Voltage-gated potassium (Kv) channels containing α-subunits of the Kv2 subfamily mediate delayed rectifier currents in excitable cells. Channels formed by Kv2.1 α-subunits inactivate from open- and closed states with both forms of inactivation serving different physiological functions. Here we show that open- and closed-state inactivation of Kv2.1 can be distinguished by the sensitivity to intracellular tetraethylammonium and extracellular potassium and lead to the same inactivated conformation. The functional properties of Kv2.1 are regulated by its association with modulatory α-subunits (Kv5, Kv6, Kv8, and Kv9). For instance, Kv9.3 changes the state preference of Kv2.1 inactivation by accelerating closed-state inactivation and inhibiting open-state inactivation. An N-terminal regulatory domain (NRD) has been suggested to determine the function of the modulatory α-subunit Kv8.1. However, when we tested the NRD of Kv9.3, we found that the functional properties of chimeric Kv2.1 channels containing the NRD of Kv9.3 (Kv2.1NRD) did not resemble those of Kv2.1/Kv9.3 heteromers, thus questioning the role of the NRD in Kv9 subunits. A further region of interest is a P motif in the sixth transmembrane segment. This motif is conserved among all α-subunits of the Kv1, Kv2, Kv3, and Kv4 subfamilies, whereas the second proline is not conserved in any modulatory α-subunit. Exchanging this proline in Kv2.1 for the corresponding residue of Kv9.3 resulted in channels (Kv2.1-P410T) that show all hallmarks of the regulation of Kv2.1 by Kv9.3. The effect prevailed in heteromeric channels following co-expression of Kv2.1-P410T with Kv2.1. These data suggest that the alteration of the PXP motif is an important determinant of the regulatory function of modulatory α-subunits.

Voltage-gated potassium (Kv) channels form the most diverse class in the ion channel superfamily, giving rise to a large variety of currents, the kinetics of which are shaped to the requirements of their physiological function (1). They are composed of four α-subunits, each containing six transmembrane segments (S1–S6), arranged around a central potassium-selective pore (2). The ability of different α-subunits to form heteromeric channels increases the diversity of K⁺ currents in native cells (3–5). Modulatory α-subunits constitute a group of proteins that are unable to build functional channels by themselves. They associate with Kv2 α-subunits forming heteromeric channels that activate, deactivate, inactivate, and recover from inactivation differently from homomeric Kv2 channels (6–12). The group of mammalian modulatory α-subunits so far consists of Kv5.1, Kv6.1–6.4, Kv8.1–2, and Kv9.1–9.3 (7, 10–15). Their selective association with Kv2 α-subunits is guided by an intracellular N-terminal domain (15, 16) initially identified in α-subunits of the subfamilies Kv1-Kv4 and named T1 for its role in tetramerization and subunit segregation (17–19). We previously described the functional properties of heteromeric channels arising from the co-expression of Kv2.1 with Kv9.3 (Kv2.1/Kv9.3) (6). Channel activation and deactivation were slowed, and their equilibrium shifted to hyperpolarized potentials when compared with homomeric Kv2.1 channels. Moreover, Kv9.3 changed the state dependence of Kv2.1 inactivation.

Kv2 α-subunits have been identified as an important component of delayed rectifier currents in a variety of excitable cells where they participate in action potential repolarization, regulation of the firing frequency, and setting the resting membrane potential (20–25). Inactivation of Kv2 channels reduces currents through these channels and thus regulates membrane excitability. Based on a model adapting the Monod-Wyman-Changeux model for allosteric proteins (26) to ion channels, Kv2.1 has been suggested to inactivate from open and closed states (27). The maximum of inactivation in this description was assigned to the last of five closed states passed by opening channels, which is linked with the open state through a transition with a voltage-independent on rate and a voltage-dependent off rate (27). Here we refer to inactivation from this last closed and from open states as open-state inactivation. The term closed-state inactivation indicates inactivation from proximal states in the activation pathway that are separated from open states by voltage-dependent transitions. For Kv2.1 open-state inactivation is fast, and closed-state inactivation slow. On the contrary, heteromeric Kv2.1/Kv9.3 channels exhibit slow open- and fast closed-state inactivation. This shift to preferential closed-state inactivation has been referred to as a change in the state dependence of inactivation (6). Preferential closed-state inactivation has been suggested to participate in the control of membrane excitability by modulating repetitive firing and back-propagation of action potentials in neurons as
well as the repolarization of cardiac action potentials (28, 29).

Here we describe a pharmacological strategy to distinguish between open- and closed-state inactivation of Kv2.1 and show that both inactivation pathways lead to the same conformation. Moreover, we investigate the structural determinants underlying the regulation of Kv2.1 by the modulatory α-subunit Kv9.3. An N-terminal regulatory domain (NRD) originally characterized in Kv8.1 (30) and suggested to govern its function was identified also in Kv9.3. However, the functional properties of chimeric Kv2.1 channels containing the NRD of Kv9.3 differed from those of Kv2.1/Kv9.3 heteromers, thereby questioning the importance of NRD in Kv9 subunits. On the contrary, we show that a single amino acid in the distal part of the pore-lining S6 determines the regulatory properties of Kv9.3.

