Voltage-Gated Potassium Channels Activated During Action Potentials in Layer V Neocortical Pyramidal Neurons

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Kang, Jian, John R. Huguenard, and David A. Prince. Voltage-gated potassium channels activated during action potentials in layer V neocortical pyramidal neurons. *J. Neurophysiol.* 83: 70–80, 2000. To investigate voltage-gated potassium channels underlying action potentials (APs), we simultaneously recorded neuronal APs and single K^+ channel activities, using dual patch-clamp recordings (1 whole cell and 1 cell-attached patch) in single-layer V neocortical pyramidal neurons of rat brain slices. A fast voltage-gated K^+ channel with a conductance of 37 pS (K_f) opened briefly during AP repolarization. Activation of K_f channels also was triggered by patch depolarization and did not require Ca^{2+} influx. Activation threshold was about −20 mV and inactivation was voltage dependent. Mean duration of channel activities after single APs was 6.1 ± 0.6 ms (mean ± SD) at resting membrane potential (−64 mV), 6.7 ± 0.7 ms at −54 mV, and 62 ± 15 ms at −24 mV. The activation and inactivation properties suggest that K_f channels function mainly in AP repolarization but not in regulation of firing. K_f channels were sensitive to a low concentration of tetraethylammonium (TEA, 1 mM) but not to charybdotoxin (ChTX, 100 nM). Activities of A-type channels (K_A) also were observed during AP repolarization. K_A channels were activated by depolarization with a threshold near −45 mV, suggesting that K_A channels function in both repolarization and timing of APs. Inactivation was voltage dependent with decay time constants of 32 ± 6 ms at −64 mV (rest), 112 ± 28 ms at −54 mV, and 367 ± 34 ms at −24 mV. K_A channels were localized in clusters and were characterized by steady-state inactivation, multiple subconductance states (36 and 19 pS), and inhibition by 5 mM 4-aminopyridine (4-AP) but not by 1 mM TEA. A delayed rectifier K^+ channel (K_{dr}) with a unique conductance of 17 pS was recorded from cell-attached patches with TEA/4-AP-filled pipettes. K_{dr} channels were activated by depolarization with a threshold near −25 mV and showed delayed long-lasting activation. K_{dr} channels were not activated by single action potentials. Large conductance Ca^{2+}-activated K^+ (BK) channels were not triggered by neuronal action potentials in normal slices and only opened as neuronal responses deteriorated (e.g., smaller or absent spikes) and in a spike-independent manner. This study provides direct evidence for different roles of various K^+ channels during action potentials in layer V neocortical pyramidal neurons. K_f and K_A channels contribute to AP repolarization, while K_A channels also regulate repetitive firing. K_{dr} channels also may function in regulating repetitive firing, whereas BK channels appear to be activated only in pathological conditions.

INTRODUCTION

Voltage-gated potassium channels play a major role in neuronal action potential (AP) repolarization and repetitive firing (Connor and Stevens 1971a; Hille 1992; Hodgkin and Huxley 1952; Kolb 1990; Rudy 1988, Schwindt et al. 1988). A delayed rectifier K^+ current (I_K) was described first by Hodgkin and Huxley (1952) and found to be responsible for AP repolarization in squid giant axons. However, the slow activation and inactivation properties of this current raised questions about its function in repolarization of more rapid mammalian neuronal APs. Subsequently, it was proposed that a transient K^+ current (I_A) contributed to neuronal repolarization and repetitive firing (Connor and Stevens 1971a,b; Hagiwara et al. 1961; Neher 1971; Schwindt et al. 1988; Storm 1987). The channels responsible for I_A (K_A channel) begin to activate below the threshold for AP generation and inactivate rapidly. Since these initial descriptions, a variety of transient K^+ currents with a wide range of voltage-dependent activation and inactivation have been recorded in a variety of neurons (Albert and Nerbonne 1995; Gestrelius and Grampp 1983; Kasai et al. 1986; Quandt 1988; Penner et al. 1986; Rudy 1988; Solaro et al. 1995; Spain et al. 1991; Stansfeld et al. 1986).

In addition to I_A, a fast Ca^{2+}-activated K^+ current (I_C) also was reported to contribute to AP repolarization and the fast spike afterhyperpolarization (AHP) in peripheral and some central neurons (Adams et al. 1982; Lancaster and Nicoll 1987; Lattorre et al. 1989; MacDermott and Weight 1982; Pennefather et al. 1985; Storm 1987). I_C was dependent on both membrane potential and intracellular Ca^{2+} levels and was very sensitive to TEA (0.2–1 mM) and charybdotoxin (ChTX) (Castle et al. 1989; Lancaster and Nicoll 1987; Storm 1987). Large-conductance K^+(BK) channels may contribute to I_C and AP repolarization in muscle cells (Pallota et al. 1981; Walsh and Singer 1983), chromaffin cells (Marty 1981), neurons of a GH_3 anterior pituitary cell line (Lang and Ritchie 1987), sympathetic neurons (Pennefather et al. 1985), Helix neurons (Crest and Gola 1993; Gola et al. 1990), and hippocampal neurons (Lancaster et al. 1991; Storm 1987; Yoshida et al. 1991). Observations in large neocortical pyramidal neurons from layer V of the cat sensorimotor cortex suggested that two transient K^+ currents contribute to AP repolarization, a fast-transient current sensitive to a 1 mM TEA that differed from I_C in terms of its Ca^{2+} dependence and a slow-transient K^+ current that inactivated slowly and was attenuated by 4-aminopyridine (4-AP) (Spain et al. 1991). A recent study showed that AP repolarization was not Ca^{2+} dependent in rat neocortical pyramidal neurons (Pineda et al. 1998). Several other groups also indicated that voltage-gated K^+ currents play more important roles in AP repolarization than Ca^{2+}-activated K^+ currents (Albert and Nerbonne 1995; Foehring and Surmeier 1993; Locke and Nerbonne 1997). The channels responsible for the

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fast- and slow-transient K^+ currents in pyramidal neurons have not yet been identified.

The application of cDNA cloning methods has identified a large number of voltage-gated K^+ channel genes and transcripts, demonstrating a molecular diversity that parallels the functional diversity. Two groups of mammalian neuronal voltage-gated K^+ channel genes have been discovered. The first group consists of homologs of *Drosophila Shaker* gene (Sh gene family), Shaker, Shab, Shaw (Kv3.x), and Shal (Jan and Jan 1990; Perney and Kaczmarek 1991; Rudy et al. 1992). The other of homologs of *Drosophila eag* gene (Warmke and Ganetzky 1994; Warmke et al. 1991). At least 20 Sh genes and 25 Sh transcripts have been identified. In addition, subunits of the same Sh subfamily can combine with each other to form heteromultimeric channels, and thereby further increase the diversity of K^+ channels (Covarrubias et al. 1991; Christie et al. 1990; Isacoff et al. 1990; Luneau et al. 1991; McCormack et al. 1990; Ruppersberg et al. 1990). Shal-related mRNAs (Kv4.1, Kv4.2, and Kv4.3) were reported to be responsible for A-type K channels in the CNS (Serodio et al. 1994), whereas Shab-related K^+ channels (Kv2.1) were reported to contribute to the delayed rectifier K^+ current (Murakoshi and Trimmer 1999). Channels, expressed from the Shaw-related gene subfamily (Kv3.3, Kv3.4, and their heteromultimers) in oocytes, are sensitive to 1 mM TEA (Luneau et al. 1991; McCormack et al. 1990; Vega-Saenz de Miera et al. 1992). Wang et al. (1998) reported that Kv3.1 in mouse auditory neurons was also sensitive to 1 mM TEA. Shaw-related K^+ channels were found to be broadly expressed in rat CNS neurons (Martina et al. 1998; Weiser et al. 1994, 1995). Identification of the subunit structure of particular native K^+ channels in specific types of CNS neurons is a challenging task. Kinetic and pharmacological properties of native K^+ channels in brain slices may provide useful data to clarify the relationship between native K^+ channels and cDNA-expressed K^+ channels.

