Modes of Regulation of Shab K⁺ Channel Activity by the Kv8.1 Subunit*

(Received for publication, October 1, 1996, and in revised form, December 30, 1996)

Miguel Salinas, Jan de Weille, Eric Guillemare, Michel Lazdunski‡, and Jean-Philippe Hugnot
From the Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, 660 route des Lucioles,
Sophia Antipolis, 06560 Valbonne, France

The Kv8.1 subunit is unable to generate K⁺ channel activity in Xenopus oocytes or in COSm6 cells. The Kv8.1 subunit expressed at high levels acts as a specific suppressor of the activity of Kv2 and Kv3 channels in Xenopus oocytes (Hugnot, J. P., Salinas, M., Lesage, F., Guillemare, E., Weille, J., Heurteaux, C., Mattéi, M. G., and Lazdunski, M. (1996) *EMBO J.* 15, 3322–3331). At lower levels, Kv8.1 associates with Kv2.1 and Kv2.2 to form hybrid Kv8.1/Kv2 channels, which have new biophysical properties and more particularly modified properties of the inactivation process as compared with homopolymers of Kv2.1 or Kv2.2 channels. The same effects have been seen by coexpressing the Kv8.1 subunit and the Kv2.2 subunit in COSm6 cells. In these cells, Kv8.1 expressed alone remains in intracellular compartments, but it can reach the plasma membrane when it associates with Kv2.2, and it then also forms new types of Kv8.1/Kv2.2 channels. Present results indicate that Kv8.1 when expressed at low concentrations acts as a modifier of Kv2.1 and Kv2.2 activity, while when expressed at high concentrations in oocytes it completely abolishes Kv2.1, Kv2.2, or Kv3.4 K⁺ channel activity. The S6 segment of Kv8.1 is atypical and contains the structural elements that modify inactivation of Kv2 channels.

Voltage-gated K⁺ channels (Kv) serve a wide range of functions including the regulation of cardiac pacemaking, action potentials, and neurotransmitter release in excitable tissues, as well as hormone secretion, cell proliferation, cell volume regulation, and lymphocyte differentiation in non-excitable tissues (2). The diversity of K⁺ channel functions is reflected by the diversity of K⁺ channel structures. Delayed rectifier K⁺ channels are constituted by 2 types of subunits called α and β (3). A large number of genes encode the different α pore-forming subunits (18 genes cloned in mammals (4)), and an increasing number of β-itory subunits are being discovered (5–8). Different gene expressions in different cells or at different times of development associated with the possible formation of heteromultimeric channels containing different types of α- and β-subunits probably allow individual cells to acquire their own characteristics of K⁺ current properties.

Sequence similarities among members of the Kv family were initially used to define 4 subfamilies (Kv1, Kv2, Kv3, Kv4) of α-subunits (9–12). The members within a given family share a large percentage of sequence identity (>70%) while this percentage falls to ~40% among members of different subfamilies. Four additional types of α-subunits (Kv5.1 (IK8 (13)), Kv6.1 (K13 (13)), Kv7.1 (14), Kv8.1 (1)) have been recently cloned. All subunits belonging to the Kv1 to Kv4 subfamilies have been functionally expressed in Xenopus oocytes. Elicited K⁺ currents display a large variety of electrophysiological characteristics reminiscent of the variety of K⁺ currents recorded in vivo. Particularly, large variations in inactivation characteristics have been observed ranging from $\tau_{\text{inact}} \approx 30$ ms for a very fast inactivating channel, such as (Kv1.4) (15), to $\tau_{\text{inact}} \approx 15$ s for a slow inactivating one, such as Kv2.1 (16). K⁺ channel inactivation occurs by at least two distinct mechanisms. The N-type inactivation is usually quite rapid with a time constant in the milliseconds range. It occurs by a “ball and chain” mechanism involving the NH₄-terminal cytoplasmic domain, which acts as a tethered blocker that occludes the pore channel in its open state and causes inactivation (17–19). C-type inactivation is generally slower and appears to involve the COOH-terminal sequence of the α-subunits (20–22).

Although the Kv5.1 and Kv6.1 proteins apparently have the structural hallmarks of functional K⁺ channel α-subunits, their expression in oocytes fails to induce K⁺ currents (13). The Kv8.1 subunit also displays the structural characteristics of a K⁺ channel subunit and is highly expressed in the brain. As Kv5.1 and Kv6.1, it does not lead to expression of K⁺ currents when produced in Xenopus oocytes. However, upon coexpression it is able to specifically abolish K⁺ currents generated by channels formed by Kv2.1, Kv2.2, and Kv3.4 subunits (1), and this inhibition is associated with the formation of multimers with these other subunits.