**MATERIALS AND METHODS**

**Site-directed Mutagenesis and cRNA Synthesis**—All of the DNA manipulations were carried out using standard recombinant DNA techniques (31). The cDNAs coding for Kv2.1 (DRK1) and Kv9.3 used in this study were identical to the ones described previously (6, 14). Point mutations were introduced into Kv2.1 cDNA using QuickChange (Stratagene, La Jolla, CA). The chimera Kv2.1NRD was generated by replacing amino acids 127–181 of Kv2.1 by the NRD domain of Kv9.3 (amino acids 111–177). Amplification by low copy PCR (15 cycles) with the polymerase Pfu (Promega, Madison, WI) was used to generate one fragment of both α-subunits. These fragments were then combined via a common introduced silent EcoRI recognition site and ligated into an appropriately cut Kv2.1. For all mutated or chimeric constructs, the sequence of the complete channel subunit was verified by sequencing with a BigDye terminator cycle sequencing kit and an ABI377 DNA sequencer (Applied Biosystems). Following linearization capped cRNAs were synthesized in vitro with mMessage mMachine (Ambion, Austin, TX). Isolation of oocytes (stages V and VI) from Xenopus laevis and cRNA injection were performed as described previously (32).

**Electrophysiological Characterization**—Whole cell currents were recorded 1–4 days after injection under two-electrode voltage-clamp control, using a Turbo TEC-10CD amplifier (NPI-Elektronik; Tamm, Germany). Intracellular electrodes had resistances of 0.3–0.8 MΩ when filled with 2 M KCl. The standard bath solution was normal frog Ringer containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 10 mM HEPES-NaOH (pH 7.2). In experiments in which KCl concentrations were raised, NaCl concentrations were lowered so that the sum of KCl and NaCl remained constant. Recordings testing the effect of internal TEA could not be performed in inside out patches because of fast rundown of Kv2.1 currents in this configuration (33).2 We therefore injected TEA into oocytes using a glass pipette filled with 105 mM KCl, 10 mM TEACL, 2.5 mM NaCl, 1.8 mM CaCl2, 10 mM HEPES-NaOH (pH 7.2). The currents were low pass filtered at 0.7–1 kHz (–3dB) and sampled at 3–5 kHz. All of the experiments were carried out at room temperature (20–22°C). Data acquisition and analysis were performed with the Pulse + PulseFit software package (HEKA Elektronik, Lambrecht, Germany), EXCELF (Microsoft), and IGOR (Wavemetrics). Boltzmann functions of the type \( P \propto \frac{1}{1 + \exp(\frac{V_i - V_{1/2}}{\alpha})} \) were used to fit steady-state activation.

**Statistical Analysis**—The data are given as the means ± S.E., with \( n \) specifying the number of independent experiments. Statistical significance was evaluated using a two-tailed Student’s t test.

**RESULTS**

Open- and Closed-state Inactivation of Kv2.1 Can Be Distinguished by Their Sensitivity to Intracellular TEA and Extracellular K+—The delayed rectifier potassium channel Kv2.1 (34) has previously been suggested to inactivate from open and closed states. This proposal was based on kinetic analyses (27) and on the observation that co-expressions of different modulatory α-subunits with Kv2.1 have opposite effects on inactivation occurring at high and low open probabilities (6, 10). Throughout this study open- and closed-state inactivation were measured using the pulse protocols \( P_{O \text{inact}} \) and \( P_{C \text{inact}} \), respectively. For \( P_{O \text{inact}} \), the membrane was depolarized for 32 s to potentials of high open probability. Time constants \( \tau_{C \text{inact}} \) derived from mono-exponential functions fit to the decay of the resulting outward currents were used to assess open-state inactivation (\( \Delta \) and \( \triangle \)) in Figs. 1, E and F, 4D, and 5, E and F. For \( P_{C \text{inact}} \), 300-ms test pulses to +40 mV were given at the beginning (P1) and end (Pn) of conditioning pulses of increasing length to potentials of low open probability. The ratio of currents elicited by the test pulses \( (I_{P1}/I_{Pn}) \) is proportional to the number of channels that did not inactivate during the conditioning pulse and declines with increasing length of the conditioning pulse. Time constants \( \tau_{C \text{inact}} \) derived from mono-exponential functions fit to this decline were used to quantify closed-state inactivation (\( \bigtriangleup \) and \( \bigtriangleup \)) in Figs. 1, E and F, 4D, and 5, E and F.