In this study, we used dual patch-clamp techniques, with one pipette in the cell-attached patch configuration and one to obtain whole cell currents (Hamill et al. 1981) to allow simultaneous recordings of single K^+ channel activities and action potentials in individual large pyramidal neurons from layer V of the rat sensorimotor cortex. We were able to identify three distinct AP-activated K channels that presumable have different functions in spike generation.

METHODS

Brain slices

Brain slices were prepared using previously described techniques (Kang et al. 1996). Eight- to 15-day-old (P8–P15) Sprague-Dawley rats of either sex (Simonsen Breeders) were anesthetized with pentobarbital sodium (55 mg/kg) and decapitated. The brain was removed rapidly, blocked in the coronal plane, and glued to the stage of a vibratome (TPI, St. Louis, MO) with the posterior surface down. The block was covered with ice-cold cutting solution (contents in *Solutions and drugs*), and 300-μm coronal slices were cut. Slices containing sensorimotor cortex (FL and Par1) (Zilles 1985) were incubated for 1–8 h in standard slice solution (see *Solutions and drugs*) gassed with 5% CO_2-95% O_2 at room temperature (23–25°C) before being transferred to the recording chamber for patch-clamp recordings.

Patch-clamp recordings

Neocortical slices were placed in a small chamber that had a volume of 1.5 ml and were superfused with standard slice solution gassed with 5% CO_2-95% O_2 at 31°C. Cells in slices were visualized with a ×63 water immersion objective and differential inference contrast (DIC) optics (Axioskop, Zeiss, Germany; Fig. 1*A*). Two electrically controlled mechanical manipulators (Newport) were mounted on the microscope stage. Recording electrodes with resistances of 3–7 MΩ were pulled from KG-33 glass capillaries (1.0 mm ID, 1.5 mm OD, Garner Glass) using a PP-83 electrode puller (Narishige, Japan). Dual patch-clamp recordings were performed on single pyramidal neurons, using an Axoclamp 2B for whole cell current-clamp and an Axopatch 200A for cell-attached patch configurations (Axon Instruments, Burlingame, CA). Neurons with seal resistances <1 GΩ were rejected. Single channel recordings were acquired using the cell-attached patch configuration (Hamill et al. 1981) and filtered through an 8-pole Bessel low-pass filter with a 1-kHz cutoff frequency. Neuronal spikes were recorded in the whole cell current-clamp configuration with 3-kHz bandwidth. The resting membrane potentials were measured in the whole cell recording. Patch membrane potentials were calculated from the equation: $V_p = RMP - V_d$, where V_d is

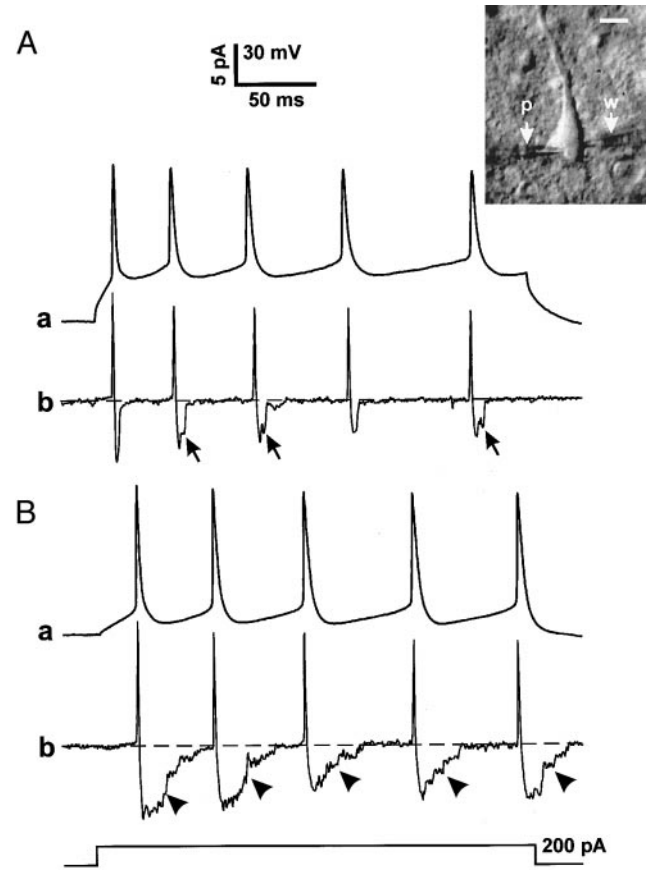

FIG. 1. *A*: dual patch-clamp recordings illustrating activities of a K_f channel during action potentials (AP) repolarization. *Inset*: large layer V neocortical pyramidal neuron in a slice visualized with DIC optics. Cell soma is patched by 2 electrodes (↓) for whole cell (w) and cell-attached patch (p) recordings. Pial surface: up. Calibration: 10 μm. *A*: depolarizing current pulse of 200 pA (— at *bottom* of *B*) was delivered to the whole cell electrode. *a*: whole cell current-clamp recording. *b*: cell-attached voltage-clamp recording showing openings of a K_f channel (←). - - -, closed state. *B*: dual patch-clamp recordings illustrating K_A channel activities during AP repolarization. *a*: train of APs activated by depolarizing current pulse. *b*: representative cell-attached patch recording showing multiple K_A channels (▲) activated by neuronal action potentials.