This paper extends previous work which was limited to Kv8.1 expression in Xenopus oocytes. It reports an analysis of Kv8.1 expression in the COS mammalian cell line. In this system, the Kv8.1 subunit is normally retained in cytoplasmic compartments. It requires coexpression with Kv2.2 to bring the subunit to the plasma membrane. Kv8.1 then induces no inhibition of the Kv2.2 current in this system but instead produces a drastic modification of the kinetic properties of Kv2.2 and particularly of its inactivation. The same effect can in fact be seen on Kv2.1 and Kv2.2 currents in Xenopus oocytes expressing moderate levels of Kv8.1, while a total inhibition is seen with higher levels of Kv8.1. Therefore, depending on its level of expression, Kv8.1 can either modify the kinetics of the Kv2 channels or completely abolish their activity. Site-directed mutagenesis has been used to show that Kv8.1 effects on the Kv2 current are mediated by the presence of singular amino acids located in the S6 domain of the Kv8.1 subunit.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection—COSm6 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and supple-
mented with 10% fetal calf serum and antibiotics (60 μg/ml penicillin, 50 μg/ml streptomycin). One day before transfection, 10^6 cells were plated onto 35-mm Petri dishes for an electrophysiology experiment and 15 x 10^3 cells onto 15-mm plates for indirect immunofluorescence microscopy. The cells were transfected by a modification of the DEAE-dextran/chloroquine method (23) using 0.6 μg of supercoiled DNA per cm^2 of cell culture. For electrophysiology studies, we co-transfected a plasmid encoding the CD8 receptor, which allows direct visualization of transfected cells by antibody-coated beads (24).

Electrophysiology on COS Cells—Voltage-clamp experiments were carried out using the whole-cell suction-pipette technique. The intracellular (pipette) solution contained 150 mM KCl, 1 mM MgCl2, 2 mM EGTA, 10 mM HEPES-KOH, pH 7.2. The extracellular solution was 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM HEPES-NaOH, pH 7.3. Pipettes were coated with Sylgard resin to reduce their capacity. Electrical signals were digitized and stored on a hard disk by a personal computer for further analysis. Experiments were carried out at room temperature (21 ± 2 °C).

Indirect Immunofluorescence Microscopy—The cells that were grown on glass coverslips were fixed for 15 min with 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS). After rinsing twice with PBS, the cells were permeabilized by incubation for 10 min with 0.1% Triton X-100. The sites of nonspecific binding were blocked by 2 h of incubation with 5% goat serum, 2% bovine serum albumin in PBS at room temperature. The cells were then incubated for 2 h with a mixture of M2 monoclonal antibody (1:50 dilution, Eastman Kodak Co.) or polyclonal antibodies against the Kv1.5 subunit (1:100) with a BS solution (2% bovine serum albumin in PBS), following by washing with PBS and incubation for 1 h with fluorescein isothiocyanate-conjugated goat antimouse Ig (1:100, Sigma) or fluorescein (5-[4,6-dichlorotiazin-2-yl]amino)fluorescein-conjugated F(ab')2 fragment of a goat anti-rabbit Ig (1:100, Immunotech) in PBS. After washing in PBS then in 1 ml Tris-HCl, pH 7.5, the cells were mounted in Vectashield Medium (Vector Laboratories, Inc.) and observed with a Leitz Aristoplan microscope (Wild Leitz) using an interference blue (fluorescein isothiocyanate filter) and a 40× or oil-immersion 100× lens.

Plasmid Constructions and Mutagenesis—The Kv8.1 hamster cDNA was cloned into the pRC/CMV vector (Invitrogen) for expression in COS cells. To introduce mutations in this plasmid, a two-step polymerase chain reaction method using sense and antisense mutant primers was used (25). The dominant-negative mutant Kv8.1 ΔA, obtained by this technique, bears a deletion from residue 426 to the COOH-terminal end of the protein. After each mutation the Kv8.1 open reading frame was entirely resequenced (dye terminator kit, Applied Biosystems). The cells that were grown on glass coverslips were transfected by a modification of the DEAE-dextran/chloroquine method (23) using 0.6 μg of supercoiled DNA per cm^2 of cell culture. One day before transfection, 10^5 cells were plated onto 15-mm plates for indirect immunofluorescence microscopy (Fig. 1).

Expression of the Kv2.2 subunit in COS cells was realized with a pcDNAI vector. To create NH2-terminal tagged Kv2.2 protein, a polym-merase chain reaction fragment (PWO Taq polymerase, Boehringer Mannheim) containing Kv2.2 open reading frame and a 3’-Apol restriction site was cloned in a HpaI- and Apol-digested Flag-pRC/CMV vector.

Kv2.1 subunit mutations were generated by oligonucleotide-directed mutagenesis on a single-stranded template (26) derived from Bluescript II vector containing Kv2.1 cDNA (27). All mutational changes were checked by sequence determination.

cRNA Synthesis, Injection, and Electrophysiological Measurement in Xenopus Oocytes—Preparation of oocytes, cRNA synthesis, injection (50 nl), and electrophysiological measurements have been previously described (1, 28). We have previously reported that in oocytes as well as in cell lines high magnitude currents could show pronounced modifications when compared with currents of lower intensity (28, 29). Therefore, in all this work, attention was paid to always compare currents of similar and relatively low intensity.

RESULTS

Modification of Kv8.1 Subcellular Localization upon Kv2.2 Coexpression in COS Cells—Despite numerous attempts to express the Kv8.1 subunit in various expression systems including Xenopus oocytes and Chinese hamster ovary and COS cell lines, K+ currents could never be recorded (1). One possible reason for this lack of success is an inadequate cellular localization of the expressed Kv8.1 protein. To check that point, an 8-aminocaproate epitope, which can be recognized by a monoclonal antibody, was added to the Kv8.1 protein, and protein localization was examined by indirect immunofluorescence on Triton-permeabilized COS cells (Fig. 1). Transfected cells expressing the Kv8.1 protein tagged on the NH2-terminal or on the COOH-terminal end show a very strong fluorescent staining localized at the perinuclear region as well as a fine reticular network extending through the cytoplasm (Fig. 1, A, B, and C). In the many experiments that have been done, a surface labeling of Kv8.1 was never observed. Confocal microscopy experiments also failed to detect the Kv8.1 protein at the plasma membrane (data not shown).