Presently our knowledge about the molecular determinants of these different inactivation pathways and the regulation by modulatory α-subunits is scarce. To have an additional tool for their analysis, we first investigated whether open- and closed-state inactivation can be separated pharmacologically. Kv2.1 channels were expressed in Xenopus oocytes, and currents were measured under two-electrode voltage clamp conditions. The quaternary ammonium ion TEA, which blocks Kv2.1 channels (34, 35), has been shown to slow C- (36, 37) and N-type inactivation (38, 39) of other Kv channels when applied to the extracellular side of the channels, respectively. Therefore, we tested whether TEA was affecting Kv2.1 inactivation when applied from either side of the membrane. After the current response was stable for at least 5 min, TEA (10 mM solution in high K+ Ringer) was injected through a third pipette impaled into the oocyte until approximately 50% of the initial current was blocked (55.3 ± 1.2%, \( n = 16 \)). This intracellular application of TEA markedly slowed the rate of open-state inactivation (Fig. 1A, \( V_{\text{inact}} = +40 \text{ mV}; E, \bigcirc \) and \( \bullet; p < 0.02 \)). On the other hand, closed-state inactivation was not altered significantly by internal TEA (Fig. 1C; \( V_{\text{inact}} = -30 \text{ mV}; E, \square \) and \( \blacksquare; p > 0.6 \)). Accordingly, Fig. 1E shows that the effect of intracellular TEA increases with more pronounced depolarizations, corresponding to increasing open probabilities of the channels. In agreement with previous publications, extracellular TEA did not affect open- or closed-state inactivation of Kv2.1 (data not shown and Ref. 27).

Additionally, we tested the influence of elevated extracellular [K+], a condition known to slow C-type inactivation of other Kv channels (40–42). Elevation of the external \([K+]_{o}\) from 2.5 to 115 mM significantly accelerated open-state inactivation. (Fig. 1B, \( V_{\text{inact}} = +40 \text{ mV}; F, \bigcirc \) and \( \bullet; p < 0.01 \)). On the contrary, closed-state inactivation was decelerated when external \([K+]_{o}\) was increased (Fig. 1D, \( V_{\text{inact}} = -30 \text{ mV}; F, \square \) and \( \blacksquare; p < 0.02 \)).

Taken together, these experiments demonstrate that the two inactivation pathways for Kv2.1 can be separated pharmacologically. Open-state inactivation is inhibited by intracellular TEA and accelerated by elevated external \([K+]_{o}\), whereas closed-state inactivation is insensitive to intracellular TEA and inhibited by elevated external \([K+]_{o}\).

Open- and Closed-state Inactivation of Kv2.1 Lead to the Same Conformation—The different sensitivities of open- and closed-state inactivation of Kv2.1 to internal TEA and external \([K+]_{o}\) could arise by either of two mechanisms. First, inactivation from open and closed states could lead to different inactivated conformations of Kv2.1 from which recovery should differ, or second, they could represent different transitions that start from open or closed channels, respectively, but lead to the same inactivated conformation. In this case recovery should be
Fig. 1. TEA, and $[K^+]_o$, can be used to distinguish open- and closed-state inactivation of Kv2.1. A, Kv2.1 currents were elicited by a 32-s depolarizing pulse to +40 mV ($P_{o,\text{inact}}$) in the presence (+TEA) or absence (-TEA) of intracellular TEA. For easier comparison, the traces are scaled to their maximum. Inset, to determine the voltage dependence of activation, Kv2.1 channels were challenged by a 300-ms pulse to potentials ranging from -80 to +70 mV, in 10-mV increments. Subsequently, the voltage was clamped to -40 mV, and the initial current was estimated from a mono-exponential fit to its decay. The relative open probabilities ($P_{o,\text{inact}}$) derived from the initial currents were plotted against the voltages of the depolarization and fit with a Boltzmann function. The data are given as the means ± S.E. (n = 9). B, Kv2.1 currents were evoked as in A with 2.5 or 115 mM K$^+$ in the extracellular solution. C, Kv2.1 closed-state inactivation evoked by $P_{c,\text{inact}}$ at an inactivating potential of -80 mV ($V_{\text{inact}} = -30$ mV). Recordings were performed either in the presence (+TEA) or absence (-TEA) of intracellular TEA. D, measurements as in C, with 2.5 or 115 mM K$^+$ in the extracellular solution. E, time constants derived from mono-exponential functions fit to the inactivation processes measured by $P_{c,\text{inact}}$ (C and D), $P_{c,\text{inact}}$ (C and D), or both (C and A) in the presence (filled symbols) or absence (open symbols) of intracellular TEA were plotted against the voltage of the inactivating pulse. F, plot of time constants of inactivation measured by $P_{c,\text{inact}}$ (C and D), $P_{c,\text{inact}}$ (C and D), or both (C and A) with 2.5 mM (open symbols) or 115 mM (filled symbols) K$^+$ in the extracellular solution. The data in E and F represent the means ± S.E. (n = 4–10).