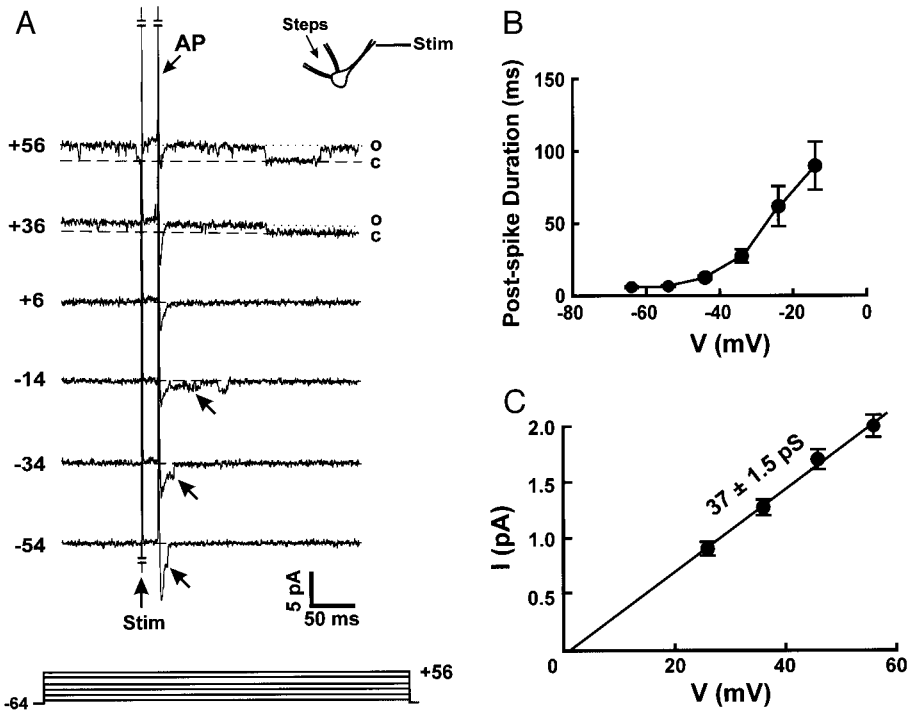

FIG. 2. *A*: action-potential-activated K_f channel activities at different patch membrane potentials. Cell-attached patch recordings (*inset*) were obtained when patches were conditioned by patch depolarization steps (400 ms, solid lines at *bottom*). Extracellular stimuli were applied to cortical layer II/II with a delay of 100 ms (arrow, Stim**;** *1st vertical line*) to evoke action potentials (AP; *2nd vertical line*). Dashed lines (c) and dotted lines (o) indicate channel closed and open states, in this and subsequent figuresy. Numbers at the *left* of traces indicate patch membrane potentials (V_p, in mV) (see METHODS). Data are from a representative patch. Arrows in −14, −34, and −54 mV traces: K_f channel openings. *B*: time course of action-potential-activated K_f channel activities at different patch membrane potentials. Postspike duration of channel activities after an AP was measured at different patch membrane potentials (*V*). At +36 and +56 mV, the K_f channel was activated by depolarization steps and inactivation started from the beginning of depolarization steps. We only include the data from AP-triggered openings of the channel. Data are means ± SE (n = 7 patches). *C*: current-voltage (*I-V*) relationship for K_f channels. Mean amplitude of outward single channel currents (*I*) was plotted against patch membrane potentials (*V*). Slope conductance calculated with linear regression was 37 ± 1.5 (pS means ± SE; n = 7 patches).

the patch depolarization step and RMP is the resting membrane potential obtained with whole cell recordings. The reported voltages were corrected for the liquid junction potential. In data from cell-attached patches with APs evoked by extracellular stimulation, an average RMP of −64 mV was used. Signals were acquired with Pclamp6.0-Clampex and stored on a video-tape recorder via a NEURO-CORDER converter (Cygnus Technology, Delaware Water Gap, PA). Extracellular stimuli (0.1 Hz) were applied through a bipolar tungsten electrode (impedance, 10 MΩ; FHC, Brunswick, ME) placed in neocortical layer II/III.

Channels permeable to K^+ ions were distinguished according to concentrations of ions across patch membrane and reversal potentials. Because the patch pipette solution contained neither Na^+ nor Ca^{2+} and intracellular levels of Na^+ and Ca^{2+} are relative low, unitary currents in cell-attached patch recordings should be carried by K^+ or Cl^-. Cell-attached patches contained 140 mM KCl, similar to the expected intracellular [K^+], thus the reversal potential for K^+ was close to 0 mV. The reversal potential for [Cl^-] was −40 to −60 mV in these neurons, as estimated from recordings of $GABA_A$ single channel currents obtained with GABA-filled cell-attached patch pipettes (unpublished data).

Solutions and drugs

The slice cutting solution contained (in mM) 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgSO_4$, 5 $CaCl_2$, 10 glucose, 26 $NaHCO_3$, and 230 sucrose. The standard slice solution contained (in mM) 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 26 $NaHCO_3$ (pH = 7.4 when gassed with 95% O_2-5% CO_2). The intracellular solution for filling whole cell electrodes contained (in mM) 117 $KMeSO_4$, 13 KCl, 0.1 EGTA, 2 $MgCl_2$, 10 HEPES, 1 ATP, 0.2 GTP, and 4 dextrose (pH adjusted to 7.2 with KOH; osmolarity 280). The solution for filling cell-attached patch electrodes contained (in mM) 130 KCl, 5 EGTA, 2 $MgCl_2$, 10 HEPES, and 4 dextrose (pH adjusted to 7.2 with KOH). Estimated final [K^+] was 140 mM after adding KOH to adjust pH. Dipotassium ATP (ATP), *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES), tetra-ethyl ammonium chloride monohydrate (TEA), ethylene glycol-bis(*b*-aminoethyl ether)*N, N,N*′*,N*′-tetraacetic acid (EGTA), poly-L-lysine, 1,2-bis(2-aminophenoxy)ethane-*N, N,N*′*,N*′-tetraacetic acid (BAPTA), and 4-AP were purchased from Sigma. Other chemicals were purchased from Mallinckrodt Specialty Chemicals (Paris, Kentucky).

Data analysis

PCLAMP 6.0 was used to plot recording traces and analyze amplitude histograms. Single channel currents were sampled every 50 μs with Fetchex and analyzed with Fetchan programs. A 50% threshold criterion was used to determine the duration of open and closed events. The collected open and closed intervals were binned with Pstat, and logarithmic distributions of the open and closed duration were exponentially fitted using the maximal likelihood method. The binwidth was determined automatically with Pstat. Amplitude histograms were analyzed via Fetchan and Pstat programs. Unitary currents for *I-V* curves were obtained from amplitude histograms. The time course of channel activities that followed single APs, "postspike duration," was measured from the positive AP peak to the end of channel activities. The mean value for postspike duration was obtained from 10 individual traces for each channel studied. Statistical data are presented as means ± SE.

RESULTS

Channel activities during action potentials

Large pyramidal neurons in layer V of the rat neocortex were visualized with DIC optics and identified by their oblong somata and single long apical dendrite extending toward to pia (Fig. 1*A*, *inset*). Dual patch-clamp recordings were performed on somata of these neurons to simultaneously acquire single channel activities (cell-attached patch) and neuronal action potentials (whole cell current clamp). The mean value of resting membrane potentials measured by whole cell electrodes was −64.0 ± 0.3 mV (n = 67 cells). When a neuronal AP was evoked with a depolarizing current pulse, openings of two

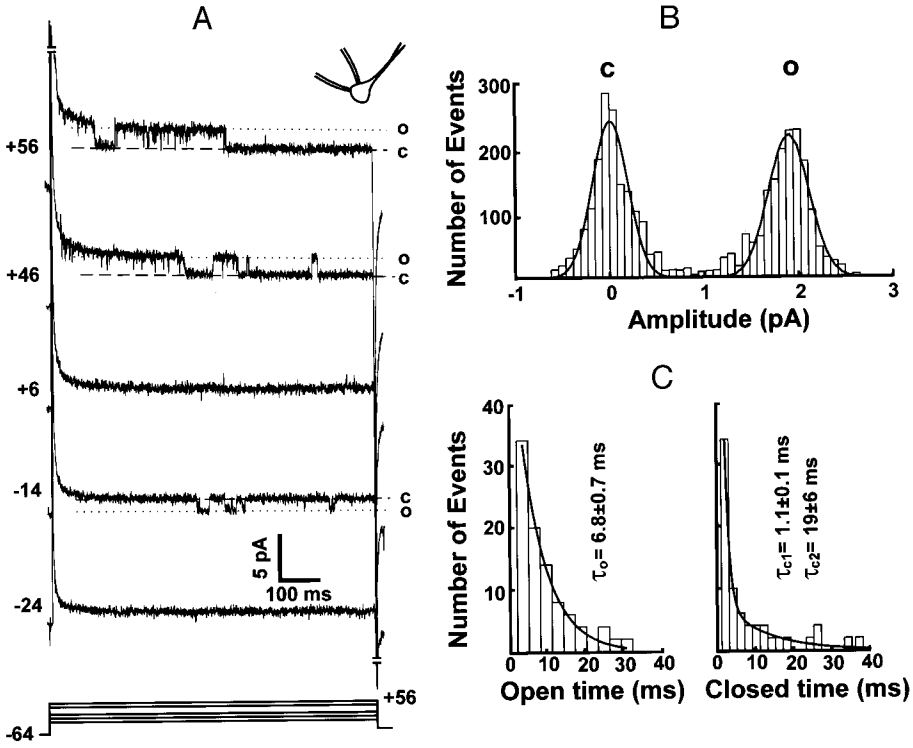

FIG. 3. Voltage-dependent activation of K_f channels. *A*: depolarization steps (— at *bottom*) were applied to cell-attached patches. Numbers at the *left* of traces are potentials across the patch membrane. - - - (c) and · · · (o), channel closed and open states, respectively. *B*: amplitude histograms at +56 mV. c, closed state. o, open state. *C*: open time and closed time distributions at +56 mV. Histograms are from a representative patch. Values for the mean open (t_o) and closed time (t_{c1} and t_{c2}) are means ± SE from 5 patches.