To verify that the COS cells were able to correctly express well known functional K+ channel α-subunits and integrate them at the plasma membrane, cells were also transfected with a Kv1.5-expressing vector (30). Protein detection in this case was made with polyclonal antibodies specific for Kv1.5. As shown in Fig. 1D, Kv1.5-expressing cells, which produce K+ currents with the expected biophysical properties (30), show a typical surface labeling unlike Kv8.1-expressing cells.

---

1 F. Lesage, unpublished data.

FIG. 1. Immunofluorescence localization of Kv subunits in COS cells. A and B, cells expressing NH2-tagged Kv8.1 subunit; C, COOH-tagged Kv8.1 subunit; D, Kv1.5 subunit; E, NH2-tagged Kv2.2 subunit; F and G, NH2-tagged Kv1.5 and Kv2.2 subunits. Detection is performed with M2 monoclonal antibody except in D where an anti-Kv1.5 rabbit polyclonal antibody was used. Arrowheads indicate perinuclear fluorescence.
Electrophysiological recording of cells transfected with this subunit show typical delayed outward rectifier $K^+$ currents with a slow activation pattern. A minority of cells showed a fluorescence distribution resembling that obtained with Kv8.1 alone, i.e. an apparent lack of surface membrane localization. In contrast, the majority of cells displayed a fluorescent staining distribution that observed for the Kv2.2 localization, i.e. a perinuclear accumulation. The Kv8.1 subunit is transported to the plasma membrane when it is coexpressed with the functional Kv2.2 subunit.

The Kv8.1 Subunit Modifies Kv2.2 Current Characteristics—

The effect of Kv8.1/Kv2.2 coexpression was then explored electrophysiologically. Expression of the Kv2.2 subunit alone resulted in a typical delayed outward rectifier $K^+$ current with a slow inactivation process (Fig. 2B). Expression of Kv8.1 alone did not give rise to a detectable $K^+$ current (not shown). Cells were then transfected with a mixture of Kv2.2 and Kv8.1-expressing vectors using 1:5 to 1:50 molecular ratios. Increasing amounts of Kv8.1 decreased the intensity of the Kv2.2 current (Fig. 2A). However, this decrease was not specific because co-transfection of Kv2.2-expressing cells with increasing amounts of $\beta$-galactosidase produced the same effect (Fig. 2A). This lack of specificity was confirmed by a coexpression of Kv2.2 with a non-functional deleted form of the Kv1.5 subunit (Kv1.5Δ30) which, in addition to being non-functional, cannot associate with Kv2.2 since Kv1 and Kv2 subunits cannot form

Fig. 3. Effect of Kv8.1 coexpression with Kv2.1, Kv2.2, and Kv3.4 subunits in Xenopus oocyte. A, influence of the molecular ratio of Kv8.1 cRNA/Kvx cRNA on $K^+$ current inhibition ($n = 10$). Current amplitudes were measured at $+30$ mV, and $100\%$ of currents corresponds to Kvx current amplitudes without Kv8.1. Kvx corresponds to Kv2.2 (●), Kv2.1 (○), and Kv3.4 (). B, C, and D, normalized mean current traces ($n = 10$) measured at $+30$ mV from oocytes co-injected with Kv8.1 cRNA and Kv3.4 cRNA (B, Kv3.4/Kv8.1, 1:1; C, Kv2.1 cRNA (Kv2.1/Kv8.1, 1:0.03); or D, Kv2.2 cRNA (Kv2.2/Kv8.1, 1:0.03)). E and F, voltage-dependent inactivation curves of Kv2.1, Kv2.2, and Kv8.1 (1:0.03) current. The duration of conditioning prepulses was 55 s, and test pulses were $+30$ mV.
Molecular ratios of injected plasmids are those indicated in Fig. 3. For Kv2.1/(NH5Kv8)-Kv1.3 cojunction with an 0.1 ratio was used, corresponding to a 50% inhibition of Kv2.1 current. Activation parameters were elicited by 500-ms depolarizing pulses from −80 mV to +60 mV in 10-mV increments with a holding potential at −80 mV. The current (I) was recorded at the end of the pulse and plotted against membrane potential (V). The membrane conductance (G) was calculated for a given command voltage (V) and peak current responses (I) from the expression \( G = I / (V - E) \), where \( E \) is the K⁺ reversal potential. G was normalized versus a maximum (Gmax) and plotted against Vmax. These plots were fitted to a Boltzmann distribution of the form, \( G / G_{\text{max}} = 1 / (1 + \exp (E_{0.5,\text{act}} - V / k_{\text{act}})) \), where \( E_{0.5,\text{act}} \) is the midpoint of activation, and \( k_{\text{act}} \) is the slope factor. \( \tau_{\text{act}} \) corresponds to the time constant of activation for a monoeponential fitting of the +40-mV activation curve. Inactivation parameters were determined by 500-ms step depolarizations to +30 mV preceded by 10-s prepulses from −120 mV to +30 mV. Current (I) was recorded at the end of 500-ms pulses, normalized by a maximum (I), and plotted against prepulse potential (V). This representation is the inactivation curve that can be fitted with a form of the Boltzmann equation, \( I / I_{\text{max}} = 1 / (1 + \exp (E_{0.5,\text{inact}} - V / k_{\text{inact}})) \), % inactivation is the midpoint of inactivation, and \( k_{\text{inact}} \) is the slope factor. The percent of inactivated current was calculated from the ratio of measured currents at 10 s and at 0.1 s from pulses at +30 mV. Values are the mean ± S.E. for five oocytes measured.