independent from the way inactivation occurred. To discriminate between the two possibilities, recovery from open- and closed-state inactivation of Kv2.1 was analyzed. As illustrated in the Fig. 2A, following a 300-ms pulse to +40 mV (P1) channels were inactivated by clamping the voltage for 40 s at either +40 mV (open-state inactivation) or -40 mV (closed-state inactivation) and a second 300-ms pulse to +40 mV (P2) was given to measure the degree of inactivation. Channels were then recovered from inactivation at hyperpolarized potentials (-80 to -120 mV) for increasing time intervals before applying another 300-ms pulse to +40 mV (Pn). The number of channels that recover during this period is proportional to the ratio of currents elicited by the short pulses ($I_{P_{1}} - I_{P_{2}}$) and increases with increasing time spent at the recovery potential. Time constants derived from mono-exponential functions fit to this process were used to quantify recovery. They are indistinguishable between recovery from open- (Rec - $I_o$) and closed-state inactivation (Rec - $I_c$) at all potentials tested (Fig. 2B, p > 0.3). Moreover, elevating the extracellular [K$^+$] accelerated recovery to a rate that was identical for open- and closed-state inactivation of Kv2.1 are separate transitions leading to the same inactivated conformation.

Identification and Characterization of the NRD of Kv9.3—We previously characterized the modulatory $\alpha$-subunit Kv9.3, which forms heteromeric channels with Kv2.1 and changes the preferred state from which inactivation occurs (6). Although Kv2.1 inactivates fast from open and slowly from closed states, heteromeric Kv2.1/Kv9.3 channels show little open- but fast and complete closed-state inactivation (Fig. 3, A and B) (6). The molecular determinants of this regulation are unknown. The inhibition of open-state inactivation of Kv2.1 by the modulatory $\alpha$-subunit Kv8.1 has been attributed to a segment of 59 amino acids preceding the first transmembrane segment (30). A comparison of this region, termed NRD (30), with the respective region in Kv9.3 showed little sequence identity. To test whether the proposed function of this domain was conserved, chimeric Kv2.1 channels containing the NRD of Kv9.3 (Kv2.1NRD) were constructed (Fig. 3C). Steady-state activation of Kv2.1NRD was shifted in the depolarized direction with respect to Kv2.1 (Kv2.1, $V_{1/2} = 3 ± 1$ mV (n = 9); Kv2.1NRD, $V_{1/2} = 12 ± 2$ mV, (n = 12) (Fig. 3D) in contrast to what has been observed for Kv2.1/Kv9.3 heteromers, which opened at more hyperpolarized potentials than Kv2.1 (6). For Kv2.1NRD, as for Kv2.1/Kv9.3 heteromers, the kinetics of activation and deactivation were slowed (data not shown), and open-state inactivation was reduced. Accordingly, at the end of 32-s depolarizing pulses to +40 mV only 34 ± 0.4% (n = 6) of the Kv2.1NRD current was inactivated, compared with 84.9 ± 1.8% (n = 24) of the Kv2.1 current (Fig. 3, E and G). However, Kv2.1NRD channels did not show the prominent closed-state inactivation that is the hallmark of Kv2.1/Kv9.3 heteromers (Fig. 3B) (6). On the contrary, they display less closed-state inactivation than Kv2.1. Thus, following 32 s at -30 mV, 84 ± 1.1% (n = 6) of the initial Kv2.1NRD current was left compared with 59.7 ± 5.5%, (n = 16) for Kv2.1 (Fig. 3, F and G).

In conclusion, the NRD of Kv9.3, rather than shifting the
maximum of inactivation from open to closed states, inhibits both inactivation pathways. Hence, another region of Kv9.3 must participate in the regulation of Kv2.1.

The Involvement of S6 in the Modulatory Effect of Kv9.3 on Channel Gating—To find such alternative regions we compared the amino acid sequences of all of modulatory α-subunits (Kv5.1, Kv6.1–6.6, Kv8.1–8.2, and Kv9.1–9.3) with the sequences of Kv1, Kv2, Kv3, and Kv4 α-subunits and thus identified the sixth transmembrane segment (S6) as a region of interest. Fig. 4A shows an alignment of the S6 in which one representative member of each subfamily was included. The second proline of a PXP motif (Fig. 4A, arrow) is conserved among all 17 α-subunits of the Kv1–Kv4 subfamilies and is replaced by serine, threonine, alanine, or histidine in all 10 modulatory α-subunits. To test the hypothesis that the alteration of this PXP motif participates in the regulatory function of Kv9.3, we mutated the proline at position 410 in Kv2.1 to the corresponding threonine of Kv9.3. The resulting α-subunit (Kv2.1-P410T) formed functional channels in Xenopus oocytes.