types of K^+ channels were observed in cell attached patches. A fast-opening channel (K_f) was characterized by very brief openings (Fig. 1*Ab*, ←), whereas a second channel (K_A) was characterized by longer openings and clustering (Fig. 1*Bb*, ▲). Twenty-three of 65 patches contained both K_f and K_A channels, 12 patches contained K_f channels without K_A channels, and 21 patches contained only K_A channels. A single K_f channel often was observed (7 of 12) in a patch (Fig. 1*Ab*, ←), whereas multiple K_A channels always were recorded (21 of 21) from one patch (Fig. 1*Bb*), indicating that K_A channels were localized in clusters.

Electrophysiological properties of K_f channels

To examine voltage-dependent properties of K_f channels, we obtained cell-attached patch recordings while evoking APs with extracellular stimulation. Cell-attached patches were conditioned by delivering voltage steps to the patch electrode (Fig. 2*A*, *bottom*), and an extracellular stimulus (200 μs) was delivered to layer II/III (Fig. 2*A*, arrow, Stim). Stimulus intensity was adjusted to reliably elicit a single spike with a delay of 7–50 ms (Fig. 2*A*, AP). Because only the patch membrane was depolarized, we could test voltage-dependent properties of

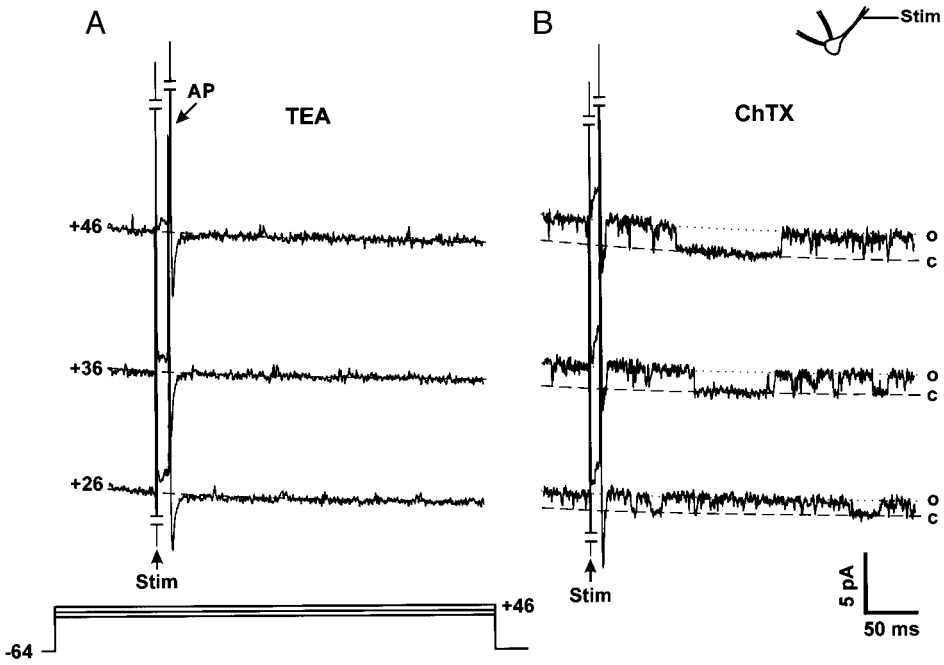

FIG. 4. Effects of 1 mM TEA and 100 nM charybdotoxin (ChTX) on K_f channels. *A*: inhibition of K_f channels by 1 mM TEA dissolved in the cell-attached patch pipette solution. Representative recordings, performed as described in Fig 2*A*, are shown. Numbers at the *left* of traces indicate patch membrane potentials. — at *bottom* indicate patch depolarization steps. *B*: K_f channels are not sensitive to 100 nM ChTX contained in the cell-attached patch pipettes. - - - and · · · , channel closed (c) and open states (o), respectively. Patch membrane potentials are indicated in *A*.

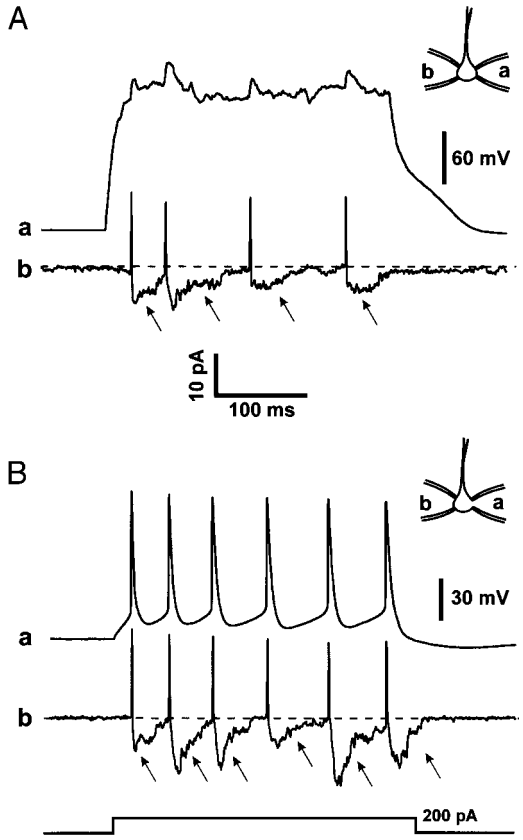

FIG. 5. K_A channels are not inhibited by chelation of intracellular Ca^{2+}. 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; 10 mM) was dissolved in the whole cell pipette solution. *A*: dual patch-clamp recordings before rupturing the whole cell patch membrane. *a*: current-clamp recordings with the whole cell pipette showing the high-input resistance before rupturing the membrane. *b*: cell-attached voltage-clamp recordings showing AP transient currents and K_A channel activities (←). *B*: dual patch-clamp recordings 2 min after rupturing the membrane. *a*: whole cell current-clamp recordings showing depolarization-induced APs. *b*: cell-attached recordings showing K_A channel activities (←). — at *bottom* is a depolarizing current pulse delivered to whole cell electrodes. - - -, closed state.