| Activation | % inactivated current at 10 s | Inactivation |
|------------|-----------------------------|--------------|
| \( \tau_{\text{act}} \) | \( E_{0.5,\text{act}} \) | \( E_{0.5,\text{inact}} \) |
| ms         | mV                          | mV           |
| Kv2.1      | 18.5 ± 2.8                  | 8.8 ± 0.6    | 61 ± 2 | −21.5 ± 0.8 |
| Kv2.1 + Kv8.1 | 27.2 ± 2.5                 | 10.2 ± 0.9   | 29 ± 1 | −62.9 ± 0.8 |
| Kv2.1 + (NH5Kv8)-Kv1.3 | 23.4 ± 2.1               | 8.5 ± 0.9    | 70 ± 1 | −19 ± 1.0  |
| Kv2.2      | 32.2 ± 2.1                  | 13.2 ± 0.5   | 60 ± 1 | −13.5 ± 0.8 |
| Kv2.2 + Kv8.1 | 45.4 ± 3.6                 | 8.5 ± 1.4    | 23 ± 1 | −55.9 ± 1.8 |
| Kv3.4      | ND*                         | 27.5 ± 0.6   | 85 ± 3 | −20.8 ± 0.8 |
| Kv3.4 + Kv8.1 | 25.5 ± 0.9                 | 29.5 ± 0.9   | 89 ± 3 | −20.4 ± 1.2 |

* ND, not determined.

heteropolymers (9, 31, 32). Again the same decrease of Kv2.2 current was observed with Kv2.2/Kv1.5 Δ mixture in a 1:50 ratio (Fig. 2A). To verify that Kv2.2 and Kv8.1 subunits interact in COS cells we created a truncated form of the Kv8.1 subunit since cells cotransfected with a mixture of the Kv2.2 and Kv8.1 plasmids (1:5 ratio) showed no detectable K⁺ channel activity (33). Again the same decrease of Kv2.2 current was observed with a Kv2.2/Kv1.5 Δ mixture in a 1:50 ratio (Fig. 2A). To verify that Kv2.2 and Kv8.1 subunits interact in COS cells we created a truncated form of the Kv8.1 subunit since cells cotransfected with a mixture of the Kv2.2 and Kv8.1 plasmids (1:5 ratio) showed no detectable K⁺ channel activity (33).

Table I. Parameters of activation and inactivation for Kv2.1, Kv2.2, and Kv3.4 expressed alone or coexpressed with the Kv8.1 subunit in Xenopus oocytes

| Channel Activity by Kv8.1 Subunit | Modification of Kv2 Currents at Low Levels of Expression and Total Inhibition of Kv2 and Kv3 Currents at Higher Concentrations |
|-----------------------------------|-------------------------------------------------------------------------------------------------------------------------------|

**Kv8.1 Expression in Xenopus Oocytes Has a Dual Effect:**
Regulation of Shab K⁺ Channel Activity by Kv8.1 Subunit

A

Kv8.1 403
Kv2.1 381
Kv2.2 389
Kv1.3 399
Kv3.4 444
Kv4.1 377
Kv7.1 429

FIG. 4. Expression of mutated Kv2.1 subunits in Xenopus oocytes and mutated Kv8.1 subunits in COSm6. A, alignment of Kv S6 transmembrane segments. Identical amino acids are indicated by negative print, and homologous residues are shaded. Kv2.1M1 to Kv2.1M8 indicate the Kv8.1 residues introduced in the Kv2.1 subunit. B, current traces recorded 2 days after oocytes injection of indicated cRNAs. The holding potential is −80 mV and −120 mV for Kv2.1M1 and Kv2.1M7 mutations. Outward currents are evoked by +10-mV increments from holding potential to +30 mV. C, an estimation of inactivation kinetics of Kv2.2-generated K⁺ currents in COSm6 cells was made by taking the ratio of the current inactivated during a 10-s pulse to +30 mV over the peak current. Co-transfection of Kv2.2 with Kv8.1 decreases the percentage of inactivation from 42 ± 5% to 16 ± 2% (p < 0.01%). Introduction of the M3M7 mutations (A428P/I416C) in Kv8.1 (mutant Kv8.1M3M7) largely restores the inactivation properties of Kv2.2 (35 ± 4% of inactivation, p < 0.1%). The inset shows two representative current traces.

upon injection of the (NH5Kv8)-Kv1/Kv2.2 mixture in a 0.1 ratio (data not shown). The steady-state activation and τact of the remaining current are the same as those of the Kv2.1 current, and the properties of inactivation are only slightly modified as compared with Kv2.1 (Table 1). Therefore the COOH-terminal end of Kv8.1 which contains the S6 segment seems to be important for the modifications of the kinetic properties of Kv2.1.

