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Identification of the Kv2.1 K^+ Channel as a Major Component of the Delayed Rectifier K^+ Current in Rat Hippocampal Neurons

Hideyuki Murakoshi and James S. Trimmer

Department of Biochemistry and Cell Biology and Institute for Cell and Developmental Biology, State University of New York, Stony Brook, New York 11794-5215

Molecular cloning studies have revealed the existence of a large family of voltage-gated K^+ channel genes expressed in mammalian brain. This molecular diversity underlies the vast repertoire of neuronal K^+ channels that regulate action potential conduction and neurotransmitter release and that are essential to the control of neuronal excitability. However, the specific contribution of individual K^+ channel gene products to these neuronal K^+ currents is poorly understood. We have shown previously, using an antibody, "KC," specific for the Kv2.1 K^+ channel α-subunit, the high-level expression of Kv2.1 protein in hippocampal neurons *in situ* and in culture. Here we show that KC is a potent blocker of K^+ currents expressed in cells transfected with the Kv2.1 cDNA, but not of currents expressed in cells transfected with other highly related K^+ channel α-subunit cDNAs. KC also blocks the majority of the slowly inactivating outward current in cultured hippocampal neurons, although antibodies to two other K^+ channel α-subunits known to be expressed in these cells did not exhibit blocking effects. In all cases the blocking effects of KC were eliminated by previous incubation with a recombinant fusion protein containing the KC antigenic sequence. Together these studies show that Kv2.1, which is expressed at high levels in most mammalian central neurons, is a major contributor to the delayed rectifier K^+ current in hippocampal neurons and that the KC antibody is a powerful tool for the elucidation of the role of the Kv2.1 K^+ channel in regulating neuronal excitability.

Key words: ion channel; CNS; hippocampus; patch clamp; immunofluorescence; potassium current; neuronal excitability; epilepsy

Voltage-sensitive K^+ channels are crucial and diverse elements in the control of electrical signaling and sensitivity in excitable tissue. K^+ channels have been characterized extensively in the mammalian hippocampus because of the important role of this brain region in information processing and cognition. A number of functionally distinct voltage-gated K^+ channel types have been identified in mammalian hippocampal neurons, including Ca^{2+}-dependent channels of low and high conductance, inward rectifiers, transient or "A"-type channels, and delayed rectifiers (Storm, 1990). These K^+ channels are the principal channel types available to hyper- or repolarize these neurons and, in concert with inward cation (i.e., Na^+ and Ca^{2+}) conductances, serve to establish and maintain the appropriate level of neuronal excitability. Pharmacological blockade (Bagetta et al., 1992) or genetic knockout (Smart et al., 1998) of hippocampal K^+ channels leads to hyperexcitability and epileptogenesis. It is clear that some of these effects are mediated by presynaptic K^+ channels important in regulating neurotransmitter release (Halliwell et al., 1986). However, recent studies have shown that the dendrites of mammalian central neurons also contain a wide variety of voltage-gated ion channels, including Na^+, Ca^{2+}, and K^+ channels (Stuart et al., 1997). Dendritic voltage-gated channels have been proposed to play a critical role in the propagation of synaptic signals to the soma as well as the backpropagation of action potentials from the axon through the dendritic tree (Stuart et al., 1997). K^+ channels in particular appear to be key elements in regulating the efficacy of both forward- and backpropagating active dendritic signaling (Hoffman et al., 1997).

The Kv2.1 K^+ channel α-subunit is expressed in virtually every neuron in mammalian brain (Trimmer, 1991; Hwang et al., 1993b; Maletic-Savatic et al., 1995; Rhodes et al., 1995, 1997; Bekele-Arcuri et al., 1996; Scannevin et al., 1996; Du et al., 1998) and is unique among Kv channels in that the cDNA was isolated originally from rat brain by expression cloning (Frech et al., 1989), exemplifying the high levels of Kv2.1 expression in mammalian brain. Kv2.1 is localized uniquely among mammalian brain K^+ channels to large clusters on the soma and on the very proximal portions of dendrites (Trimmer, 1991; Scannevin et al., 1996; Du et al., 1998). This is especially evident in the hippocampus, where Kv2.1 is found at high levels in CA1–CA3 pyramidal cells, dentate granule cells, and interneurons (Maletic-Savatic et al., 1995; Rhodes et al., 1997; Du et al., 1998). Hippocampal neurons in culture also exhibit robust Kv2.1 expression (Maletic-Savatic et al., 1995). The high levels of Kv2.1 expression in the soma and proximal dendrites *in situ*, combined with the observed effects of phosphorylation on the activation properties of Kv2.1 (Murakoshi et al., 1997), suggest that Kv2.1 may play a pivotal and dynamic role in regulating the transmission of electrical signals into and out of the neuronal soma. However, despite this large body of indirect evidence implicating Kv2.1 as a major component of delayed rectifier current in neurons, the specific contribution of this channel has not been feasible because of a lack of pharmacological agents that selectively act on Kv2.1.

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Correspondence should be addressed to Dr. James S. Trimmer, Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794-5215.

We have raised a panel of subtype-specific polyclonal and monoclonal antibodies against Kv2.1 (Trimmer, 1991; Bekele-Arcuri et al., 1996; Nakahira et al., 1996). Here we show that one of these antibodies, the rabbit polyclonal anti-Kv2.1 antibody "KC," is a selective and potent inhibitor of recombinant Kv2.1 channels expressed in transfected cells. We then use this antibody to define the contribution of Kv2.1 to the slowly inactivating K^+ current in hippocampal neurons.

MATERIALS AND METHODS

Materials. All materials not specifically identified were purchased from Sigma (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN).

