM, and the solution was gently mixed by pipetting to allow efficient reaction of TMO with the surface of oocytes. TMO-treated oocytes were washed before transferring to the recording chamber. Command voltage pulses were controlled and data were acquired using pClamp10 software (Molecular Devices).

Data Analysis—Due to channel rundown in single-channel patches, the numbers of open and closed events were not sufficiently large to allow construction of open and closed histograms from each individual patch. Therefore, all single-channel events at each Vₘ were pooled together on square root-log coordinates to construct single open and closed time distributions, which were best-fitted with two and three exponential components, respectively, using the maximum-likelihood method (22). The mean open and closed times obtained from this method have been shown to be similar to those determined by averaging the mean open and mean closed times of each patch (23). Opening events were sufficiently long and well resolved (mean open time ≥ 5 ms) that data correction for missed events was not required (dead time = 360 μs). Single-channel current variances (σ²) in open and closed states were calculated using Clampfit basic statistics.
RESULTS

The Steep Dependence of $I_{K1}$ on $[K^+]_o$ Requires the Presence of Extracellular Na$^+$ and Ca$^{2+}$—Fig. 1A shows $I-V_m$ relationships recorded from one outside-out patch expressing Kir2.1 channels with $K^+$ (1–150 mM) as the only extracellular cation. $I-V_m$ curves were almost linear at $V_m$ negative to $E_K$. In contrast, at $V_m$ positive to $E_K$, $I_{K1}$ reached a maximum value, which we refer to as peak outward $I_{K1}$. Consistent with previous findings (6), $I-V_m$ relationships shifted in parallel with changes in $E_K$ in the hyperpolarization direction as $[K^+]_o$ decreased from 150 to 1 mM. Neither inward rectification nor outward $I_{K1}$ amplitude was changed markedly when $[K^+]_o$ was decreased by 150-fold. Fig. 1B and 1C show the $[K^+]_o$ dependence of $I_{K1}$ in the presence of physiological $[Na^+]_o$ and $[Ca^{2+}]_o$ (1.8 mM). The effects of $[K^+]_o$ on the peak outward $I_{K1}$ ratio (peak $I_{K1}$ at different $[K^+]_o$ divided by peak $I_{K1}$ at 150 mM $[K^+]_o$) are summarized in Fig. 1D. In the absence of extracellular Na$^+$ and Ca$^{2+}$, the peak outward $I_{K1}$ ratio was decreased by 27% (from 1 to 0.73 ± 0.04) when $[K^+]_o$ was reduced from 150 to 1 mM. However, in the presence of Na$^+$, or Na$^+$ and Ca$^{2+}$, the same decrease in $[K^+]_o$ reduced the peak outward $I_{K1}$ ratio by about 80%. Addition of 1.8 mM $[Ca^{2+}]_o$ in the presence of 140 mM $[Na^+]_o$ further decreased the peak outward $I_{K1}$ ratio at 20 mM $[K^+]_o$ but not at lower $[K^+]_o$ probably due to the saturating effect of 140 mM $[Na^+]_o$ alone at low $[K^+]_o$. It might be argued that the effects of extracellular Na$^+$/$Ca^{2+}$ on outward $I_{K1}$ were not accurately determined because peak outward $I_{K1}$ did not occur at a constant $V_m$. To exclude this possibility, we also examined the dependence of normalized $I_{K1}$ (peak $I_{K1}$ divided by peak $I_{K1}$ at 150 mM $[K^+]_o$) on $[K^+]_o$ at $V_m - E_K = +25$ mV. Fig. 1D shows that the effects of $[K^+]_o$ on the peak outward $I_{K1}$ ratio and normalized $I_{K1}$ at a fixed $V_m - E_K$ were not significantly different ($p > 0.05$). Thus, in subsequent experiments, we focused on the effects of $[K^+]_o$ on the peak outward $I_{K1}$ ratio.