channels without changing RMP or neuronal excitability. In addition, AP-activated channel activities could be measured at different patch membrane potentials to examine voltage-dependent inactivation of the channel. Data from seven patches, which contained only K_f channels without K_A channels, were analyzed. When cell-attached patches were depolarized to a potential below −14 mV, the K_f channel was not activated by depolarization steps but opened by APs (Fig. 2*A*). Brief openings of the channel indicate fast inactivation (Fig. 2, *A* and *B*). The mean postspike duration of K_f channel activities was 6.1 ± 0.6 ms at −64 mV, 6.7 ± 0.7 ms at −54 mV, and 62 ± 15 ms at −24 mV (Fig. 2*B*, $n = 7$ patches). The results show that inactivation of K_f channels is voltage dependent. The current-voltage relationship (*I-V*) corresponded to a slope conductance of 37 ± 1.5 pS (Fig. 2*C*, $n = 7$ patches). Single channel currents were reversed at +1.3 ± 2.5 mV ($n = 7$ patches), which was close to estimated K^+ equilibrium potential for cell-attached patches ($E_K \approx 0$ mV). K_f channels were activated by strong patch depolarization (Fig. 2*A*, +36 and +56 mV), indicating that they are voltage-gated channels. To further test voltage-dependent activation of K_f channels, cell-attached patches were depolarized without extracellular stimulation. Patch depolarization induced openings of K_f channels (Fig. 3*A*) with a threshold of −22 ± 2.5 mV ($n = 7$ patches). Because only a small piece of membrane in the patch pipette was depolarized, no AP or AP-induced Ca^{2+} influx occurred. Therefore activation of K_f channels was independent of AP-induced Ca^{2+} influx. The amplitude histogram for K_f channel openings had a single peak (Fig. 3*B*, o), reflecting a single conductance state. Kinetic analysis demonstrated a mean open time (τ_o) of 6.8 ± 0.7 ms at membrane potential of +56 mV ($n = 5$ patches), which was close to the postspike duration of K_f channel activities at −64 mV (6.1 ± 0.6 ms), suggesting that the channel might open once, or very few times, during AP repolarization. A double exponential with a fast time constant (τ_{c1}) of 1.1 ± 0.1 ms and slow time constant (τ_{c2}) of 19 ± 6 ms was needed to best fit the closed time distribution (Fig. 3*C*, $n = 5$ patches).

Pharmacological properties of K_f channels

In rat neocortical pyramidal neurons, both AP repolarization and the fast-transient K^+ current were inhibited by low concentrations of TEA (1 mM, data not shown), similar to obser-

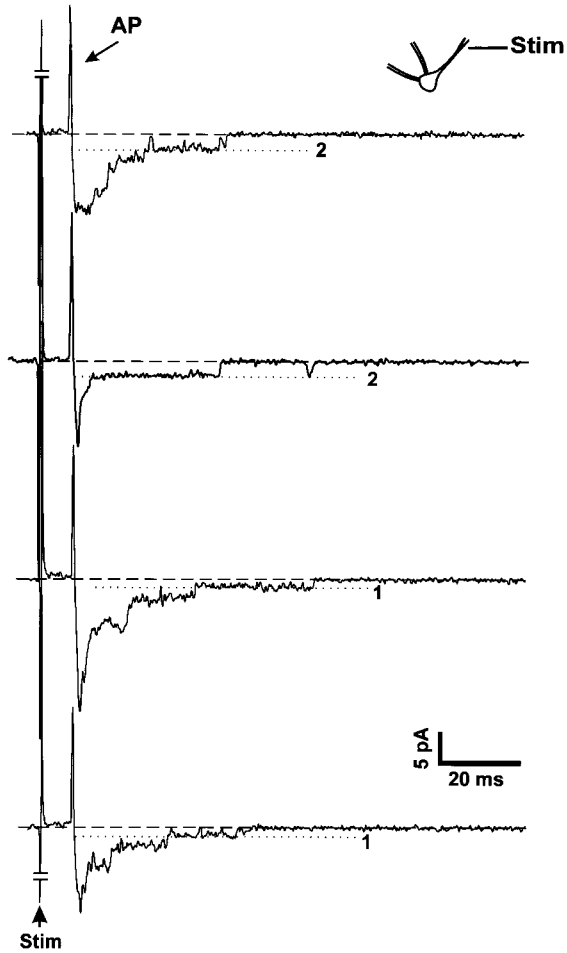

FIG. 6. Multiple subconductances of K_A channels. Four recording traces from a representative cell-attached patch illustrate at least 2 subconductance states (1 and 2) of K_A channels (dotted lines). Action potentials were evoked by extracellular stimuli (arrow, Stim). Patch was held at 0 mV. Amplitudes for the 2 open states are 1.2 ± 0.03 pA ($n = 15$ patches) and 2.3 ± 0.04 pA ($n = 15$ patches), respectively.

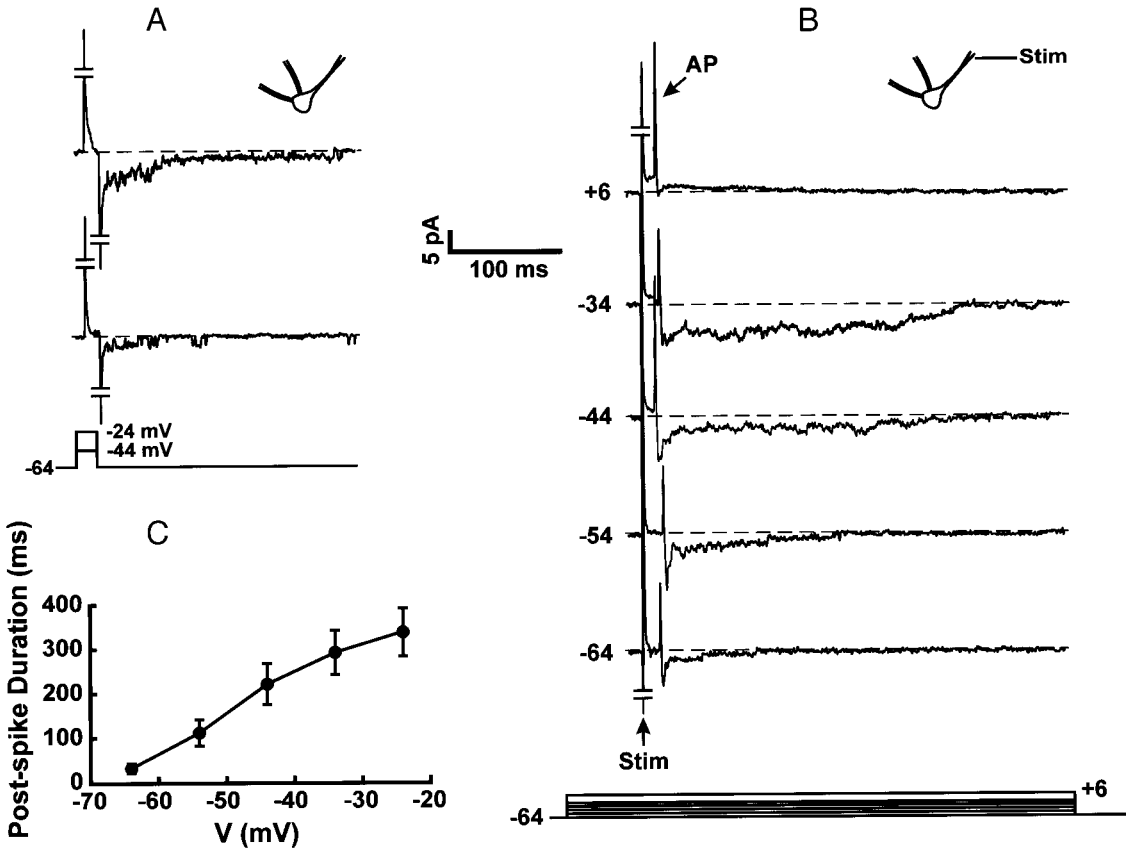

FIG. 7. Voltage-dependent activation and inactivation of K_A channels. *A:* activation of K_A channels by patch depolarization steps (*bottom lines*). Patch depolarization from rest (−64 mV) to −44 mV activated K_A channels (*bottom*). Larger depolarization (to −22 mV) activated more channels (*top*). *B*: voltage-dependent inactivation of K_A channels. Action potentials (AP) were evoked by extracellular stimuli (arrow, Stim) when cell-attached patches were window-depolarized by applying voltage steps with a 500-ms duration and a 20-mV increment (*bottom lines*). Postspike duration of K_A channel activities was longer at more depolarized compared with less depolarized patch membrane potentials (cf. −34- and −54-mV traces). Dashed lines indicate closed state. *C*: postspike duration of K_A channel activities at different potentials. This value was determined by calculating the decay time constant of averaged K_A channel activities. Decay constant was obtained by fitting the averaged trace (average of 10 traces) from the negative peak of the AP to the end of K_A channel activities with a single exponential.

vations in cat neocortical pyramidal neurons (Spain et al. 1991). To further determine whether K_f channels contribute to the TEA-sensitive component of AP repolarization, we attempted to block K_f channels by including 1 mM TEA in the patch pipette solution. K_f channel activities were not detected in any of the 35 TEA-containing cell-attached patches (Fig. 4*A*), indicating that K_f channels were TEA sensitive. Because Ca^{2+}-activated BK channels are very sensitive to both TEA (1 mM) and charybdotoxin (ChTX, 100 nM), we tested sensitivity of K_f channels to ChTX. K_f channel activities were not blocked in five cell-attached patch recordings obtained with pipettes containing 100 nM ChTX (Fig. 4*B*), demonstrating that K_f channels are pharmacologically distinct from BK channels (Kang et al. 1996).