Antibodies and fusion proteins. Generation and characterization of rabbit polyclonal antibodies to the cytoplasmic C termini of Kv2.1 [KC; amino acids 837–853 (Trimmer, 1991)] and Kv4.2 [Kv4.2C; amino acids 484–502 (Nakahira et al., 1996)] have been described previously. The generation and purification of the recombinant Kv2.1 fusion proteins GST–TC (pGEX–KC; amino acids 822–853) and GSTdrk1 (pGEX–drk1; amino acids 516–533) also have been described previously (Trimmer, 1991). An antibody to the external domain of Kv1.4 was generated by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein GST–Kv1.4 containing amino acids 336–370 of the S1–S2 linker of Kv1.4. KC and Kv1.4E antibodies were affinity-purified on a nitrocellulose strips containing the corresponding fusion proteins (Trimmer, 1991); anti-Kv4.2 antibodies were affinity-purified on a column of agarose beads with bound Kv4.2C peptide (Nakahira et al., 1996). Monoclonal antibodies have been described previously (Bekele-Arcuri et al., 1996).

Transient transfection of COS-1 cells. COS-1 cells were transfected with the mammalian expression vector pRBG4 (Lee et al., 1991) containing full-length cDNAs for Kv2.1 (Frech et al., 1989), Kv1.5 (Swanson et al., 1990), or Kv2.2 (Hwang et al., 1992) by the calcium phosphate precipitation method (Trimmer, 1998). Cells were cotransfected with an expression plasmid containing a cDNA-encoding CD8 surface antigen to identify transfected cells visually by using anti-CD8 antibody-coated beads (Jurman et al., 1994). The $CaPO_4$–DNA mixture was prepared at a final concentration of 4 μg/ml of K^+ channel DNA and 0.8 μg/ml of cDNA-encoding CD8 antigen (Murakoshi et al., 1997). Coexpression of CD8 had no effect on the properties of expressed Kv2.1 in transiently transfected COS-1 cells (our unpublished observations). Cells were seeded at 1% confluence and grown at 37°C in DMEM containing 10% calf serum. The calcium phosphate–DNA mixture was added within 24 hr of seeding, when cells were approximately twice the original plating density, and then left for 18–24 hr. The transfection medium was removed, and fresh medium was added and then incubated at 37°C for an additional 24 hr. Immunofluorescence staining of transfected cells was performed essentially as described (Shi et al., 1994).

Generation of the drk1CGN/l(tk) stable cell line. Mouse fibroblast L(tk^-) cells were transfected with a plasmid (drk1/CGN) containing the full-length Kv2.1 cDNA (Frech et al., 1989) under the control of the CMV promoter and containing an N-terminal influenza hemagglutinin epitope tag (Tanaka and Herr, 1990). A plasmid ($p17^{neo}$; Ballivet et al., 1988) carrying neomycin resistance was cotransfected, and the transfectants were identified by growth on 400 μg/ml G418. Drug-resistant colonies were screened by dot blot with the KC antibody, and positive clones were rescreened by immunoblot to verify the molecular identity of the immunoreactive species. Positive colonies were subcloned by limiting dilution.

Primary culture of hippocampal neurons. Preparation of primary cultures was done according to the method of Banker and Cowan (1977). Briefly, embryonic day 19 (E19) hippocampi were digested with 0.25% trypsin for 15 min at 37°C and dispersed by trituration with a constricted Pasteur pipette 15–20 times to produce a homogeneous suspension. Cells were plated on coverslips coated with 1 mg/ml poly-L-lysine at 17,200 cells per coverslip in MEM containing 10% horse serum/0.06% glucose. After 4 hr, when the cells were adhered to the substrate, coverslips were transferred inverted into six-well tissue culture plates containing a confluent layer of astrocytes prepared from cerebral hemispheres of neonatal rat pups (postnatal day 1; P1) in neuronal maintenance medium (modified Eagle's medium, 10% horse serum, 0.06% glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin). Neurons did not contact the glia because of the presence of paraffin wax pedestals. After 24 hr, the medium was changed to serum-free MEM with N2 supplements, 0.1% ovalbumin, and 0.1 mM sodium pyruvate (Goslin and Banker, 1991). After 3 d, 5 μM cytosine arabinoside was added to inhibit the proliferation of non-neuronal cells. Cultures were kept at 37°C in a humidified atmosphere of 95% air/5% CO_2. One-third of the culture medium was changed weekly.

Electrophysiological analyses. Recordings were made via the whole-cell patch-clamp configuration (Hamill et al., 1981). For COS-1 cells, recordings were made 36 hr after transfection. Before use the cotransfected cells were incubated with external solution containing 1000-times-diluted anti-CD8 antibody-coated beads for 3–5 min to allow for CD8-transfected cells to be decorated with beads, which made visual identification of transfected cells possible (Jurman et al., 1994; Murakoshi et al., 1997). Electrodes (1–3 MΩ) pulled from borosilicate glass were fire-polished and filled with a pipette solution (see below). Currents were recorded with a patch-clamp amplifier (EPC-7), sampled at 10 kHz on an ITC-16 A/D converter, and filtered at 2 kHz by a digital Bessel filter. All currents were capacitance- and leak-subtracted, using the *P*/*n* procedure (Heinemann, 1983). All experiments were performed at room temperature. The membrane potential was held at −80 mV and depolarized to a maximum of +50 mV for 200 msec by 10 mV increments. The pipette solution contained (in mM): 140 KCl, 1 $CaCl_2$, 10 Na-EGTA, and 10 Na-HEPES, pH 7.2. For antibody block experiments the pipette solution also contained KC or other test antibodies; the whole-cell recording configuration allows for exchange of the pipette solution with the internal solution of the cell being recorded and thus access of the KC antibody to its intracellular epitope. The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, and 10 Na-HEPES, pH 7.2. The current (I) was converted into conductance (G), using the following equation: $G = I/(V - E_K)$. The Nernst K^+ equilibrium potential E_K was calculated as −84 mV. Then the normalized conductances were plotted against the test potential, V, and fit to a single Boltzmann equation: $G = G_{max}/(1 + \exp(-[V - V_{1/2})/k)]$. G_{max} is the maximum conductance, $V_{1/2}$ is the test potential at which the channel has a half-maximal conductance, and k is the slope parameter that represents the slope of the activation curve. Data were presented as mean ± SEM. Statistical significance was evaluated by paired or nonpaired Student's t test between two groups. If $p < 0.05$, the value was considered to be statistically significant.