The results shown in Fig. 1A were obtained from experiments performed by varying $[K^+]_o$ without adjusting osmolarity. To investigate whether an osmolarity imbalance contributed to the $[K^+]_o$ dependence of the peak outward $I_{K1}$ ratio, we performed experiments in which 280 mM sucrose was added to solutions containing $[K^+]_o = 20$ mM. Fig. 1D shows that the $[K^+]_o$ dependence of the peak outward $I_{K1}$ ratio was the same in the presence and absence of sucrose, indicating that an osmolarity imbalance does not account for the $[K^+]_o$ dependence of the peak outward $I_{K1}$ ratio. Therefore, in subsequent experiments osmolarity was not adjusted in solutions containing $[K^+]_o = 20$ mM. In summary, Fig. 1D shows that the $[K^+]_o$ dependence of the peak outward $I_{K1}$ ratio was shifted to the right by extracellular Na$^+$, either alone or in combination with Ca$^{2+}$. As a result, the amplitude of the outward $I_{K1}$ marked $[K^+]_o$ in the pathophysiological range (3–18 mM) at physiological $[Na^+]_o$ and $[Ca^{2+}]_o$.

![Diagram](image-url)
\( [K^+]_o \)-dependent Inhibition of Outward \( I_{K1} \) by Extracellular \( Na^+ \) and \( Ca^{2+} \) Is Independent of Intracellular Block—Next, we investigated the mechanisms underlying the inhibition of outward \( I_{K1} \) by extracellular \( Na^+ \) and \( Ca^{2+} \). It has been proposed that extracellular \( K^+ \) may bind to a high affinity site in the Kir2.1 channel pore and electrostatically weaken the pore blockade by intracellular Mg\(^{2+}\) or polyamines such that inward rectification is weaker at higher \( [K^+]_o \) (13, 14). The experiments shown in Fig. 1 were conducted in outside-out patches where perfusion was limited so that intracellular blockers could not be completely removed. Therefore, it is possible that extracellular \( Na^+ \) and \( Ca^{2+} \) might inhibit or weaken the \( K^+ \)-blocker interaction, thereby increasing the apparent block by intracellular blockers and shifting the \( [K^+]_o \) dependence of the peak outward \( I_{K1} \) ratio to the right. If such \( K^+ \)-blocker interactions indeed determine the outward \( I_{K1} \) amplitude, the \( [K^+]_o \) dependence of the peak outward \( I_{K1} \) ratio should also be shifted to the right when the intracellular blockade is stronger. In this study, we found that the degrees of inward rectification (1 − (peak outward \( I_{K1} \)/peak \( I_{K1} \) recorded at −40 mV)) varied in outside-out patches exposed to 150 mM \( [K^+]_o \), indicating various degrees of washout of endogenous intracellular blockers (see “Discussion”). To examine whether blockade by intracellular blockers plays a role in the inhibition of \( I_{K1} \) by extracellular \( Na^+ \) and \( Ca^{2+} \), we divided the data into two groups based on the degree of inward rectification at 150 mM \( [K^+]_o \). Fig. 2A shows \( I-V \_m \) relationships recorded at the indicated \( [K^+]_o \) from two different outside-out patches; in one, the degree of inward rectification was 0.88 (upper panel), and in the other, the degree of rectification was 0.78 (lower panel). Fig. 2C shows that the averaged \( [K^+]_o \) dependence of the peak outward \( I_{K1} \) ratio in the presence of 140 mM \( [Na^+]_o \) was not significantly different between the two groups with different degrees of inward rectification, indicating that the inhibition caused by extracellular \( Na^+ \) is not related to a \( K^+ \)-blocker interaction mechanism. To further test this idea, we performed experiments using a D172N mutant with reduced sensitivity to intracellular block (24, 25). Fig. 2B (upper panel) shows the \( I-V \_m \) relationships recorded in the D172N mutant (degree of inward rectification = 0.54) at different \( [K^+]_o \) in the presence of 140 mM \( [Na^+]_o \). The \( [K^+]_o \) dependence of the peak outward \( I_{K1} \) ratio was not significantly different in the D172N compared with that in the wild-type Kir2.1 channel, even though the degree of inward rectification was smaller in the D172N mutant (Fig. 2C). To further study whether the effect of extracellular \( Na^+ \) is related to intracellular block, we examined whether 100 \( \mu \)M spermine added in the pipette (intracellular) solution affected the \( [K^+]_o \) dependence of the peak outward \( I_{K1} \) ratio in the D172N mutant (Fig. 2B, lower panel).