The alignment of the sequence of the S6 segment of Kv8.1 with S6 segments of other functional Kv subunits is presented in Fig. 4A. Kv8.1 is atypical in 4 positions in this segment: (i) an alanine is localized at position 412 instead of a glycine in all other functional Kv subunits (M1, Fig. 4A); (ii) an isoleucine at 420 replaces a conserved valine residue (M2); (iii) an alanine is found at 428 instead of a proline (M3) in other functional Kv subunits; (iv) at position 433, an arginine is found instead of a glycine in all other functional Kv subunits (M1, Fig. 4B, C).

To explore the role of these amino acids in K⁺ channel properties, the Kv2.1 subunit itself was mutated to introduce these single residues peculiar to the Kv8.1 sequence (mutants Kv2.1M1 to Kv2.1M4, Fig. 4, A and B). Four additional mutations were carried out corresponding to other amino acids specific to the Kv8.1 sequence, but not situated at necessarily conserved positions in other sequences. These mutations are L384T/L385T (Kv2.1M5, Fig. 4A), G391F/L392M (Kv2.1M6), C394I (Kv2.1M7), and I401L (Kv2.1M8).

Current traces recorded after expression of mutated Kv2.1 proteins in Xenopus oocytes are shown in Fig. 4B. Corresponding electrophysiological parameters are reported in Table II. All the mutations significantly alter current properties.

Channel opening is most dramatically affected by mutation Kv2.1M3 (Pro replaced by Ala). This mutation produces a 30-fold decrease of the rate of activation (τact 501.5 ms versus 17.7 ms for wild type Kv2.1) together with a +17.6 mV shift of the activation midpoint (+5.4 mV versus −10.5 mV for wild type). Other mutations induce smaller modifications of the activation process ranging from a 1.6-fold acceleration for Kv2.1M2 (τact 10.9 ms) to a 1.5-fold decrease for Kv2.1M1 (τact 26.3 ms). Changes in activation midpoints ranged from a −13.7 mV shift for Kv2.1M7 (E0.5(τact) = +4.7 mV) to a +17.6 mV change for Kv2.1M3 (E0.5(τact) = +26.8 mV).

Except for the Kv2.1M5 mutation, all mutations produced a reduction of the inactivation process of the Kv2.1 channel. The most spectacular modifications are observed for K⁺ currents produced by Kv2.1M3 (P406A) and Kv2.1M7 (C394I) mutants that inactivate only by 2–10% during a 10-s depolarizing pulse to +30 mV, while the control Kv2.1 current inactivates by 67% in the same conditions (Fig. 4B). Both mutants also show a considerable shift of the inactivation midpoint toward positive (Kv2.1M3, +56 mV shift) or negative potentials (Kv2.1M7, −42 mV shift).

Since the Kv2.1M3 (P406A) and Kv2.1M7 (C394I) mutations strongly altered the Kv2.1 current, the influence of these positions on the effect of Kv8.1 on Kv2 expression was also examined. Three mutated Kv8.1 subunits were prepared, Kv8.1M3 (A428P), Kv8.1M7 (I416C), and Kv8.1M3M7 (A428P, I416C), and their effect on Kv2.2 current was tested in COS cells. None of these mutants produced a K⁺ current by itself. The Kv8.1M3 as well as the Kv8.1M7 mutants influenced the Kv2.2 current exactly as the wild type Kv8.1. Particularly, the effect of these Kv8.1 mutants on the inactivation of Kv2.2 currents was not significantly different from the effect of Kv8.1 itself on Kv2.2 current inactivation (Fig. 4C). The Kv8.1 subunit carrying both A428P and I416C mutations (Kv8.1M3M7) behaved differently. Activation kinetics for Kv8.1M3M7/Kv2.2 currents were intermediate between those of Kv2.2 and Kv8.1/Kv2.2 currents (not shown), the E0.5 of inactivation was similar to that of the Kv2.2 current (Kv2.2, −37.1 mV; Kv2.2/Kv8.1, −48.1 mV; Kv2.2/Kv8.1M3M7, −36.0 mV), and K⁺ current inactivation was more pronounced than for the Kv8.1/Kv2.2 current (Fig. 4C).

**DISCUSSION**

We recently reported the cloning of Kv8.1, a brain-expressed K⁺ channel α-subunit sharing only 40% identity with other subunits of the Kv channel family (1). Kv8.1 cannot by itself generate currents in Xenopus oocytes. However, Kv8.1 can specifically inhibit currents mediated by Kv2 (Kv2.1 and
Oocyte currents were analyzed as described in Table I. Activation parameters were elicited by 500-ms pulses (3 s for M3) from −80 mV to +60 mV in 10-mV increments with a holding potential at −80 mV (−120 mV for M1 and M7). $E_{0.5\text{act}}$ is the midpoint of activation, and $k_{\text{act}}$ is the slope factor. The time constants for activation ($\tau_{\text{act}}$) are determined at +40 mV. Inactivation parameters were determined by 500-ms step depolarizations from −80 mV to +30 mV preceded by 20-s prepulses from −80 mV (−120 mV for M1 and M7) to +30 mV (+70 mV for M3). $E_{0.5\text{inact}}$ is the midpoint of inactivation, and $k_{\text{inact}}$ is the slope factor. The percent of inactivated current has been calculated for a pulse at +30 mV at 10 s after the peak current. Values are the mean ± S.D. for 6 to 11 oocytes measured.