RESULTS

The KC rabbit polyclonal antibody is specific for Kv2.1

The Kv2.1 K^+ channel α-subunit polypeptide has the longest cytoplasmic C-terminal domain of any Kv channel at 440 amino acids. The vast majority of this domain is unique to Kv2.1; thus antibodies raised against sequences within this domain would be expected to recognize Kv2.1 selectively. We have raised rabbit polyclonal (Trimmer, 1991) and mouse monoclonal (Bekele-Arcuri et al., 1996) antibodies against synthetic peptides and recombinant fusion protein fragments corresponding to the unique C terminus of Kv2.1. One of the rabbit polyclonal antibodies, termed KC, was raised against a synthetic peptide corresponding to the last 17 amino acids (837–853) of Kv2.1 (Trimmer, 1991) located at the distal end of the 440-amino-acid-long cytoplasmic tail of Kv2.1. On immunoblots the KC antibody recognizes a single pool of M_r = 110–130 kDa polypeptides in adult rat brain; this immunoreactivity is abolished by preincubating the antibody with a recombinant fusion protein, GST–KC, containing a Kv2.1 fragment corresponding to the carboxyl 27 amino acids, but not with a fusion protein, GST–drk1, corresponding to another region of Kv2.1 (Trimmer, 1991). KC also recognizes full-length recombinant Kv2.1 expressed in transfected COS-1 cells (Shi et al., 1994), but not recombinant Kv2.2 or Kv1.5 expressed in the same cell background (Fig. 1).

KC blocks Kv2.1 currents in transfected cells

Voltage-dependent outward currents in transiently transfected COS-1 cells expressing various K^+ channel α-subunit polypeptides were analyzed by whole-cell patch clamp as previously described (Murakoshi et al., 1997), with the exception that the patch pipette internal solution contained 3 nM = 0.5 μg/ml

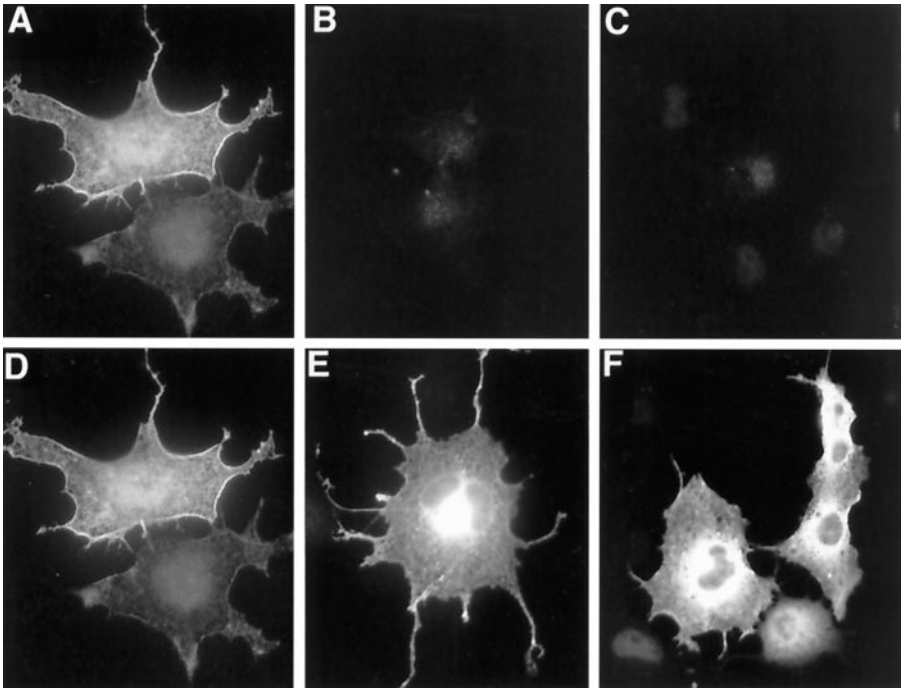

Figure 1. Immunofluorescence staining of recombinant K^+ channel α-subunit polypeptides expressed in COS-1 cells. COS-1 cells were transfected with 8 μg/ml of Kv2.1/RBG4 (in *A*, *D*), Kv2.2/RBG4 (in *B*, *E*), and Kv1.5/RBG4 (in *C*, *F*). Then the transfected cells were fixed, permeabilized, and stained with affinity-purified KC IgG plus anti-Kv2.1 monoclonal D4/11 (in *A*, *D*), anti-Kv2.2 monoclonal K37/89 (in *B*, *E*), and anti-Kv1.5 monoclonal K7/45 (in *C*, *F*). Finally, the cells were incubated with fluorescein-conjugated goat-anti rabbit and Texas Red goat anti-mouse secondary antibodies. Photographs show fluorescein (*A–C*) and Texas Red (*D–F*) staining of double-labeled cells.

affinity-purified KC IgG. Recording in the whole-cell configuration allowed for access of the KC antibody to its intracellular epitope via exchange of the pipette solution with the inside of the cell. Voltage-dependent outward currents were recorded at the time the seal was made and at successive 1 min intervals thereafter. COS-1 cells transfected with the Kv2.1 cDNA expressed large outward currents under whole-cell patch clamp at the time the seal was made (Fig. 2). Exposure of Kv2.1-transfected cells to the patch pipette containing KC antibody caused a marked reduction in the expressed voltage-dependent outward currents after 10 min (Fig. 2). Data from a number of cells showed that the currents from COS-1 cells transiently expressing recombinant Kv2.1 were inhibited to ~53% of the original amplitude after a 10 min exposure to 3 nM KC IgG within the patch pipette (Table 1). As expected from the lack of immunological cross-reactivity (see Fig. 1), exposure to 3 nM KC IgG had no significant effect on voltage-dependent outward currents in COS-1 cells transiently expressing the Kv1.5 or Kv2.2 K^+ channel α-subunits (Table 1), which are the Kv α-subunits most similar to Kv2.1. In addition, KC does not block currents from a Kv2.1 C-terminal truncation mutant, ΔC318, which lacks the last 318 amino acids of Kv2.1 and the KC epitope (our unpublished observations).