Outward IK$_1$ by Extracellular Na$^+$ and Ca$^{2+}$—The inward IK$_1$ channel probability of outward iK$_1$ through the D172N mutant was higher than through the wild-type Kir2.1 channel (33), and because the effects of extracellular Na$^+$ and Ca$^{2+}$ act as open channel blockers, they should either decrease mean open time without affecting iK$_1$ (intermediate blocker) or reduce iK$_1$ and introduce extra “noise” to the open state (fast blocker) (32). Because the open channel probability of outward iK$_1$ through the D172N mutant was the same (Fig. 2), we examined the effects of extracellular Na$^+$ or Ca$^{2+}$ on iK$_1$ in the D172N mutant. Fig. 3A shows outward iK$_1$ recorded from two different inside-out patches (used to allow efficient washout of intracellular solution), one at V$_m$ = +30 mV (V$_m$ - E$_k$ = +70 mV) and 20 mM [K$^+$]$_o$ [6, 26–31]. To study whether extracellular Na$^+$ and Ca$^{2+}$ inhibit the outward IK$_1$ by occluding the channel, we examined outward single-channel currents (iK$_1$). If extracellular Na$^+$ and Ca$^{2+}$ act as open channel blockers, they should either decrease mean open time without affecting iK$_1$ (intermediate blocker) or reduce iK$_1$ and introduce extra “noise” to the open state (fast blocker) (32). Because the open channel probability of outward iK$_1$ through the D172N mutant was higher than through the wild-type Kir2.1 channel (33), and because the effects of extracellular Na$^+$ and Ca$^{2+}$ on the wild-type and D172N mutant were the same (Fig. 2), we examined the effects of extracellular Na$^+$ or Ca$^{2+}$ on iK$_1$ in the D172N mutant. Fig. 3A shows outward iK$_1$ recorded from two different inside-out patches (used to allow efficient washout of intracellular solution), one at V$_m$ = +30 mV (V$_m$ - E$_k$ = +70 mV) and 20 mM [K$^+$]$_o$ (left), and the other at V$_m$ = 0 mV (V$_m$ - E$_k$ = +70 mV) and 5 mM [K$^+$]$_o$ (right). In the absence of extracellular Na$^+$ and Ca$^{2+}$ (control condition), iK$_1$ was +1.97 ± 0.04 pA (n = 11) and +1.82 ± 0.03 (n = 3) pA at 20 and 5 mM [K$^+$]$_o$, respectively. In the presence of 140 mM [Na$^+$]$_o$ and 1.8 mM [Ca$^{2+}$]$_o$, iK$_1$ decreased to 1.45 ± 0.02 pA (n = 6) and 0.85 ± 0.01 (n = 4) pA at 20 and 5 mM [K$^+$]$_o$, respectively. Thus, the reduction in iK$_1$ by Na$^+$ and Ca$^{2+}$ was greater at lower [K$^+$]$_o$. Furthermore, extracellular Na$^+$ and Ca$^{2+}$ did not change the normalized open channel noise [σ(O)/σ(C)] at 5 or 20 mM [K$^+$]$_o$ (see Fig. 3A, legend, for σ(O)/σ(C) values). These results indicate that the decreased outward K$^+$ conductance was not due to fast open channel block by extracellular Na$^+$ or Ca$^{2+}$. Fig. 3B shows that the distributions of open and closed dwell times at V$_m$ = +30
**[K+]o Dependence of Outward I<sub>K1</sub>**

**FIGURE 4.** Concentration-dependent effects of extracellular cations on the normalized peak outward I<sub>K1</sub>. Effects of different concentrations of Na<sup>+</sup>, Li<sup>+</sup>, NMG<sup>+</sup>, Ca<sup>2+</sup>, and Sr<sup>2+</sup> on the normalized peak outward I<sub>K1</sub> (n = 2–10). The ionic compositions of the solutions containing 5 mM [K<sup>+</sup>]o and various cation concentrations ([X]<sub>i</sub>) are described in Table 1. Peak outward I<sub>K1</sub> recorded from outside-out patches were normalized to that obtained in the absence of Na<sup>+</sup>, Li<sup>+</sup>, NMG<sup>+</sup>, Sr<sup>2+</sup>, and Ca<sup>2+</sup>.