Steady-state activation of Kv2.1-P410T was shifted 12 mV toward hyperpolarized potentials compared with Kv2.1 (Fig. 1, Kv2.1, $V_{50} = 3 \pm 1$ mV, $n = 9$; Kv2.1-P410T, $V_{50} = -9 \pm 2$ mV, $n = 10$; Fig. 4B, inset), and activation and deactivation kinetics decelerated, similar to what has been observed for heteromeric Kv2.1/Kv9.3 channels (6). Moreover, like Kv2.1/Kv9.3 heteromers, Kv2.1-P410T channels showed little open-state inactivation (Fig. 4B, $V_{nacl} = +40$ mV, and D) while inactivating fast from closed states (Fig. 4C, $V_{nacl} = -40$ mV, and D). Plotting the time constants of inactivation as a function of the voltage for both Kv2.1 and Kv2.1-P410T shows their inverse state preference for inactivation (Fig. 4D). The curves cross in the intermediate voltage range where channels start to open. At voltages negative to the activation threshold (closed-state inactivation), Kv2.1 inactivation is slow and incomplete, whereas Kv2.1-P410T channels inactivate in a fast and complete manner. At potentials positive to the activation threshold (open-state inactivation) Kv2.1 inactivation is fast, whereas only a few Kv2.1-P410T channels inactivate. Recovery from inactivation, as for Kv2.1/Kv9.3 heteromeric channels, was accelerated for Kv2.1-P410T and showed less pronounced voltage and K+ dependence compared with Kv2.1 (data not shown). Thus, the single amino acid exchange P410T in Kv2.1-P410T conveys all of the functional properties that Kv9.3 brings to Kv2.1/Kv9.3 heteromers.

Preferred Closed-state Inactivation of Kv2.1-P410T Is Confirmed by Its Pharmacological Profile—The voltage dependence of Kv2.1-P410T inactivation suggested that these channels inactivate preferentially from closed states. Therefore, if the inactivation mechanism is preserved, inactivation of Kv2.1-P410T should be slowed by high extracellular [K+] but remain unchanged upon application of internal TEA, which selectively slowed open-state inactivation of Kv2.1 (Fig. 1, A, C, and E). Indeed, injection of intracellular TEA blocking approximately 50% of the maximal current (47.2 ± 2.9%, $n = 8$) was without significant effect on inactivation of Kv2.1-P410T measured at voltages between −60 mV and +60 mV (Fig. 5, A, C, and E; $p > 0.1$). When the extracellular K+ concentration was increased to 115 mM, closed-state inactivation was inhibited (Fig. 5, D and F; $p < 0.01$) as for Kv2.1, and open-state inactivation was not significantly affected (Fig. 5, D and F; $p = 0.15$). These data corroborate the idea that Kv2.1-P410T channels inactivate by the same mechanism as Kv2.1 but preferentially from closed states.

The Effect of Kv2.1-P410T Prevails upon Co-expression with Kv2.1—The functional properties of Kv2.1-P410T channels resembled those of Kv2.1/Kv9.3 heteromers in all aspects that we have analyzed. To further assess the importance of this residue in the regulatory function of Kv9.3, Kv2.1-P410T was co-expressed with Kv2.1. We assumed a random assembly of both subunits, because the region guiding this process, the T1 domain, is identical for both subunits. Therefore, to favor a 2:2 stoichiometry that has been suggested to predominate in the assembly of Kv2.1 with modulatory α-subunits (30), Xenopus oocytes were co-injected with cRNA dilutions that gave a current ratio of 1:1 (Fig. 6A, at +40 mV Kv2.1 = 20.1 ± 2.6 nA; Kv2.1-P410T = 19.4 ± 3 µA; Kv2.1/Kv2.1-P410T = 18.1 ± 0.5 µA, $n = 7$–10). steady-state activation of channels resulting from the co-expression of Kv2.1 with Kv2.1-P410T was intermediate when compared with the respective homomeric channels (Fig. 6B; Kv2.1/Kv2.1-P410T $V_{50} = -4 ± 1.5$ mV, $n = 7$). Open-state inactivation of Kv2.1, evaluated as the fraction of the initial current that is left at the end of 32 s depolarizations to +40 mV ($I_{32s}/I_{0}$), was inhibited by co-expression of Kv2.1-P410T (Fig. 6, C and F; Kv2.1 current left = 15.1 ± 1.8%, $n = 24$; Kv2.1/Kv2.1-P410T current left = 42 ± 1.6%, $n = 7$). On the contrary, closed-state inactivation of Kv2.1 was accelerated by co-expression of Kv2.1-P410T (Fig. 6, E and F, after a 32-s pulse to −30 mV Kv2.1, current left = 59.7 ± 5.5%, $n = 16$;
Kv2.1/Kv2.1-P410T current left = 30 ± 1.8%, n = 6).