Voltage-dependent outward currents in a stable mouse fibroblast cell line, drk1CGN/l(tk$^-$), expressing the Kv2.1 α-subunit then were analyzed by whole-cell patch clamp. The drk1CGN/l(tk$^-$) cell line expresses large outward currents under whole-cell patch clamp (Fig. 3*A*). Voltage-dependent outward currents were recorded at the time the seal was made and at successive 1 min intervals thereafter. Exposure of drk1CGN/l(tk$^-$) cells to the patch pipette containing pipette solution alone (Fig. 3*A, Control*) yielded little time-dependent reduction in current amplitude over the course of the experiment, with only statistically insignificant and time-independent variations in current amplitude observed. The inclusion of 3 nM KC IgG (Fig. 3*A, + Antibody*) in the patch pipette caused a marked time-dependent reduction in the expressed voltage-dependent outward currents, with some inhibition observed even at the earliest time point (1 min exposure) and statistically significant inhibition achieved at 9 min after exposure to the antibody solution (Fig. 3*B*). When 3 nM KC IgG was present in the patch pipette, the currents were inhibited to >50% of their original amplitude after 10 min exposure to the antibody solution (Fig. 3*A,B*, Table 1). Dose–response data (Fig. 3*C*) obtained after 10 min exposure to KC IgG revealed that inhibition of currents in drk1CGN/l(tk$^-$) cells first was seen at 300 pM KC IgG, with increasing inhibition up to the highest concentration tested (3 nM). KC-mediated inhibition of Kv2.1 current was suppressed by preincubating the KC antibody with the GST–KC fusion protein containing the KC epitope, but not with the GST–drk1 fusion protein that is outside the region of the KC epitope (data not shown). A 10 min exposure to affinity-purified rabbit polyclonal IgGs specific for either the Kv1.4 (3 nM) or Kv4.2 (200 nM) K^+ channel α-subunits had no significant effect on the amplitude of Kv2.1 currents in this stable Kv2.1 cell line (our unpublished observations).

Kv2.1 is expressed in the cell body and proximal dendrites of cultured hippocampal neurons

Kv2.1 expression is widespread in the rat hippocampus, with prominent KC antibody staining in the cell bodies and proximal dendrites of dentate granule cells, CA1–CA3 pyramidal cells, and interneurons throughout the hippocampal formation (Maletic-Savatic et al., 1995; Rhodes et al., 1995, 1997; Du et al., 1998). Cultured hippocampal neurons also express Kv2.1 (Maletic-Savatic et al., 1995). KC staining of such hippocampal cultures reveals the discrete localization of Kv2.1 in clusters on the soma and proximal dendrites (Fig. 4) similar to what is seen for KC antibody staining in the hippocampus *in situ*. Thus, these cells present an attractive culture model for determining the contribution of Kv2.1 K^+ channels to neuronal K^+ currents.

KC specifically inhibits a slowly activating component of outward current in hippocampal neurons

To determine the contribution of Kv2.1 K^+ channel to the K^+ currents in cultured hippocampal neurons, we analyzed currents

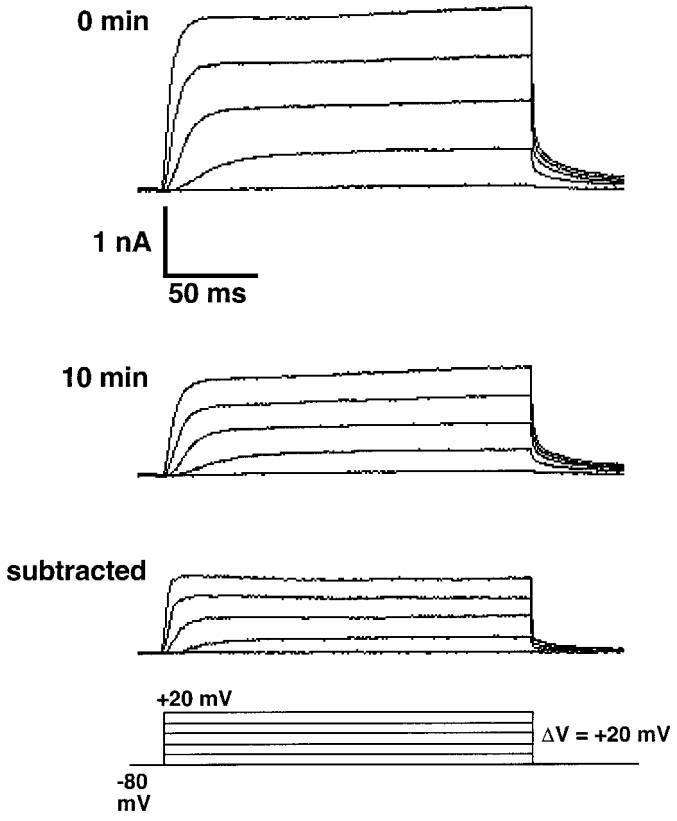

Figure 2. KC antibody block of voltage-dependent currents in transiently transfected COS-1 cells. Shown are typical membrane currents from COS-1 cells expressing recombinant Kv2.1 recorded 0 min (*top*) and 10 min (*middle*) after a seal was made in whole-cell patch-clamp configuration with 3 nM KC IgG in the pipette internal solution. The *bottom trace* shows the resultant subtracted current. The current traces that are shown were recorded by a step depolarization from a holding potential of −80 to +20 mV in 20 mV steps for 200 msec.

under whole-cell patch clamp. Rapidly inactivating transient A-type currents, which were found to exhibit antibody-independent rundown during the course of the 10 min experiments, were eliminated by holding the cells at −30 mV. Then the remaining voltage-dependent outward currents were recorded at the time the patch was made and at successive 1 min intervals thereafter. Figure 5*A* shows current traces obtained 0 and 10 min after exposure of the cells to 3 nM KC IgG and the subtracted current that reveals the characteristics of the KC-sensitive component of the neuronal outward current. Analysis of such data revealed that there are differences between the macroscopic current–voltage relationships of the KC-sensitive and KC-resistant components of the outward current (Table 2) as well as differences in the inactivation kinetics (Fig. 5*A*).