mV and 20 mM [K+]o fit well with two and three exponentials, respectively, in the presence and absence of 140 mM [Na+]o and 1.8 mM [Ca<sup>2+</sup>]o. Fig. 3C summarizes the effects of Na<sup>+</sup> and Ca<sup>2+</sup> on the single-channel properties at 20 mM [K+]o. Extracellular Na<sup>+</sup> and Ca<sup>2+</sup> decreased the outward iK<sub>1</sub> and shifted the single-channel i-V<sub>o</sub> relationship in the direction of depolarization (Fig. 3C, panel a). Furthermore, extracellular Na<sup>+</sup> and Ca<sup>2+</sup> did not decrease the open probability (Fig. 3C, panel b) or affect the mean open time at V<sub>o</sub> = 0 or +20 mV, but did increase the mean open time at V<sub>o</sub> = +30 and +40 mV (Fig. 3C, panel c). The closed time was decreased at 0 mV by extracellular Na<sup>+</sup> and Ca<sup>2+</sup>, but was increased at +30 and +40 mV (Fig. 3C, panel d). Taken together, the results suggest that even if extracellular Na<sup>+</sup> and Ca<sup>2+</sup> block the channels, the effect is overwhelmed by a surface electrostatic mechanism as discussed below.

**Extracellular Na<sup>+</sup> and Ca<sup>2+</sup> Inhibit Outward I<sub>K1</sub> in Part via a Surface Electrostatic Effect**—In addition to blocking channels, ions such as H<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup> have been shown to exert a surface electrostatic effect on various ion channels (34, 35). We next examined whether extracellular Na<sup>+</sup> and Ca<sup>2+</sup> regulate the outward I<sub>K1</sub> through such a surface electrostatic effect. Demonstrating that I<sub>K1</sub> inhibition is dependent on the valence of the screening ion, but not on the particular ionic species, is a good test for the existence of a surface electrostatic effect (35).

**FIGURE 5.** Effects of extracellular K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup> on the I-V<sub>o</sub> curves of TMO-treated wild-type Kir2.1 channels and D114N and E153Q mutant channels. A, I-V<sub>o</sub> curves recorded from an outside-out patch (with TMO treatment) exposed to different [K+]o with K<sup>+</sup> as the only cation. B, I-V<sub>o</sub> curves obtained in the presence of 140 mM [Na+]o and 1.8 mM [Ca<sup>2+</sup>]o with TMO treatment. C, effects of [K+]o on the peak outward I<sub>K1</sub> ratio in the wild-type Kir2.1 channel with and without TMO treatment, and in D114N, E125N, or E153Q mutant channels in the indicated solutions without TMO treatment. ***, p < 0.001, and ****, p < 0.001 for comparisons of data obtained in the wild-type Kir2.1 channel without (○) and with TMO treatment (▲) in the absence of Na<sup>+</sup> and Ca<sup>2+</sup>; n = 2–8.
To further investigate the idea that surface potential is responsible for the \([K^+]_o\) dependence of the outward \(I_{K1}\), we examined the effect of extracellular TMO, a carboxylate esterifying agent that neutralizes negative surface charges (35, 36). The \(I-V_{m}\) relationship recorded with \(K^+\) as the only extracellular cation in an outside-out patch from an oocyte pretreated with 50 mM TMO is presented in Fig. 5A. Outward currents in TMO-treated oocytes were more sensitive to changes in \([K^+]_o\) than were those recorded from oocytes not treated with TMO (Fig. 5C). When \([K^+]_o\) was decreased from 150 to 1 mM in patches from TMO-treated oocytes, outward \(I_{K1}\) decreased by 57% compared with a 27% reduction without TMO treatment. These results support the conclusion that screening of negative surface charges can indeed decrease outward \(I_{K1}\), and indicate that extracellular \(Na^+\) and \(Ca^{2+}\) may inhibit outward \(I_{K1}\) by a surface-charge effect. In the presence of physiological \([Na^+]_o\) and \([Ca^{2+}]_o\), outward \(I_{K1}\) in TMO-treated oocytes was further reduced (Fig. 5, B and C). In fact, \([K^+]_o\) dependence of the outward peak \(I_{K1}\) ratio in the presence of extracellular \(Na^+\) and \(Ca^{2+}\) was the same with and without TMO treatment. The fact that \([K^+]_o\) dependence of the peak outward \(I_{K1}\) ratio was further decreased by extracellular \(Na^+\) and \(Ca^{2+}\) in the TMO-treated Kir2.1 channel indicates that extracellular \(Na^+\)/\(Ca^{2+}\) have effects in addition to surface-charge screening effects.