Thus, Kv2.1/Kv2.1-P410T heteromers inactivate faster from closed than open states, thereby recapitulating the shift in the state preference of inactivation that is central to the modulation of Kv2.1 by Kv9.3. The point mutation P410T therefore introduces a P domain (20–22, 43) and cardiac (23, 25) action potentials, hypoxic vasoconstriction of pulmonary arteries (7, 24), and insulin secretion of pancreatic β cells (44, 45). Open and closed Kv2.1 channels can inactivate (27), reducing the pool of available channels in a voltage-dependent manner. We show that both

**FIG. 3. The NRD of Kv9.3.** A, Kv2.1 and Kv2.1/Kv9.3 currents elicited by 10-s pulses to +40 mV. Kv2.1 shows pronounced inactivation from the open state, whereas Kv2.1/Kv9.3 does not. B, closed-state inactivation elicited by $P_{\text{c,inact}}$, at an inactivating potential of −30 mV ($V_{\text{inact}} = −30$ mV). The noninactivated fraction of the current ($I/I_{\text{max}}$) through Kv2.1 (○) and Kv2.1/Kv9.3 (●) is plotted as a function of the time spent at the inactivating potential. Closed-state inactivation is slow and incomplete for Kv2.1, whereas it is fast and complete for Kv2.1/Kv9.3. C, schematic drawing of the chimera Kv2.1$_{NRD}$ indicating the region of Kv2.1 that has been replaced by the corresponding amino acids (111–177) of Kv9.3. D, to analyze the voltage dependence of activation, Kv2.1 (○) and Kv2.1$_{NRD}$ (●) were activated by a 300-ms pulse to potentials ranging from −80 to +70 mV, applied in increments of 10 mV. Subsequently, the voltage was clamped at −40 mV, and the initial current in this segment was estimated from a mono-exponential fit to its decay. The relative open probabilities ($P_{\omega}/P_{\omega_{\text{max}}}$) derived from the initial currents were plotted against the voltage of the depolarization and fit with a Boltzmann function. The data are given as the means ± S.E. (n = 9–12). E, representative scaled currents of Kv2.1 and Kv2.1$_{NRD}$ elicited by a 32-s depolarizing pulse to +40 mV showing decreased open-state inactivation for K2.1$_{NRD}$ compared with Kv2.1. F, closed-state inactivation of Kv2.1 and Kv2.1$_{NRD}$ was measured by $P_{\text{c,inact}}$ at an inactivation potential of −30 mV ($V_{\text{inact}} = −30$ mV). G, bar diagram showing the fraction of currents ($I/I_{\text{max}}$) through Kv2.1 (white bars) and Kv2.1$_{NRD}$ channels (black bars) that did not inactivate from closed (−30 mV) or open states (+40 mV) during a 32-s pulse to the respective potential. The error bars represent the means ± S.E. (n = 6–24). The current traces in A and E are scaled to their maximum.

**DISCUSSION**

Ion channels containing Kv2.1 α-subunits mediate delayed rectifier K+ currents in a variety of cells, contributing to such diverse physiological processes as the repolarization of neuronal (20–22, 43) and cardiac (23, 25) action potentials, hypoxic vasoconstriction of pulmonary arteries (7, 24), and insulin secretion of pancreatic β cells (44, 45). Open and closed Kv2.1 channels can inactivate (27), reducing the pool of available channels in a voltage-dependent manner. We show that both
in this study should facilitate further analysis of the role of open- and closed-state inactivation in native currents.

Different proteins have evolved that contribute to the functional diversity of Kv channels. For example, α-subunits of the Kv1 and Kv4 subfamilies associate with Kv β-subunits (46) and Ca²⁺-binding proteins (47), respectively. The interaction with these intracellular proteins changes the surface expression (47–50) as well as the gating characteristics of the resulting channel complexes (46, 47, 51, 52). Kv2 α-subunits form heterotetrameric channels with modulatory α-subunits. These transmembrane proteins are the biggest group of Kv channel modifying proteins, with 10 members so far (7, 10–15), and their malfunction has been implied in the pathogenesis of such diverse conditions as epilepsy (53) and pulmonary hypertension (7). We previously characterized the modulatory α-subunit Kv9.3, which changes the state dependence of Kv2.1 inactivation, inhibiting open-state inactivation and accelerating closed-state inactivation. Such preferential closed-state inactivation has been suggested to participate in the control membrane excitability by modulating repetitive firing and back-propagation of action potentials in neurons as well as the repolarization of cardiac action potentials (28, 29). In this study we wanted to identify the structural determinants of this regulation.

**Fig. 4.** The involvement of S6 in the regulatory function of Kv9.3. A, alignment of the last transmembrane segment (S6) for one representative member from each Kv subfamily. Kv subunits were assigned into subfamilies following the guidelines of the HUGO Gene Nomenclature Committee (www.gene.ucl.ac.uk/nomenclature/genefamily/KCN.shtml) B, currents through Kv2.1 and Kv2.1-P410T elicited by a 32-s depolarizing pulse to +40 mV ($V_{\text{inact}}$). To determine voltage dependence of activation (inset) for Kv2.1 (●) and Kv2.1-P410T (○), 300-ms pulses from −80 to +70 mV were applied in 10-mV increments. Subsequently, the voltage was clamped to −40 mV, and the initial current in this segment was estimated from a mono-exponential fit to its decay. The relative open probabilities ($P_\text{open}/P_{\text{inact}}$) derived from the initial currents were plotted against the voltage of the conditioning pulses and fit with a Boltzmann function ($n = 9$–10). C, closed-state inactivation of Kv2.1 and Kv2.1-P410T measured by the pulse protocol illustrated ($P_{\text{inact}}, V_{\text{inact}} = −40$ mV). The currents in $B$ and $C$ were scaled to their maximum. D, time constants of inactivation of Kv2.1 (open symbols) and Kv2.1-P410T (filled symbols) measured by $P_{\text{inact}}$ (● and ○), $P_{\text{inact}}$ (● and ○), or both (● and ○), plotted as a function of the voltage of the inactivating pulse. The data in $D$ are represented as the means ± S.E. ($n = 4$–10).
Molecular Determinants of Modulatory K⁺ Channel Function