Analysis of the extent of antibody block at 1 min intervals after the seal was made showed that exposure to 3 nM KC IgG caused a detectable block of current after 1 min; statistically significant inhibition of the slowly inactivating current was observed after 5 min (Fig. 5*B*). The increase in KC-dependent current block was fairly linear up to 10 min of exposure, after which the rate of inhibition slowed and became more variable such that no additional significant differences were observed at time points after 10 min (Fig. 5*B*). Pooled data from five different neurons showed that the outward currents were inhibited to ~56% of the original amplitude after a 10 min exposure to 3 nM KC IgG within the patch pipette (Table 3). KC IgG-mediated inhibition of outward currents in hippocampal neurons had a similar dose–response (Fig. 5*C*) to that observed in the drk1CGN/l(tk$^-$) cells. Exposure to patch pipettes containing, in the internal solution, 3 nM anti-Kv1.4 IgG or 200 nM anti-Kv4.2 IgG had no significant effect on the slowly inactivating or sustained components of the outward current (Table 3).

Table 1. K^+ current inhibition in transfected cells

Cells	Treatment	I/I_0	n
Kv2.1 in COS	3 nM KC IgG	53.1 ± 12.3	3
Kv2.2 in COS	3 nM KC IgG	107.6 ± 4.9[b]	3
Kv1.5 in COS	3 nM KC IgG	100.3 ± 8.7[b]	5
drk1CGN/L(tk−)	Control	100.2 ± 7.5	5
drk1CGN/L(tk−)	3 nM KC IgG	46.9 ± 10.2[a]	4

[a] $p < 0.005$ versus control.

[b] $p < 0.05$ versus Kv2.1 in COS + KC.

Cells were maintained at a holding potential of −80 mV and depolarized to +20 mV for 200 msec once per minute. Currents at 10 min were expressed as a percentage of the current at time zero (I/I_0).

Preincubation of affinity-purified KC IgG with a recombinant fusion protein, pGEX–KC, containing amino acids 822–853 of Kv2.1 eliminated immunoblot reactivity to rat brain membranes (Trimmer, 1991). A 1 hr incubation in the pGEX–KC fusion protein eliminated most of the blocking activity of the KC IgG toward the slowly inactivating current in cultured hippocampal neurons (Table 3). Incubation with a fusion protein containing a Kv2.1 fragment (amino acids 507–533) distinct from the KC binding site had no such competitive effect on KC current inhibition (Table 3). These results together show that KC specifically inhibits a slowly inactivating component of the outward current in hippocampal neurons and thus represents a novel Kv2.1-specific reagent to investigate further the function of this K^+ channel in neuronal function.

DISCUSSION

We have used the KC antibody previously to identify and characterize the Kv2.1 K^+ channel α-subunit polypeptide in rat brain (Trimmer, 1991, 1993; Rhodes et al., 1995, 1997; Scannevin et al., 1996), in hippocampal cultures (Maletic-Savatic et al., 1995), in developing cardiac myocytes (Barry et al., 1995; Xu et al., 1996), and in transfected cells (Shi et al., 1994; Nakahira et al., 1996; Scannevin et al., 1996; Murakoshi et al., 1997). These studies and those by other laboratories using the KC antibody (Archer et al., 1998; Du et al., 1998) or an antibody (SP13) made to the same Kv2.1 peptide (Hwang et al., 1993a,b) together support the monospecific reaction of this antibody with native and recombinant Kv2.1 α-subunits. Pharmacological characterization of recombinant Kv2.1 has revealed unexceptional sensitivity to classical K^+ channel blockers (tetramethylammonium, 1–10 mM; 4-aminopyridine, 0.5–3 mM) and insensitivity to apamin, charybdotoxin, and dendrotoxin (Frech et al., 1989). Thus, these agents cannot be used selectively to block Kv2.1 in neurons. A peptide toxin from the venom of a Chilean tarantula, termed hanatoxin, has been isolated, which blocks Kv2.1 and Kv4.2 with an affinity (~100 nM) similar to that found here for the KC antibody (Swartz and MacKinnon, 1995). However, subsequent mapping of the binding site (Swartz and MacKinnon, 1997) reveals that Kv2.2, which was not tested for pharmacological blockade, is identical to Kv2.1 in the S3–S4 linker region critical for hanatoxin binding and would be predicted to be blocked by this toxin as well; similar arguments