Next, we sought to identify the source of the surface charges. There are five negatively charged residues (Asp\(^{112}\), Asp\(^{114}\), Glu\(^{125}\), Asp\(^{152}\), and Glu\(^{153}\)) located at the extracellular surface of the Kir2.1 channel (26, 37, 38). One of these residues, Glu\(^{125}\), has been shown to be involved in the block of inward \(I_{K1}\) by extracellular \(Ba^{2+}\) and \(Mg^{2+}\), and in the permeation of \(K^+\) (26, 39), probably via a surface charge-screening effect (26). Glu\(^{153}\) has also been shown to be the primary source of surface charges for the inward \(K^+\) conductance of the Kir2.1 channel (38). To gain a better understanding of the physical location of these residues, we built a homology model of the Kir2.1 channel based on the crystal structure of the closely related Kir2.2 channel (40). The homology model (Fig. 6) suggests that Asp\(^{114}\), Glu\(^{125}\), and Glu\(^{153}\) are most likely exposed to the extracellular milieu. In addition, the model predicts that Asp\(^{112}\) is buried within each subunit and Glu\(^{152}\) is buried at the interface of adjacent subunits. Therefore, we used site-directed mutagenesis to examine whether the Asp\(^{114}\), Glu\(^{125}\), and Glu\(^{153}\) sites are involved in regulating the outward \(I_{K1}\) of the Kir2.1 channel. Fig. 5C shows that with \(K^+\) as the only extracellular cation, the \([K^+]_o\) dependence of the peak outward \(I_{K1}\) ratio was not changed in D114N, E125N, or E153Q mutants. Because it is possible that more than one surface charge is involved in the surface electrostatic effects, we examined whether the D114N/E153Q double mutation and/or D114N/E125N/E153Q triple mutation had a greater effect on outward \(I_{K1}\). Fig. 7, A and B, show the I-\(V_{m}\) relationships for Kir2.1 mutants D114N/E153Q and D114N/E125N/E153Q, respectively, recorded with \(K^+\) as the only extracellular cation. Outward \(I_{K1}\) in each of these mutants was more sensitive to changes in \([K^+]_o\) than recorded from the wild-type (Fig. 1A). In fact, as shown in Fig. 7C, the \([K^+]_o\) dependence of the peak outward \(I_{K1}\) ratio in the D114N/E153Q and D114N/E125N/E153Q mutants was statistically indistinguishable from recordings of the TMO-treated wild-type Kir2.1 channel in the absence of extracellular \(Na^+\) and \(Ca^{2+}\). These results indicate that both Asp\(^{114}\) and Glu\(^{153}\) are involved in the surface electrostatic effects of extracellular \(Na^+\) and \(Ca^{2+}\). Fig. 7C also shows that the \([K^+]_o/peak outward I_{K1}\) ratio relationships for the D114N/E153Q and D114N/E125N/E153Q mutants were the same as for the wild-type Kir2.1 channel in the presence of extracellular \(Na^+\) and \(Ca^{2+}\). These results indicate that extracellular \(Na^+\) and \(Ca^{2+}\) inhibit the Kir2.1 channel by an additional mechanism in which Asp\(^{114}\), Glu\(^{125}\), and Glu\(^{153}\) are not involved.