Electrophysiological properties of K_A channels

To distinguish K_A channels from Ca^{2+}-activated K^+ channels, we dissolved 10 mM BAPTA, a calcium chelator (Tsien 1980), in the whole cell pipette solution. Before the whole cell patch membrane was ruptured, a depolarizing current pulse induced a large voltage jump (>200 mV, Fig. 5*Aa*), due to the high-input resistance (>1 GΩ) of the intact of patch membrane, together with *a* train of AP transient currents that showed frequency adaptation in cell-attached patch-clamp recordings (Fig. 5A*b*). Action potentials were followed by activities of multiple K_A channels (Fig. 5*Ab*). Once the patch membrane was ruptured, spike frequency adaptation was reduced significantly (Fig. 5*B, a* and *b*), presumably due to inactivation of Ca^{2+}-dependent channel activities by chelation of intracellular Ca^{2+}. However, K_A channel activities were not inhibited (Fig. 5*Bb*, ←), demonstrating that K_A channels are not Ca^{2+} dependent.

K_A channels showed multiple subconductances, a finding similar to that reported in tissue cultures (Forsythe et al. 1992). Because each patch often contained multiple K_A channels, it was difficult to distinguish all subconductance states from simultaneous openings of multiple channels. However, we could clearly distinguish at least two single channel current levels of 1.2 ± 0.03 pA and 2.3 ± 0.04 pA after evoked APs (n = 15 patches), especially in late openings (Fig. 6, 1 and 2). The slope conductance of K_A channels was not resolvable because channels were localized in clusters and multiple channels were activated when patches were depolarized (Fig. 7, *A* and *B*). When conductance was estimated by dividing the mean

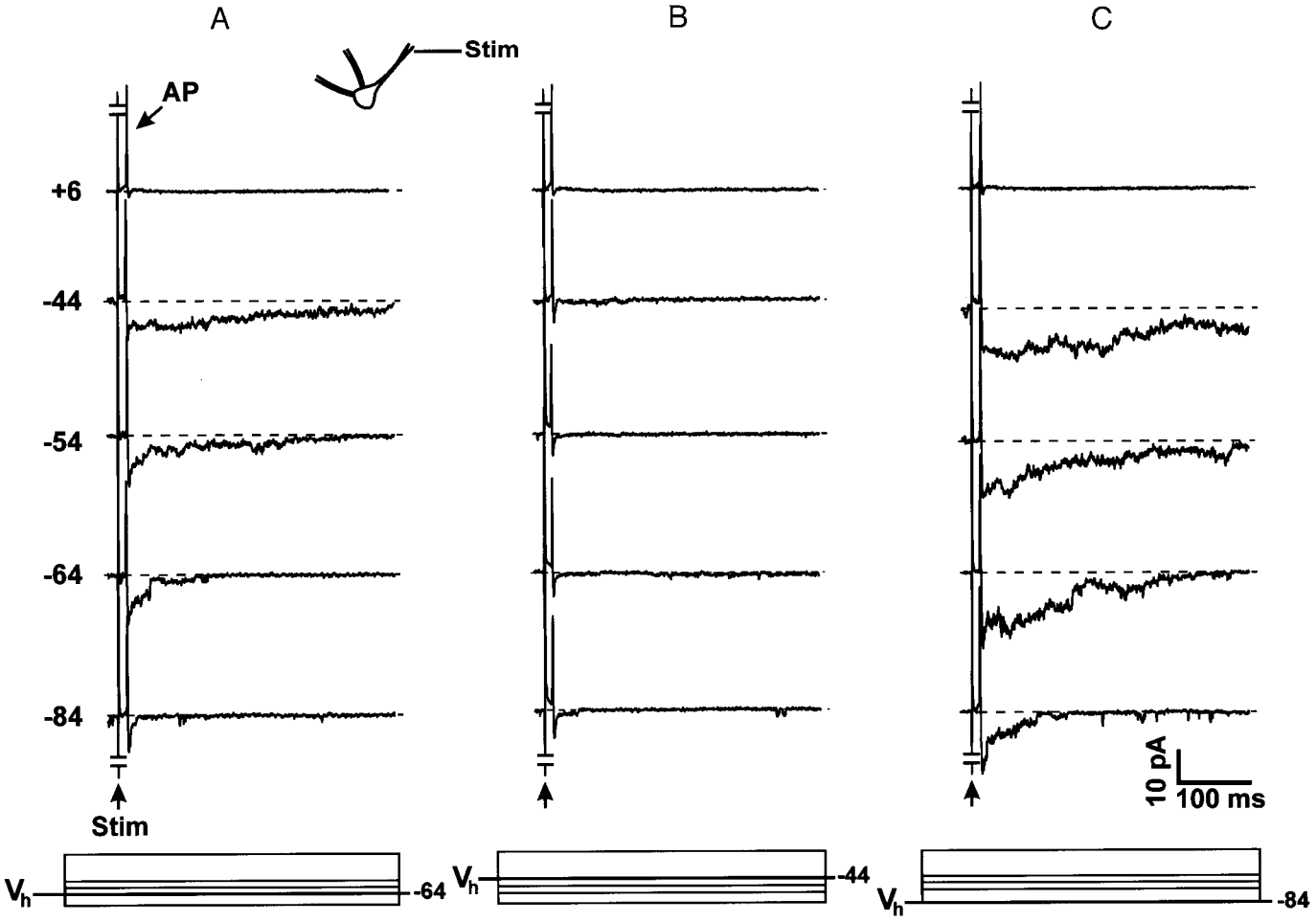

FIG. 8. Steady-state inactivation of K_A channels. Recordings of channel activities were performed as described in Fig. 7*B*. Cell-attached patches first were held (V_h) at rest (*A*, −64 mV), then 20 mV depolarized (*B*, −44 mV), and finally 20 mV hyperpolarized (*C*, −84 mV). More K_A channel activities were generated after APs when the patch was held at hyperpolarized potential (*C*). Solid lines below each column of traces indicate patch depolarization steps. Dashed lines indicate closed state. Data are representative of those obtained in 10 experiments.

current amplitude by −64 mV (assumed mean resting membrane potential from whole cell recordings), two major subconductances of 19 and 36 pS were obtained. The different conductances are likely to represent two subconductances of the K_A channel, rather than two subtypes of K_A channels because these two conductances always coexisted in patches containing K_A channels; transition between two states could be seen occasionally; they showed similar activation and inactivation; 4-AP blocks both of them; and similar subconductances of K_A channels have been reported previously in neuronal tissue cultures (Forsythe et al. 1992).