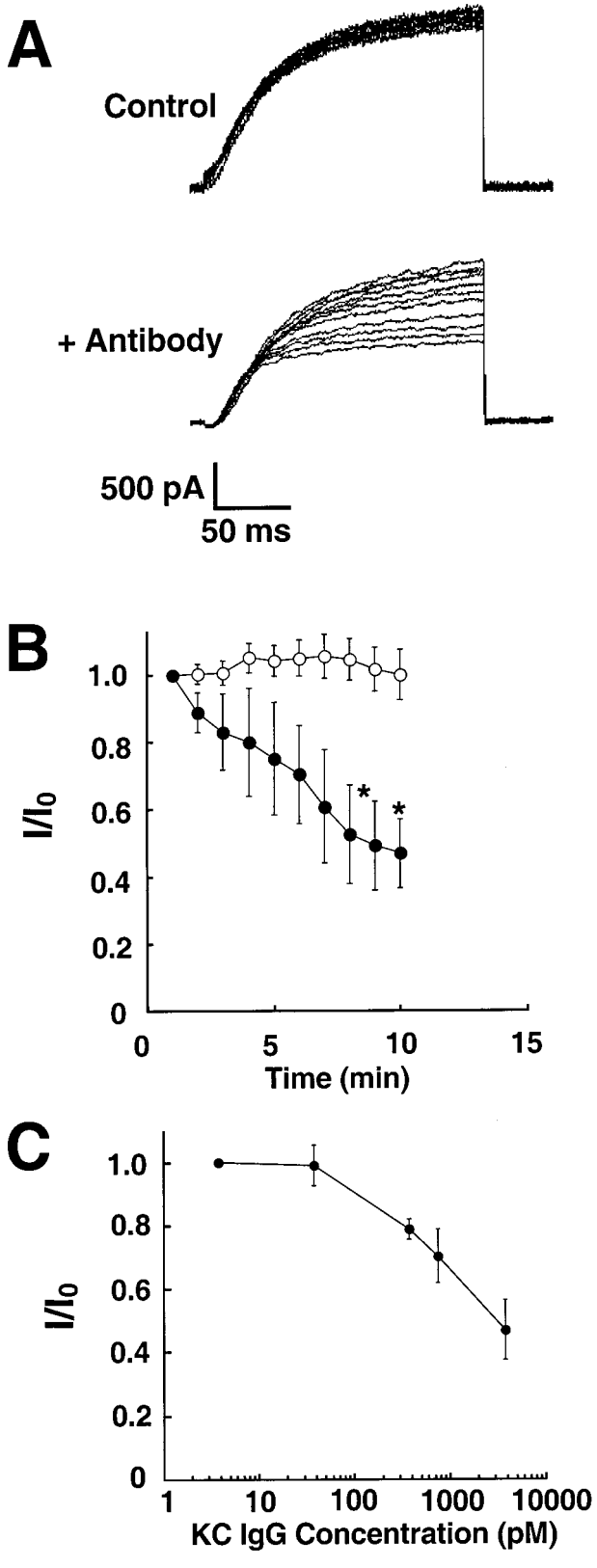

Figure 3. KC antibody block of voltage-dependent currents in the drk1CGN/l(tk$^-$) stable cell line. *A*, Membrane currents were recorded at the time the seal was made in whole-cell patch-clamp configuration and at successive 1 min intervals thereafter for 10 min. Currents were recorded from drk1cgn/l(tk$^-$) cells expressing recombinant Kv2.1 after a seal was made with pipette solution alone (*Control*, *top trace*) or 3 nM KC IgG (+ *Antibody*, *bottom trace*) in the pipette internal solution. The current traces that are shown were recorded by a step depolarization from a holding potential of −80 mV to a test potential of +20 mV for 200 msec. *B*, Time course of the effects of antibody treatment on current amplitude in drk1cgn/l(tk$^-$) cells. The current amplitude in the cells was recorded with 3 nM KC IgG (*filled circles*) or no antibody (*open circles*) present in the patch pipette internal solution. Currents were evoked once every minute by a depolarization from −80 to +20 mV for 200 msec. Current amplitudes (I) are expressed relative to those obtained at time 0 (I_0); mean ± SEM of four cells for each treatment. $^*p < 0.05$ versus time 0. *C*, Dose–response of KC block of current in drk1cgn/l(tk$^-$) cells. Currents were recorded as in *A* and *B* at the time the seal was made in whole-cell patch-clamp configuration (time *0*) and 10 min later, using patch pipettes with different amounts of KC IgG in the patch pipette internal solution. Current amplitudes after 10 min (I) are expressed as relative to those obtained at time 0 (I_0); mean ± SEM of four cells for each treatment.

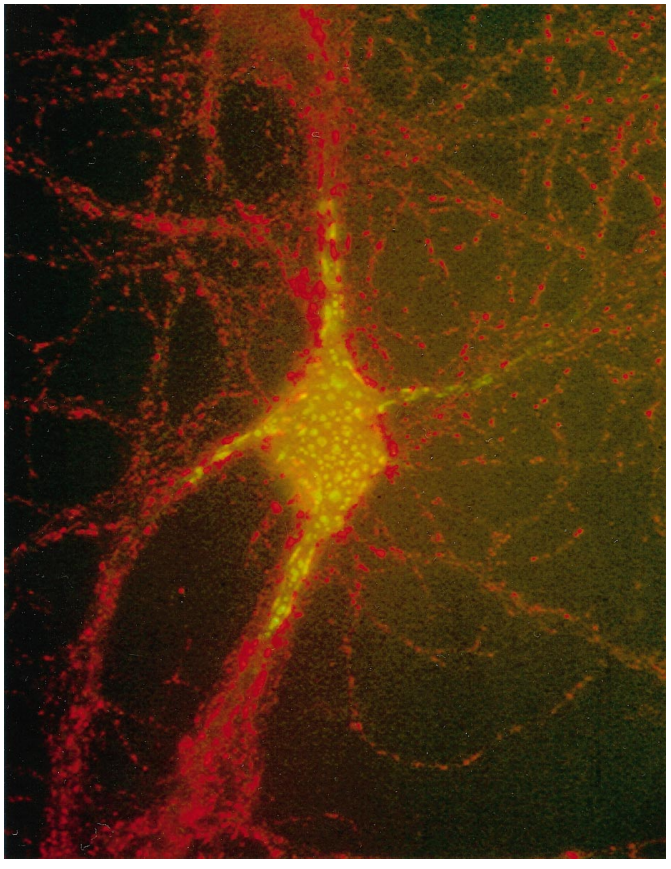

Figure 4. Kv2.1 expression in cultured hippocampal neurons. Shown is immunofluorescence staining of E19 hippocampal neurons after 14 d in culture, using 0.6 nM KC IgG and an anti-synaptophysin mouse monoclonal antibody. The cultured cells were fixed, permeabilized, and stained with primary antibodies, followed by incubation with fluorescein-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse secondary antibodies.

could be made to predict hanatoxin block of Kv4.1 and Kv4.3. Thus, compared with the Kv2.1-specific sequence at the binding site of KC, the binding site for hanatoxin seems rather well conserved among Kv2 and Kv4 channels.