\(K^+\) Activation Contributes to the \([K^+]_o\) Dependence of Outward \(I_{K1}\) and Its Regulation by Extracellular \(Na^+\) and \(Ca^{2+}\)—Results presented in Figs. 5 and 7 show that although a surface electrostatic mechanism is engaged, additional mechanisms are involved in the action of extracellular \(Na^+\) and \(Ca^{2+}\). Fig. 1D shows that increases in \([K^+]_o\) can relieve the inhibition of outward \(I_{K1}\) by extracellular \(Na^+\) and \(Ca^{2+}\), suggesting that \(Na^+\) and \(Ca^{2+}\) may compete with \(K^+\) for the same binding site(s) and generate opposite effects. It is possible that \(Na^+\) and \(Ca^{2+}\) compete with \(K^+\) for a channel-activation site. Previously, it has been shown that \([K^+]_o\)-dependent increases in inward \(I_{K1}\) were reduced in R148Y mutants compared with those in wild-type Kir2.1 channels (3). In addition, we have shown that neutralization of Arg\(^{148}\) reduced the increases in the inward single-channel conductance of Kir2.1 channels induced by elevated \([K^+]_o\) (41). These results indicate that a \(K^+\) activation mecha-
Type channel, where increases in \([K^+]_o\) increased outward \(I_{K_1}\). This observation suggests that \(K^+\)-mediated channel activation exists in the Kir2.1 channel and that this mechanism is greatly reduced in the R148Y mutant. Therefore, when \([K^+]_o\) is increased, the surface charge screening effect of extracellular \(K^+\) is manifested and outward \(I_{K_1}\) decreases. Increased \([K^+]_o\) may produce a great surface charge screening effect and thus substantially reduce outward \(I_{K_1}\). If this argument is correct, removing the surface charge in the R148Y mutant with TMO should reduce the inhibitory effect of high \([K^+]_o\) on outward \(I_{K_1}\). Indeed, as shown in Fig. 8B, \([K^+]_o\) dependence of outward \(I_{K_1}\) was much reduced in the R148Y mutant pretreated with TMO, supporting our view that the surface charge effect is dominant in the R148Y mutant. Fig. 8C shows the \([K^+]_o\) dependence of peak outward \(I_{K_1}\) in the presence of \(Na^+\) and \(Ca^{2+}\) in the R148Y mutant pretreated with TMO. The hump-shaped outward \(I_{K_1}\) that occurred near \(E_N\) was completely abolished, and the outward \(I_{K_1}\) was about the same when \([K^+]_o\) was increased. In addition, the outward \(I_{K_1}\) formed a plateau that extended over a broad \(V_m\) range. The mechanism for this plateauing of outward \(I_{K_1}\) is unknown, but it was also observed in the absence of extracellular \(Na^+\) and \(Ca^{2+}\). Therefore, the effect is likely related to the R148Y mutation rather than the presence of \(Na^+\) and \(Ca^{2+}\). Fig. 8E shows the averaged effects of \([K^+]_o\) on the peak outward \(I_{K_1}\) ratio in the R148Y mutant. Because a sharp peak outward \(I_{K_1}\) was not obvious in the presence of \(Na^+\) and \(Ca^{2+}\), the peak outward \(I_{K_1}\) ratio was calculated by normalizing the outward \(I_{K_1}\) at \(V_m - E_K = +20\) mV at different \([K^+]_o\), to that at 150 mM \([K^+]_o\). Both the presence of \(Na^+\) and \(Ca^{2+}\) and pretreatment with TMO greatly reduced the dependence of the peak outward \(I_{K_1}\) ratio on \([K^+]_o\) elevation. It is noted that the outward \(I_{K_1}\)-reducing effects of \(Na^+\) and \(Ca^{2+}\) were slightly, but significantly, larger than that of TMO pretreatment in the R148Y mutant (Fig. 8E). Previous several studies have shown that extracellular \(Na^+\) could induce time-dependent decay of inward currents through Kir channels of skeletal muscle (28), egg cells (29), and cardiac myocytes (27, 30, 31). The inhibitory effect of \(Na^+\) has been attributed to \(V_m\)-dependent block of the Kir channel by extracellular \(Na^+\) (28–31) or to stabilizing the channel at a closed state (27) (this may be relevant to \(K^+\)-activation mechanisms). Indeed, we found that, extracellular \(Na^+\)/\(Ca^{2+}\) reduced the inward \(K^+\) conductance in both the wild-type and R148Y channel (Fig. 8, D and F). It is possible that extracellular \(Na^+\)/\(Ca^{2+}\) may also inhibit outward \(K^+\) conductance by blocking the channel pore and the effect is preserved in the R148Y mutant.