K_A channels are voltage-gated and were opened by patch depolarization with a threshold of −44.8 ± 2.9 mV (Fig. 7*A*, $n = 13$ patches). Channel activities were larger during tail currents than during the depolarization period because of the larger driving force at −64 mV than at −44 mV (Fig. 7*A*, *bottom*). Stronger patch depolarizations induced opening of more channels (Fig. 7*A*, *top*). To test the voltage-dependence of K_A channel inactivation, cell-attached patches were step depolarized (Fig. 7*B*, *bottom*) and APs were evoked by extracellular stimulation (Fig. 7*B*, arrow, Stim). Action-potential-activated K_A channel activities lasted much longer when the patch membrane potential was more positive than resting potential (Fig. 7, *B* and *C*, −54, −44, and −34). Postspike duration of K_A channel activities was 32 ± 6 ms at −64 mV, 112 ± 28 ms at −54 mV, and 367 ± 34 ms at −24 mV ($n = 5$ patches). The results indicated that inactivation from the opening state of K_A channels is voltage dependent. The time course of K_A channel activities would allow the channel to play roles in both AP repolarization and regulation of the repetitive firing rate. The steady-state inactivation property, previously described for I_A (Connor and Stevens 1971a,b), also was observed in K_A channel activities. When patch membrane was held at rest (−64 mV), AP-activated K_A channel activities were similar to those in Fig. 7*B* (Fig. 8*A*). When the patch membrane was held at −44 mV (5–15 s), K_A channels were steady-state inactivated (Fig. 8*B*). When the patch membrane then was held at −84 mV (10–20 s), K_A channels recovered from inactivation, and even more channels were activated by the AP than at rest (cf traces at V_h −64 mV, Fig. 8*A* and V_h −84 mV Fig. 8*C*), suggesting that some channels had been inactivated at rest. Steady-state inactivation occurs from the closed state of K_A channels and is different from inactivation occurring from opening state of the channel (Fig. 7, *B* and *C*). Steady-state inactivation is also voltage dependent and occurs in less channels when the patch membrane is more hyperpo-

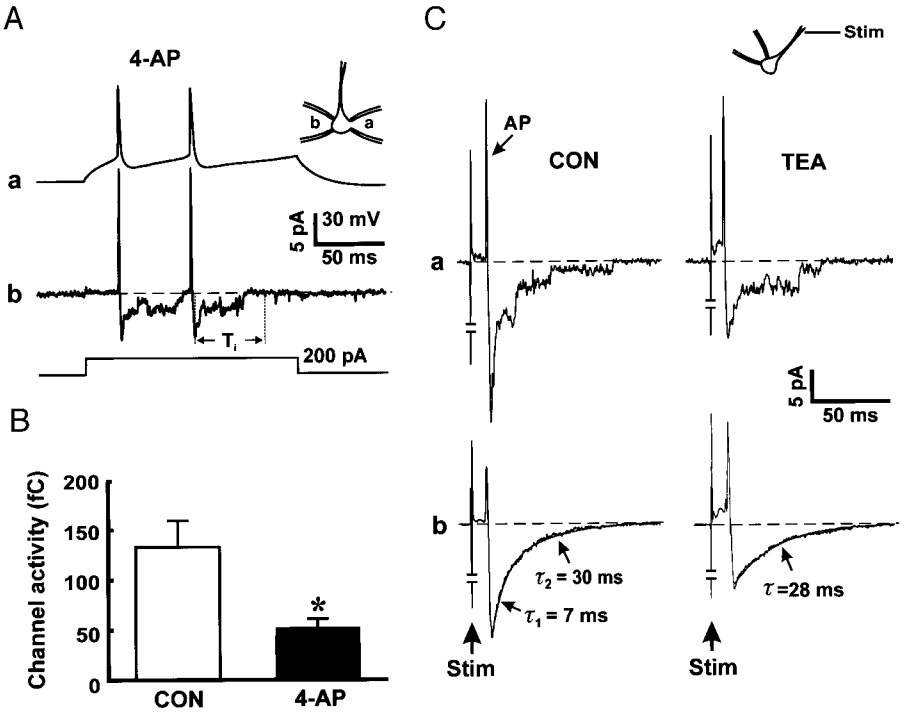

FIG. 9. Sensitivity of K_A channels to 4-aminopyridine (4-AP) and TEA. *A*: dual patch-clamp recordings in the presence of 4-AP. 4-AP (5 mM) and 1 mM TEA were predissolved in the cell-attached patch pipette solution, and 10 mM BAPTA was predissolved in the whole cell pipette solution. Recordings were performed as described in Fig. 1. T_i, integration time. *B*: integrated charge for channel activities in cell-attached patch recordings in the absence (CON) or presence (4-AP) of 4-AP. Control recordings (CON) were obtained in the presence of 1 mM TEA and 10 mM BAPTA. A 50-ms period of traces starting from the negative peak of the AP (T_i in *Ab*) was integrated. $*P < 0.05$ compared with control, *t* test. $n = 5$ patches (showing channel activity) for the control group and 4 patches (showing channel activity) for the 4-AP group. *C*: cell-attached patch recordings with extracellular stimulation-induced AP in the absence (CON) or presence (TEA) of 1 mM TEA. TEA was dissolved in the patch pipette solution. Recordings were obtained as described in Fig. 2*A*. *a*: representative traces from patches obtained with control (CON) and TEA-containing (TEA) patch pipettes. *b*: average of 20 traces. Decay was fitted by a double exponential (τ_1 and τ_2) in control (*left*) and a single exponential (τ) in TEA conditions (*right*). - - -, closed state. Data are representative from 21 patches.

larized (Fig. 8), whereas inactivation from opening state of the channel is faster when the patch membrane is more hyperpolarized (Fig. 7, *B* and *C*). Therefore voltage dependence for steady-state inactivation is opposite in direction to that for inactivation from the opening state of K_A channels.

Pharmacological properties of K_A channels

To examine sensitivity of K_A channels to 4-AP, dual patch-clamp recordings were performed in the absence or presence of 5 mM 4-AP in cell-attached patch pipettes. BAPTA (10 mM) and 1 mM TEA were added to the whole cell and cell-attached pipettes, respectively, to eliminate activities of Ca^{2+}-activated K^+ and K_f channels. Six of 10 control patches showed K_A channel activities, and 4/10 patches obtained with 4-AP-filled pipettes showed 4-AP-insensitive K_A channel activities (Fig. 9*A*). Measurements of the integrated charge for channel currents over a period of 50 ms indicated that 4-AP significantly inhibited K_A channel activities (Fig. 9*B*, $P < 0.05$, *t*-test, $n =$