The effect of the modulatory α-subunit Kv8.1 was assigned to the 59 amino acids preceding S1 (30). This region called NRD contains the T1-S1 linker and layer 4, the membrane-facing surface, of the T1 domain, with part of its Zn²⁺ coordination site (54). The NRD of Kv8.1 in chimeric Kv2.1 channels caused slowing of activation and deactivation kinetics. Inactivation at potentials of maximal open probability was inhibited, whereas closed-state inactivation was not analyzed (30). Transferring the equivalent region of the Kv9.3 to Kv2.1 (Kv2.1NRD) had the same effect (Fig. 3). Activation, deactivation, and open-state inactivation were slowed. However, unlike Kv2.1/Kv9.3 heteromers, Kv2.1NRD channels show no significant closed-state inactivation. To our understanding, the effect of NRD on channel gating results from a disruption of the normal T1 function of Kv2.1 that has been observed following a number of different manipulations. For instance, deletion of a part of the N terminus (55), application of methylmethanesulfonate attaching a thiomethyl group to native N-terminal cysteines involved in Zn²⁺ coordination (56), as well as replacing part of the Kv2.1 N terminus by corresponding regions of Kv1.5 (57), Kv8.1 (30), and Kv9.3 (Fig. 3) all result in channels with slow activation, deactivation, and open-state inactivation. Where investigated (Kv9.3 and Kv1.5), closed-state inactivation of the corresponding channels was inhibited (Fig. 3, C and D) (57). This implies that NRD participates in the regulation of Kv2.1 channel gating by modulatory α-subunits but is alone insufficient to explain the effect of Kv9 subunits.

Conserved among all of the α-subunits from the subfamilies Kv1–Kv4 is a PXP motif in the distal part of S6. This region projects to the bundle crossing in the KcsA structure (58) and was proposed to cause a bend in the S6 of Kv channels based on sequence analysis, as well as blocker protection and Cd²⁺ bridging of introduced cysteines (59, 60). The intracellular activation gate of Kv channels is thought to coincide with this bend (61). Proline residues, which are potent helix breakers in aqueous solutions, are commonly found in transmembrane α-helices, where multiple but not single prolines cause bends (62). In modulatory α-subunits the second proline of the PXP motif is replaced by threonine, serine, alanine, or histidine, predicting the S6 to be straight. To investigate whether this exchange participates in the regulation of Kv2.1 by Kv9.3, the second proline of the PXP motif in Kv2.1 was mutated to the corresponding threonine of Kv9.3. The resulting channels, Kv2.1-P410T, show all hallmarks of Kv2.1/Kv9.3 heteromers (6). Steady-state activation is shifted to hyperpolarized potentials, and activation and deactivation kinetics are slowed. In particular, the state dependence of inactivation is reversed, causing Kv2.1-P410T to inactivate in a fast and complete manner from closed states, whereas open-state inactivation is inhibited. The inactivation mechanism appears to be conserved, because closed-state inactivation remains sensitive to extracellular K⁺ and insensitive to intracellular TEA. In addition, like for Kv2.1/Kv9.3 heteromers, recovery from inactivation is accelerated and displays reduced voltage and K⁺ dependence. These effects on channel gating prevail upon co-expression of Kv2.1-P410T with Kv2.1 in conditions favoring a 2:2 stoichiometry, or a 1:1 mix of the respective dilutions, Kv2.1/Kv2.1-P410T (gray bar), or a 1:1 mix of the respective dilutions, Kv2.1/Kv2.1-P410T (black bar) or a 1:1 mix of the respective dilutions, Kv2.1/Kv2.1-P410T (black circle).

The mechanism of Kv2.1 inactivation, which has been called U-type inactivation for its nonmonotonic voltage dependence (27), is unknown. It has been suggested to involve a narrowing of the selectivity filter, allowing Na⁺ to permeate inactivated channels (63), similar to what has been observed for C-type inactivation of Shaker channels (64). However, these data re-
main protein because the apparent Na⁺ conductance could be accounted for by changed intracellular K⁺ concentrations and thus changed K⁺ reversal potential under the particular experimental conditions (65). Here we show that mutation of the second proline of the S6 PXXP motif, thought to comprise the activation gate, accelerates closed-state inactivation and inhibits open-state inactivation, which in turn are suggested to lead to the same inactivated conformation. Interestingly, the mutations in the two residues surrounding the second proline of the S6 PXXP motif of Kv4.1 have been shown to disrupt closed-state inactivation of the respective channels, which was suggested to occur at the internal vestibule of the pore (66). However, internal TEA and extracellular K⁺ are thought to bind on either side of the selectivity filter (33, 39), as well as disruption of the intracellular T1 domain and T1-S1 linker affect Kv2.1 inactivation. Thus, further experiments delineating which of the above manipulations affects the inactivation gate directly and which can be accounted for by allosteric effects on a distant gate are needed.