| Kv2.1 | 17.7 ± 1.6 | 9.2 ± 1.2 | 11.3 ± 0.3 | 67 ± 7 | −24.6 ± 2.4 | −7.4 ± 0.9 |
| Kv2.1M1 | 26.3 ± 8.2 | 13.6 ± 2.5 | 18.4 ± 1.0 | 41 ± 6 | −58.5 ± 4.1 | −13.4 ± 1.8 |
| Kv2.1M2 | 10.9 ± 1.4 | −2.4 ± 2.8 | 16.6 ± 0.8 | 19 ± 2 | −40.5 ± 2.8 | −7.9 ± 0.6 |
| Kv2.1M3 | 50.1 ± 85.8 | 26.8 ± 2.0 | 12.1 ± 0.6 | 10 ± 3 | +31.4 ± 7.2 | −15.8 ± 0.7 |
| Kv2.2 | 11.6 ± 1.1 | 14.3 ± 1.2 | 13.0 ± 0.7 | 57 ± 2 | +1.0 ± 1.9 | −13.9 ± 1.2 |
| Kv2.5M | 15.2 ± 0.8 | 6.8 ± 0.9 | 11.4 ± 0.4 | 76 ± 6 | −30.8 ± 2.0 | −9.8 ± 1.7 |
| Kv2.6M | 20.1 ± 1.4 | 12.6 ± 1.9 | 13.8 ± 1.5 | 14 ± 3 | −37.0 ± 3.4 | 12.5 ± 1.0 |
| Kv2.7M | 11.1 ± 1.3 | −4.7 ± 5.3 | 20.0 ± 1.6 | 2 ± 1 | −66.1 ± 4.4 | 13.3 ± 1.0 |
| Kv2.1M8 | 12.8 ± 1.0 | 3.0 ± 5.3 | 9.9 ± 1.6 | 26 ± 4 | −24.2 ± 2.7 | −9.6 ± 2.3 |

Kv2.2) and Kv3 (Kv3.4) subunits, and immunoprecipitation studies have shown that this is due to the formation of Kv8.1/Kv2 heteropolymers. This paper first analyzes the properties of expression of Kv8.1 in the mammalian COS cell line. Again, no K⁺ current could be recorded from these cells when they expressed only Kv8.1. One important reason for this lack of expression is an inadequate subcellular localization of Kv8.1. Identification of the Kv8.1 protein with antibodies indicates that it remains in the endoplasmic reticulum and is apparently unable to reach the surface membrane (Fig. 1, A, B, and C). This is unlike other Kv subunits that generate K⁺ currents in these COS cells such as Kv1.5 and Kv2.2 which, as expected, are found to be localized at the plasma membrane (Fig. 1, D and E). Intracellular accumulation of Kv8.1 corresponds to an intrinsic property of this particular Kv channel subunit. However, Kv8.1 is transferred to the plasma membrane, if it is coexpressed with Kv2.2 (Fig. 1, F and G), a subunit which interacts with Kv8.1 (1), and which, when expressed alone, forms functional K⁺ channels at the surface membrane. Similar situations where a channel protein is not able by itself to reach the plasma membrane and requires the association with other subunits for proper movement to the surface are not unusual. One similar example concerns subunits of the amiloride-sensitive Na⁺ channel (35). Since no known endoplasmic reticulum localization sequence is found in the Kv8.1 subunit, it is probable that endoplasmic reticulum retention arises from protein misfolding and/or inefficient assembly. Association with the Kv2.2 subunit, which has access by itself to the plasma membrane, corrects the defect and facilitates Kv8.1 transport. The Kv2.2/Kv8.1 complex, once it reaches the surface membrane, forms a functional K⁺ channel with activation and inactivation properties that are different from those of the Kv2.2 channel.

Coexpression of Kv8.1 and Kv2.2 proteins in COS cells does not lead to a specific K⁺ current reduction as compared with expression of Kv2.2 alone. Only large Kv8.1/Kv2.2 plasmid ratios (50:1) lead to smaller K⁺ currents. However, the latter effects are not specific since they are also observed in Kv2.2-expressing cells transfected with a high β-galactosidase/Kv2.2. The reduction of the K⁺ current magnitude is then due to a decrease of Kv2.2 expression due to competition between the two plasmids at the transfection, transcription, and/or translation levels. Conversely, in Xenopus oocytes, dramatic K⁺ current reductions eventually leading to complete inhibition of Kv2.1, Kv2.2, and Kv3.4 currents were observed upon injection of Kv8.1 with sufficiently high Kv8.1/Kv2.1, Kv8.1/Kv2.2, or Kv8.1/Kv3.4 cRNA ratios (see Fig. 5 in Ref. 1). In that case, inhibitions of K⁺ currents correspond to a very specific process and are linked to the heteropolymeric interaction of the Kv8.1 subunit with Kv2 and Kv3 subunits (1). It is probable that, as in COS cells, Kv8.1 normally stays in intracellular compartments in Xenopus oocytes, and that when expressed at a high level Kv8.1 acts as a dominant trapping subunit that retains Kv2 and Kv3 subunits in intracellular membranes and prevents them from reaching the surface membrane.