The electrophysiological properties of recombinant Kv2.1 channels heterologously expressed in *Xenopus* oocytes (Frech et al., 1989; VanDongen et al., 1990; Benndorf et al., 1994) and mammalian cells (Ikeda et al., 1992; Shi et al., 1994; Scannevin et al., 1996; Murakoshi et al., 1997) have been well characterized.

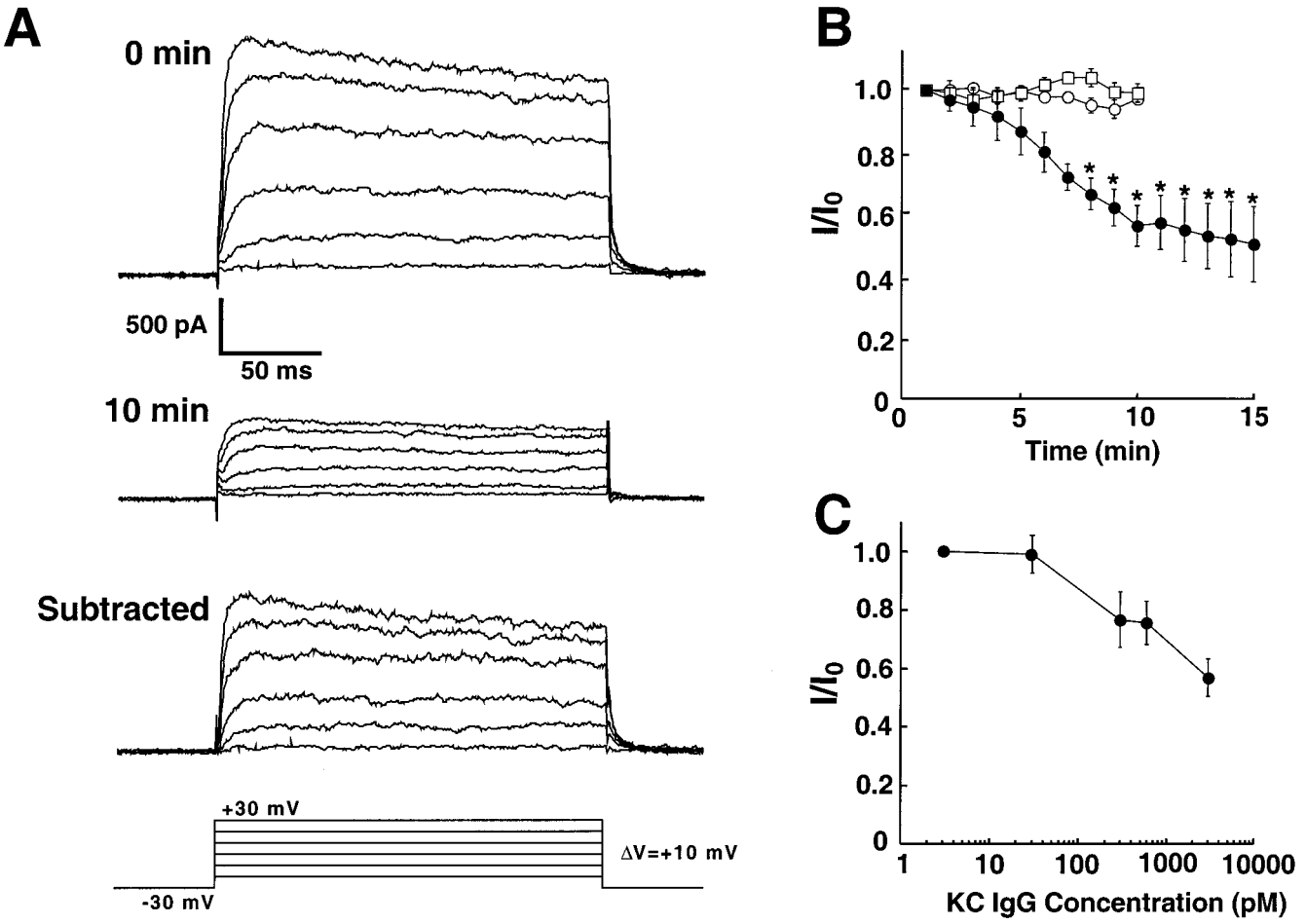

Figure 5. KC antibody block of slowly inactivating voltage-dependent currents in cultured hippocampal neurons. *A*, Typical membrane currents recorded from E19 rat hippocampal neurons after 14 d in culture. The current traces that are shown were recorded at the time the seal was made in whole-cell patch-clamp configuration (time *0*) and 10 min later by step depolarizations from a holding potential of −30 mV to voltages ranging from −30 to +20 mV in increments of 10 mV for 200 msec. *B*, Time course of the effects of antibody treatment on current amplitude in hippocampal neurons. The current amplitude in cells was recorded with 3 nM KC IgG (*filled circles*), 3 nM Kv1.4E IgG (*open squares*), or no antibody (*open circles*) present in the patch pipette internal solution. Currents were evoked once every minute by a depolarization from −30 to +20 mV for 200 msec. Current amplitudes (I) are expressed relative to those obtained at time 0 (I_0); mean ± SEM of four cells for each treatment. $^*p < 0.05$ versus time 0. *C*, Dose–response of KC block of neuronal outward currents. Currents were recorded as in *A* and *B* at 0 and 10 min after the seal was made in whole-cell patch-clamp configuration and with different amounts of KC IgG in the patch pipette internal solution. Current amplitudes after 10 min (I) are expressed as relative to those obtained at the time the seal was made (I_0); mean ± SEM of four cells for each treatment.