Acknowledgments—We are very grateful to Walter Stuhmer for generous support. We thank the technical personnel in the Research Group of Molecular & Cellular Neuropharmacology and S. Schapermeier for help with the oocyte preparation and R. Schliephacke for computer support. We thank Drs. Florentina Soto and Paola Pedarzani for critical help with the manuscript. We thank Dr. E. Posada, Dr. M. Camacho, Dr. E. Rey, and I. Rugeles for support.

REFERENCES

1. Hille, B. (2001) Ionic Channels of Excitable Membranes, 3rd Ed., Sinauer Associates, Sunderland, MA.
2. MacKinnon, R. (1991) Nature 350, 232–235
3. Christie, M. J., North, R. A., Osborne, P. B., Douglass, J., and Adelman, J. P. (1990) Neuron 4, 405–411
4. Isacoff, E. Y., Jan, Y. N., and Jan, L. Y. (1990) Nature 345, 530–534
5. Ruppersberg, J. P., Schroter, K. H., Sakmann, B., Stocker, M., Sewing, S., and Ruppersberg, J. P. (1998) J. Physiol. 517, 473–498
6. Kerschensteiner, D., and Stocker, M. (1999) J. Neurosci. 19, 8685–8693
7. Patel, A. J., Lazdunski, M., and Honore, E. (1997) Nature 389, 248–257
8. Hugnot, J. P., and Tager, J. M. (1997) J. Physiol. 509, 553–557
9. Kirschensteiner, D., and Stocker, M. (1999) Biophys. J. 77, 248–257
10. Patel, A. J., Lazdunski, M., and Honore, E. (1997) Biophys. J. 76, 6615–6625
11. Salinas, M., de Weille, J., Guilleumaire, E., Lazdunski, M., and Hugnot, J. P. (1997) J. Biol. Chem. 272, 8774–8780
12. Salinas, M., Duprat, F., Heurtault, C., Hugnot, J. P., and Lazdunski, M. (1997) J. Biol. Chem. 272, 24371–24379
13. Kramer, J. W., Post, M. A., Brown, A. M., and Kirsch, G. E. (1998) Am. J. Physiol. 274, C1501–C1510
14. Castilloano, A., Chiara, M. D., Mellstrom, B., Molina, A., Monje, F., and Lu, G. W. (1997) J. Neurosci. 17, 4652–4661
15. Ottevanger, N. A., Roos, A., and Denys, D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7986–7991
16. Hugnot, J. P., Salinas, M., Lasage, P., Guilleumaire, E., de Weille, J., Heurtault, C., Mattei, M. G., and Lazdunski, M. (1996) EMBO J. 15, 3322–3331
17. Stocker, M., and Kirschensteiner, D. (1998) Biochem. Biophys. Res. Commun. 248, 927–934
18. Stocker, M., Hugnot, J., and Kirschensteiner, D. (1999) J. Neurosci. 19, 1725–1734
19. Post, M. A., Kirsch, G. E., and Brown, A. M. (1996) FEBS Lett. 399, 177–182
20. Li, M., Jan, Y. N., and Jan, L. Y. (1992) Science 257, 1225–1230
21. Shen, N. V., Chen, X., Boyer, M. M., and Pfaffinger, P. J. (1995) Neuron 11, 67–76
22. Kirsch, A., Paffinger, P. J., Stevens, C. F., and Choe, S. (1998) Nature 392, 945–948
23. Da, J., Haak, L. L., Phillips-Tansy, E., Russell, J. T., and Mcbain, C. J. (2000) J. Physiol. 522, 19–31
24. Blaine, J. T., and Ribera, A. B. (2001) J. Neurosci. 21, 1473–1480
25. Quattrochi, E. A., Marshall, J., and Kaczmarek, L. K. (1994) Neuron 12, 73–86
26. Schultz, J. H., Volk, T., and Ehme, H. (2001) Circ. Res. 88, 483–490
27. Smirnov, S. V., Beck, N., Tammaro, P., Ishii, T., and Aaronson, P. I. (2002) J. Physiol. 538, 867–878
28. Xu, H., Barry, D. M., Li, H., Brunet, S., Guo, W., and Nérbonne, J. M. (1999) Circ. Res. 85, 623–633
29. Monod, J., Wyman, J., and Changeux, J.-P. (1965) J. Mol. Biol. 12, 88–118