In summary, these results show that Arg148 is crucial for \(K^+\) activation of the Kir2.1 channel, and demonstrate that both \(K^+\) activation and surface charge effects regulate the amplitude of the outward \(I_{K_1}\). In addition, reducing \(K^+\) activation and surface electrostatic effects mimicked the action of extracellular \(Na^+\) and \(Ca^{2+}\), suggesting that \(Na^+\) and \(Ca^{2+}\) may inhibit outward \(I_{K_1}\) by affecting both \(K^+\) activation and surface electrostatic mechanisms.

**DISCUSSION**

The modulation of Kir channels can profoundly affect health, in some cases causing diseases, but it also provides a possible

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**FIGURE 7. Effects of extracellular \(K^+\), \(Na^+\), and \(Ca^{2+}\) on the \(I-V_m\) curves of D114N/E153Q and D114N/E125N/E153Q mutants.**

(A) \(I-V_m\) curves for the D114N/E153Q mutant recorded from an outside-out patch exposed to different \([K^+]_o\) with \(K^+\) as the only cation. \(B\) \(I-V_m\) curves recorded at different \([K^+]_o\) in the absence of extracellular \(Na^+\)/\(Ca^{2+}\) in the D114N/E125N/E153Q mutant. \(C\) effects of \([K^+]_o\) on the peak outward \(I_{K_1}\) ratio in the wild-type Kir2.1 channel with and without TMO treatment, and in D114N/E153Q and D114N/E125N/E153Q mutant channels in the indicated solutions (\(n = 3–8\)).

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nism exists in the Kir2.1 channel and that residue Arg148 may be involved in the mechanism (3). Thus, to examine whether \(Na^+\) and \(Ca^{2+}\) compete with \(K^+\) for an activation site, we examined the \([K^+]_o/\)peak outward \(I_{K_1}\) ratio relationships in the R148Y mutant.

Fig. 8A shows that increases in \([K^+]_o\) led to decreases in outward \(I_{K_1}\) in the R148Y mutant; this was in contrast to the wild-
[K^+]_o Dependence of Outward I_{K1}

Target for therapeutic intervention. I_{K1} depends on [K^+]_o, with increased [K^+]_o leading to increases in I_{K1}. In this study, we found that extracellular Na^+ and Ca^{2+} inhibited outward I_{K1} to less extent at higher [K^+]_o such that the [K^+]_o dependence of outward I_{K1} was steep over the pathophysiological [K^+]_o range (3–18 mM).
Regulation of Outward $I_{K_1}$ by Extracellular Na$^+$ and Ca$^{2+}$—We present several lines of evidence supporting the conclusion that Na$^+$ and Ca$^{2+}$ inhibit outward $I_{K_1}$ by a surface charge screening effect. First, extracellular Na$^+$ and Ca$^{2+}$ reduced the outward single-channel current, an effect that was greater at lower ionic strength. Second, extracellular Na$^+$ and Ca$^{2+}$ did not decrease the mean open time. Third, TMO modification and neutralizing both Asp$^{114}$ and Glu$^{153}$ by site-directed mutagenesis decreased outward $I_{K_1}$, in the absence of extracellular Na$^+$ and Ca$^{2+}$.

In addition to a surface-electrostatic effect, extracellular Na$^+$ and Ca$^{2+}$ also inhibit the outward $I_{K_1}$ through at least another mechanism (Fig. 5). Because the inhibitory effect of reducing both K$^+$ activation and surface charges (R148Y mutant pre-treated with TMO) on the outward $I_{K_1}$ was similar to the effects of extracellular Na$^+$ and Ca$^{2+}$, we propose that extracellular Na$^+$ and Ca$^{2+}$ may also reduce outward $I_{K_1}$ by inhibiting the K$^+$ activation mechanism. Finally, we cannot rule out that extracellular Na$^+$/Ca$^{2+}$ might also inhibit outward K$^+$ conductance by blocking the channel pore. The multiple mechanisms involved in extracellular Na$^+$ and Ca$^{2+}$ inhibition may explain why $V_m$ dependence of the open mean and mean closed times of the $i_{K_1}$ are complicated.