Table 2. Electrophysiological parameters of K^+ currents in hippocampal neurons

Component	I (nA)	$V_{½}$ (mV)	k	n
Total	2.8 ± 0.5	17.8 ± 3.7	13.5 ± 0.8	4
KC-resistant	1.1 ± 0.1	9.3 ± 3.4	16.4 ± 1.2	4
KC-sensitive	2.0 ± 0.4	15.3 ± 2.1	13.6 ± 0.8	4

Table 3. K^+ current inhibition in hippocampal neurons

Treatment	I/I_0	n
Control	97.2 ± 2.1	4
3 nM KC IgG	56.4 ± 6.6[a]	5
3 nM Kv1.4E IgG	99.0 ± 2.9	4
200 nM Kv4.2C IgG	99.4 ± 2.9	4
3 nM KC IgG plus pGEX-KC	90.1 ± 4.3	4
3 nM KC IgG plus pGEX-drk1	56.9 ± 4.9[a]	2

[a] $p < 0.005$ versus control.

Cells were maintained at a holding potential of −30 mV and depolarized to +20 mV for 200 msec once per minute. Currents at 10 min were expressed as a percentage of the current at time zero (I/I_0).

The only other member of the Kv2 or *Shab* subfamily expressed in neurons with which Kv2.1 could coassemble is Kv2.2. However, in mammalian central neurons Kv2.2 has a distinct, nonoverlapping distribution (Hwang et al., 1992, 1993b) and is thus unlikely to be present in hetero-oligomeric K^+ channel complexes with Kv2.1. Thus, at least a portion of neuronal delayed rectifier channels is formed as homotetramers of Kv2.1 α-subunits, perhaps with associated auxiliary subunits (Trimmer, 1991). Channels formed by homotetramers of recombinant Kv2.1 expressed in a variety of heterologous expression systems have generally consistent properties, with the exception of their voltage dependence of activation. The $V_{½}$ of activation of macroscopic currents can vary from −9.2 mV in *Xenopus* oocytes (VanDongen et al., 1990) to +6.1 mV in canine polarized Madin-Darby canine kidney

(MDCK) epithelial cells (Murakoshi et al., 1997), apparently because of differences in phosphorylation state, with channels exhibiting increased phosphorylation having increased $V_{1/2}$ values (Murakoshi et al., 1997). The $V_{1/2}$ of the KC-sensitive current in cultured hippocampal neurons was 15.3 mV (see Table 2), consistent with the hyperphosphorylated state of native Kv2.1 in brain relative to Kv2.1 expressed in heterologous systems (Murakoshi et al., 1997). The fact that the M_r of Kv2.1 in brain changes with development (Trimmer, 1993), combined with the localization of Kv2.1 on the soma and proximal dendrites, raises the possibility that developmentally regulated modulation of the voltage-dependent activation of Kv2.1 by differential phosphorylation could confer plasticity to signal integration and processing in developing neurons. Using KC antibody blockade will allow for a direct determination of the contribution of Kv2.1 channels to shaping neuronal excitability at different stages of development and in various models of plasticity in adult neurons, such as long-term potentiation.

Few reports of functional antibody block of ion channels exist in the literature. Recently, an antibody raised against a peptide corresponding to the pore domain of Kv1.2 was shown to block currents expressed from recombinant Kv1.2, but not Kv1.3, K^+ channel α-subunits heterologously expressed in mammalian cells (Zhou et al., 1998). This antibody also blocked voltage-dependent outward currents in neuronal NG108–15 cells (Zhou et al., 1998), known to express Kv1.2 (Yokoyama et al., 1989). However, this antibody shares extensive sequence identity (12 of 15 residues = 80% identity) with Kv1.6; thus this antibody could not be used as a selective probe for Kv1.2 channels in native neurons. Antibodies from the serum of patients with the autoimmune diseases myasthenia gravis [against nicotinic acetylcholine receptor channels (Bufler et al., 1996)] and Lambert–Eaton myasthenic syndrome [against the α_{1A} calcium channel subunit (Pinto et al., 1998)] inhibit currents from their target channels, as does an anti-peptide antibody specific for the anti-α_{1D} calcium channel subunit (Wyatt et al., 1997). In each of these cases the antibodies recognize determinants on the external face of the channel, presumably near the pore, and inhibit on external application. The KC antibody, for which the immunoreactivity and inhibition are specific for the Kv2.1 K^+ channel α-subunit, is unique in its action via its binding to the cytoplasmic C terminus of this polypeptide.

The blocking effects of KC are somewhat surprising in that a number of studies have used truncation of the cytoplasmic C terminus of Kv α-subunits to show that, in general, this domain does not play a critical role in channel gating (VanDongen et al., 1990; Hopkins et al., 1994; Uebele et al., 1994; Scannevin et al., 1996; Murakoshi et al., 1997), although changes in Kv2.1 activation related to the deletion of phosphorylation sites have been reported (Murakoshi et al., 1997). Recent studies have provided biochemical evidence for interaction between the C and N termini of KV α-subunits (Schulteis et al., 1996; Jerng and Covarrubias, 1997), supporting the model of interacting cytoplasmic domains proposed originally from studies of Kv2.1 truncation mutants (VanDongen et al., 1990). Cross-linking of multiple C termini within the channel tetramer by divalent KC IgG could immobilize both the C and N termini, locking the channels in a closed or inactivated conformation, resulting in a decreased number of available channels and smaller currents. Studies with univalent Fab fragments of KC may shed further light on the mechanism whereby KC inhibits Kv2.1 currents.

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