The major effect of the coexpression of Kv8.1 with Kv2.2 in COS cells concerns the inactivation process. Its voltage-dependence is shifted toward negative potentials, and its rate of inactivation is considerably slowed down. Clearly then, the Kv2.2/Kv8.1 heterotetramer has kinetic properties different from Kv2.2 homotetramers. Interestingly enough, when low concentrations of Kv8.1 are expressed in Xenopus oocytes to produce only a partial inhibition of Kv2.1 or Kv2.2 channels, the remaining K⁺ channel activity is unlike the activity observed for Kv2.1 or Kv2.2 channels expressed in the absence of Kv8.1. The kinetics are drastically altered indicating that new types of channels are formed corresponding to Kv2.1/Kv8.1 or Kv2.2/Kv8.1 heterotetramers. The characteristics of these new channels are similar to those observed upon expression of Kv2.2 and Kv8.1 in COS cells. They have a changed voltage dependence of their inactivation (a shift as large as −47 to −42 mV for both the Kv2.1/Kv8.1 and Kv2.2/Kv8.1 combinations), and they have a largely decreased rate of inactivation. Even if results obtained in oocytes and in COS cells are not absolutely identical, they are qualitatively and strikingly similar. The differences between the two systems, for example in the voltage shift of the inactivation process, might be due to factors such as a different lipid composition of the membrane, different associations with the cytoskeleton elements, different phosphorylation processes, etc. Altogether, these results indicate that when expressed in relatively low concentrations Kv8.1, even in Xenopus oocytes, can reach the surface membrane via its association with Kv2.1 or Kv2.2 and form there new types of channels with new kinetics properties. One reasonable possibility is that heterotetramers containing a dominant number of Kv8.1 subunits versus Kv2.1, Kv2.2, or Kv3.4 subunits are unable to reach the plasma membrane, whereas tetramers containing a dominant proportion of Kv2.1, Kv2.2, or Kv3.4 subunits reach the plasma membrane and are expressed there with new kinetic properties. It is known that a single subunit in a heterologous tetramer can impose new kinetics (36–38).

Several observations strongly suggest that the S6 segment in Kv8.1 is a key element in the Kv2 current modification. First, coexpression of Kv2.1 with a chimeric subunit in which the S6
COOH terminus domain of Kv8.1 has been replaced by the corresponding region of Kv1.3 ((NH5Kv8)-Kv1 (Table I)) suppresses the induction of a slow inactivation rate. Moreover the shift of the voltage dependence of the steady-state inactivation observed for Kv8.1/Kv2.1 currents is absent for the (NH5Kv8)-Kv1/Kv2.1 currents. The second indication comes from the fact that following coexpression of Kv3.4 with Kv8.1, the inactivation of the Kv3.4 current which is of the N-type ball and chain mechanism involving the NH2-terminal part of the protein (39), is not significantly modified. Since the S6 domain of Kv8.1 appears to play a central role in imposing new inactivation characteristics to the Kv2 current, identification of important amino acids for this phenomenon was achieved by a mutagenesis approach of Kv2.1. The strategy was to introduce singular Kv8.1 residues not present in the S6 region of other expressing Kv channels into the Kv2.1 S6 segment. This led to K+ currents with new characteristics. Particularly spectacular effects on inactivation were observed when Pro-406 was replaced by an alanine (Ala-428 in the Kv8.1 sequence, mutant Kv2.1M3) and Cys-394 was replaced by an isoleucine (Ile-416 in the Kv8.1 sequence, mutant Kv2.1M7). These 2 mutants of Kv2.1 generate nearly non-inactivating K+ channels. Clearly then, the S6 segment of Kv8.1 appears to contain structural elements, especially Ile-416 and Ala-428, that change the kinetics of inactivation of the Kv2 channels when it forms multimers with the Kv8.1 subunit. Finally, a last indication for the involvement of S6 residues is provided by mutations of the S6 segment of Kv8.1. A mutant of Kv8.1, in which Ile-416 and Ala-428 have been replaced by a cysteine and a proline, respectively (mutant Kv8.1M3M7), essentially loses its properties to modify the inactivation kinetics of Kv2.2.

The inactivation process of voltage-gated K+ channels is important for defining the shape and for the integration of electrical signals and can occur over a wide range of timescales. It is probable that in the regions of the mammalian functioning nervous system both the inactivation effect of Kv8.1 channels together with its partners of the Kv2 and Kv3 families. Since the Kv8.1 subunit is abundantly expressed in the hippocampus, its capacity to produce long term changes in electrical signals might have a role in long term potentiation and memory processes.

Acknowledgments—We are very grateful to Dr. Hwang and Dr. Li (The John Hopkins University, Baltimore) for the gift of Kv2.2 clones, to Dr. John Baylor College of Medicine, Houston) for the gift of Kv2.1. We gratefully thank Y. Benhamou, D. Doume, C. Le Calvez, M. Jodar, and G. Jarretou for expert technical assistance and F. Lesage for the gift of polyclonal antibodies against Kv1.5.