It is noted that various degrees of inward rectification were recorded in outside-out patches, indicating that washout of endogenous intracellular blockers was variable even though the pipette solution did not contain Mg$^{2+}$ or polyamines. However, it should be emphasized that the incomplete washout of endogenous blockers would not affect our interpretations of the inhibitory effects of extracellular Na$^+$ and Ca$^{2+}$ on outward $I_{K_1}$. First, we found that varying degrees of intracellular block and addition of 100 μM spermine did not affect the inhibition of outward $I_{K_1}$ by extracellular Na$^+$. Second, we showed that extracellular Na$^+$ and Ca$^{2+}$ reduced single-channel conductance and slightly increased open probability. In contrast, it has been shown that increases in the intracellular spermine concentration decrease the open probability of outward single-channel currents through the Kir2.1 channel but do not change the single-channel conductance (33). The simplest interpretation of these results is that the regulation of [K$^+$]$_o$-dependent outward $I_{K_1}$ by extracellular Na$^+$ and Ca$^{2+}$ is not related to intracellular block.

Regulation of Outward $I_{K_1}$ by Extracellular K$^+$—This study shows that extracellular K$^+$ has two opposite effects on the outward $I_{K_1}$ through the Kir2.1 channel. First, increases of [K$^+$]$_o$ increase outward K$^+$ conductance (as shown in Fig. 1A). Direct activation of K$^+$ channels by K$^+$ has been proposed as an explanation for the increase in K$^+$ channel activity caused by increased [K$^+$]$_o$ in various types of K$^+$ channels (3, 3, 13, 17, 43, 44). Based on site-directed mutagenesis studies, it has been suggested that K$^+$ occupancy at extracellular site(s) is involved in the K$^+$ activation of inward rectifier K$^+$ channels and $V_m$-gated K$^+$ channels (3, 17, 26, 44). It is possible that extracellular K$^+$ binds to a site (or sites) stabilized by salt bridges formed by residues Glu$^{138}$ and Arg$^{148}$ (45) and then activate channel activity in the Kir2.1 channel. Indeed, the recently resolved crystal structure of the Kir2.2 channel shows that such an ion pair “staples” the pore region together (40). The homology model of the Kir2.1 channel also revealed that Arg$^{148}$ and Glu$^{138}$ are located close to each other (Fig. 6B). Another hypothesis to account for the [K$^+$]$_o$ dependence of Kir channel activation is that the block of outward $I_{K_1}$ by polyamines is relieved by increased [K$^+$]$_o$ (14). It has been shown that changes in [K$^+$]$_o$ shift $V_m$ dependence of polyamine block (14). This finding explains why the voltages at which the peak outward $I_{K_1}$ occur depend on $V_m - E_K$ when [K$^+$]$_o$ is changed (6).

Second, increases of [K$^+$]$_o$ decrease outward K$^+$ conductance by a surface electrostatic mechanism. Because extracellular Na$^+$/Ca$^{2+}$ can screen extracellular surface charges and thus reduce outward $I_{K_1}$, extracellular K$^+$ should also have such an effect. Indeed, this effect was demonstrated by the steep reduction of outward $I_{K_1}$ when [K$^+$]$_o$ were increased in the R148Y mutant (Fig. 7A). Usually, this mechanism is concealed by the K$^+$ activation effect but is unraveled in the R148Y mutant where [K$^+$]$_o$ dependence of Kir channel activation is reduced.

Conclusion—K$^+$ activation has been described for several K$^+$ channels, including Kir and $V_m$-gated K$^+$ channels, and is an important mechanism for regulating K$^+$ channel function. Likewise, surface electrostatic effects modulate many properties of ion channels. However, few studies have addressed the relevance of these effects in terms of the pathophysiological functions of ion channels. In this study, we showed that the [K$^+$]$_o$ dependence of the outward $I_{K_1}$ is controlled by both K$^+$ activation and surface electrostatic mechanisms. In addition, extracellular Na$^+$ and Ca$^{2+}$ inhibit outward $I_{K_1}$ in a [K$^+$]$_o$-dependent fashion, at least in part by a surface electrostatic effect, resulting in a steep [K$^+$]$_o$ dependence of the outward $I_{K_1}$ in the pathophysiological [K$^+$]$_o$ range. Our work suggests that surface charge screeners by inhibiting the outward $I_{K_1}$ may be potential candidates for diseases related to elevated outward $I_{K_1}$.

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