REFERENCES
1. Hugnot, J. P., Salinas, M., Lesage, F., Guillaumere, E., Weille, J., Heurteaux, C., Mattei, M. G., and Lazdunski, M. (1996) EMBO J. 15, 3222–3231
2. Hille, B. (1992) Ionic Channels of Excitable Membranes, 2nd Ed., Sinauer Associates Inc., Sunderland, Massachusetts
3. Rehm, H., and Lazdunski, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4919–4925
4. Chandy, K. G., and Gutman, G. A. (1995) Voltage-gated K+ Channels, CRC Press Inc., Boca Raton, FL
5. Adelman, J. P. (1995) Curr. Opin. Neurobiol. 5, 286–295
6. Pongs, O. (1995) Semin. Neurosci. 7, 137–146
7. Fink, M., Duprat, F., Lesage, F., Heurteaux, C., Romey, G., Barhanin, J., and Lazdunski, M. (1996) J. Biol. Chem. 271, 26341–26348
8. Shi, G., Nakohira, K., Hammond, S., Rhodes, K. J., Schechter, L. E., and Trimmer, J. S. (1996) Neuron 16, 843–52
9. Salkoff, L., Baker, K., Butler, A., Covarrubias, M., Pak, M. D., and Wei, A. G. (1992) Trends Neurosci. 15, 161–166
10. Jan, L. Y., and Jan, Y. N. (1990) Trends Neurosci. 13, 415–419
11. Pongs, O. (1992) Physiol. Rev. 72, 569–888
12. Pongs, O. (1993) Trends Pharmacol. Sci. 14, 435
13. Drewes, J. A., Verma, S., F reeh, G., and Joho, R. H. (1992) J. Neurosci. 12, 538–548
14. Zhao, B., Rassendren, F., Kaang, B. K., Furukawa, Y., Kubo, T., and Kandel, E. R. (1994) Neuron 13, 1205–1213
15. Tseng-Crank, C. J., Teng, G. N., Schwartz, A., and Tonouye, M. A. (1990) FEBS Lett. 268, 63–68
16. Albrecht, B., Lorra, C., Stocker, M., and Pongs, O. (1993) Recept. Channels 1, 99–110
17. Demo, S., and Yellen, G. (1991) Neuron 7, 743–753
18. Hoshi, T., Zagotta, W. N., and Aldrich, R. W. (1990) Science 250, 533–538
19. Zagotta, W. N., Hoshi, T., and Aldrich, R. W. (1990) Science 250, 568–571
20. Hoshi, T., Zagotta, W. N., and Aldrich, R. W. (1991) Neuron 7, 547–556
21. Choi, K. L., Aldrich, R. W., and Yellen, G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5092–5095
22. Liu, Y., Jurman, M. E., and Yellen, G. (1996) Neuron 16, 859–867
23. Lopata, M. A., Cleveland, D. W., and Solnier-Webb, B. (1984) Nucleic Acids Res. 12, 5707–17
24. Jurman, M. E., Boland, L. M., Liu, Y., and Yellen, G. (1994) BioTechniques 17, 876–881
25. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 61–68
26. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
27. F reeh, G., Van Dungen, A. M. J., Schuster, G., Brown, A. M., and Joho, R. H. (1989) Nature 340, 642–645
28. Guillaumere, E., Honore, E., Pradier, L., Lesage, F., Schweitz, H., Attali, B., Barhanin, J., and Lazdunski, M. (1992) Biochemistry 31, 12463–12468
29. Honore, E., Attali, B., Romey, G., Lesage, F., Barhanin, J., and Lazdunski, M. (1992) EMBO J. 11, 2465–2471
30. Attali, B., Lesage, F., Ziliani, P., Guillaumere, E., Honore, E., Waldmann, R., Hugnot, J. P., Mattei, M. G., Lazdunski, M., and Barhanin, J. (1993) J. Biol. Chem. 268, 24283–24289
31. Christie, M. J., North, R. A., Osborne, P. B., and Adelman, J. P. (1990) Neuron 5, 405–411
32. Covarrubias, M., Wei, A., and Salkoff, L. (1991) Neuron 7, 763–773
33. Hodgkin, A. L., and Huxley, A. F. (1952) J. Physiol. (Lond.) 116, 473–496
34. Grissmer, S., and Cahalan, M. (1989) Biophys. J. 55, 203–206
35. Languedel, E., Renard, S., Waldmann, R., Voilley, N., Champigny, G., Plass, H., Lazdunski, M., and Barpy, P. (1994) J. Biol. Chem. 269, 13736–13739
36. Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Hipuchi, M., Loyedi, H., Burnashev, N., Sakmann, B., and Seeburg, P. H. (1994) Science 265, 127–1272
37. Waldmann, R., Champigny, G., Bassilana, F., Voilley, N., and Lazdunski, M. (1995) J. Biol. Chem. 270, 27411–27414
38. Duprat, F., Lesage, F., Guillaumere, E., Fink, M., Hugnot, J. P., Bigay, J., Lazdunski, M., Romey, G., and Barbanin, J. (1995) Biochem. Biophys. Res. Commun. 212, 657–663
39. Stephens, G. J., and Robertson, B. (1995) J. Physiol. (Lond.) 148, 1–13
40. Hwang, P. M., Cunningham, A. M., Peng, Y. W., and Snyder, S. H. (1993) Neuroscience 55, 613–620
41. Luthi, A., Galwiler, B. H., and Gerber, U. (1996) J. Neurosci. 16, 586–594