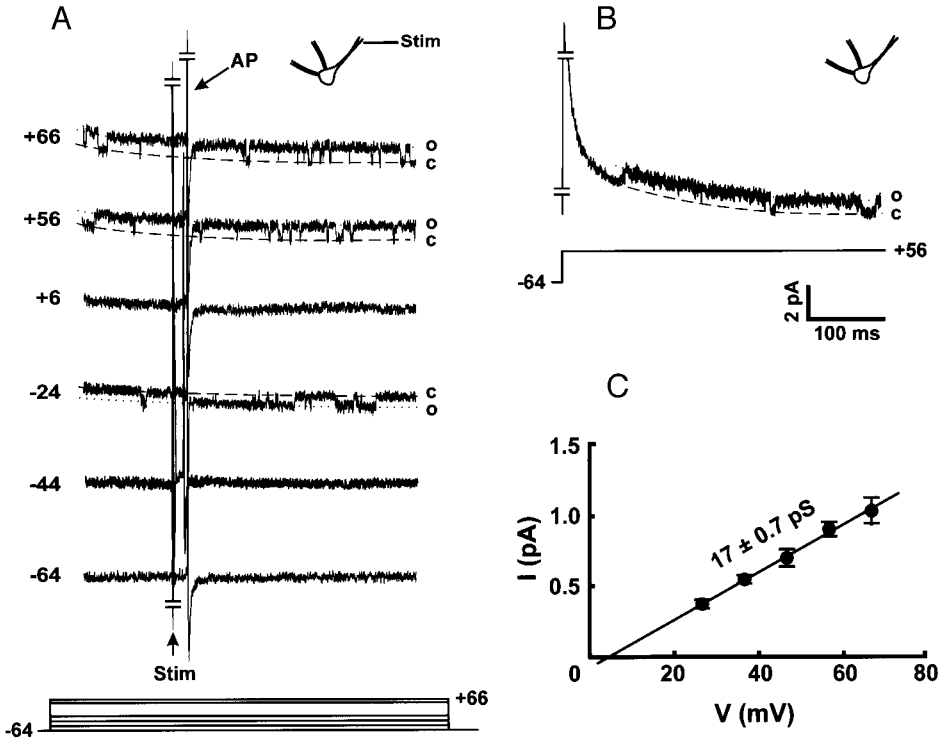

FIG. 10. *A*: K_{dr} channel activities activated by patch depolarization steps. Cell-attached patch recordings combined with extracellular stimulation-induced APs were performed as described in Fig. 2*A*. TEA (1 mM) and 5 mM 4-AP were predissolved in the patch pipette solution. K_{dr} channels were not activated by single action potentials but were activated by patch depolarization to −24 mV. *B*: activation of a K_{dr} channel by patch depolarization to +56 mV. — (*below* trace), patch depolarization step. Data in *A* and *B* are from a representative patch. *C*: *I-V* curve for K_{dr} channels. Number *above* to the regression line is mean ± SE for the slope conductance ($n = 5$ patches).

5 and 4 for control and 4-AP groups, respectively). K_A channels that were insensitive to 4-AP had properties similar to those of 4-AP-sensitive K_A channels, including subconductances, clustered localization, voltage-dependent activation and inactivation, and steady-state inactivation (data not shown). From these data, we cannot determine whether there are two types of K_A channel that are differentially sensitive to 4-AP or one type the sensitivity of which to 4-AP is relatively low, so that 5 mM 4-AP could not block all channels. K_A channel activities were not blocked by 1 mM TEA, although the fast component of decay component of postspike ensemble channel activity (τ1), which reflected K_f channel activities, was eliminated (Fig. 9*Cb*).

Activities of delayed rectifier K^+ channels (K_{dr})

Activities of a K_{dr} channel were observed in 5/10 patches recorded with TEA/4-AP-filled pipettes. K_{dr} channels were not activated by single APs when the patch membrane potential was more negative than −24 mV (Fig. 10*A*). Strong patch depolarization induced delayed long-lasting openings of K_{dr} channels (Fig. 10*B*). The threshold for K_{dr} channel openings was −23 ± 2.4 mV ($n = 5$ patches). A unique slope conductance of 17 ± 0.7 pS for K_{dr} channels was determined from *I-V* curves (Fig. 10*C*, $n = 5$ patches). The channel could not be a subconductance state of K_A channels because K_{dr} channels never showed multiple conductances; K_{dr} channels were not distributed in clusters; activation and inactivation of K_{dr} channels were slower than those of K_A channels; the opening threshold for K_{dr} channels (−23 ± 2.4 mV) was higher than that of K_A channels (−44.8 ± 2.9 mV); and insensitivity to 1 mM TEA or 5 mM 4-AP. The results suggest that K_{dr} channels may not contribute to repolarization of a single AP because of their delayed activation but, with a slow inactivation time course, may play a role in regulation of AP wave form during repetitive firing.

Activities of BK channels

Although BK channels have been hypothesized to contribute to AP repolarization and the fast AHP in hippocampal pyramidal neurons (Lancaster and Nicoll 1987; Storm 1987; Yoshida et al. 1991), we did not observe BK channel openings in any of the 65 "normal" cell-attached patches in rat neocortical pyramidal neurons even under conditions where Ca^{2+} (2 mM) was included in the patch pipette ($n = 5$). Further, BK channels were not activated when >20 APs were evoked by the depolarizing current (data not shown). However, after patches were excised from neurons and changed into the inside-out configuration (8/8 of patches), BK channel openings were observed, indicating that patches contained BK channels, but they did not open in intact neurons under our recording conditions. These results are in accordance with previous observations by other groups that no I_C was found in whole cell currents of cat neocortical pyramidal neurons (Spain et al. 1991) and spike repolarization was Ca^{2+} independent (Pineda et al. 1998; Schwindt et al. 1988). In six experiments, BK channels were not activated when neuronal APs appeared normal (Fig. 11, 20 min, a). However, BK channels started to open in a spike-independent manner when neuronal responses deteriorated as evidenced by smaller or absent spikes (Fig. 11, 40 and 42 min).

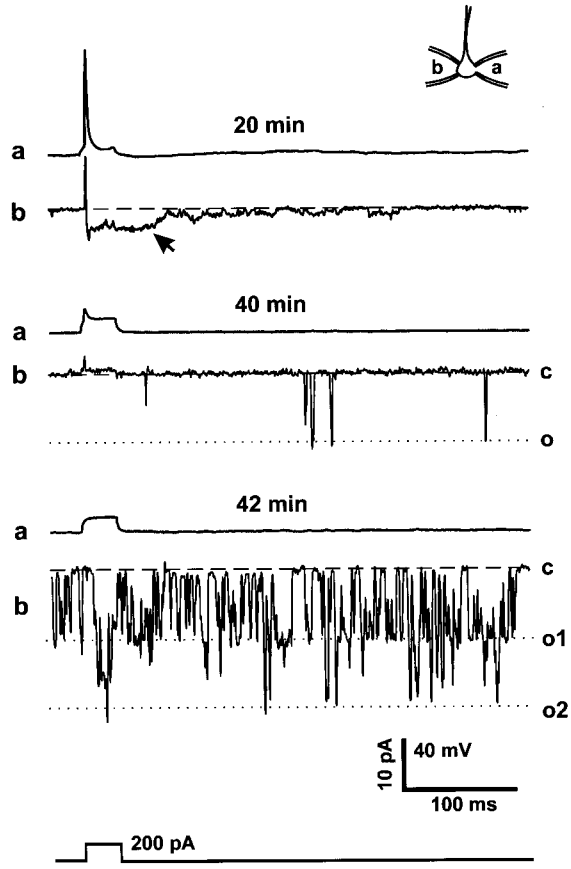

FIG. 11. Large conductance Ca^{2+}-activated K^+ (BK) channels were not activated during APs under normal conditions. Dual patch-clamp recordings were performed as described in Fig. 1. Times in minutes at the *top* of each set of double traces indicate times after rupturing the whole cell patch membrane. Arrow, K_A channel activities. *a*: whole cell recordings. *b*: cell-attached patch recordings. Dashed lines (c), indicate closed state. Dotted lines o1 and o2, indicate openings of 2 BK channels. Solid line at the *bottom* indicates the depolarization current delivered to the wholecell electrode. Data are representative for 6 experiments.

Initiation of BK channel activities often occurred over a short time (1–2 min) accompanied by a change in cellular morphology observed under DIC optics, implying that some untoward intracellular events might have occurred. The results suggest that somatic BK channels do not contribute to AP repolarization and the fast AHP in these cells but may play roles under pathological conditions.

DISCUSSION

In these experiments, we examined the properties of two voltage-gated K^+ channels activated by APs, K_f and K_A, using dual patch-clamp techniques applied to large layer V pyramidal neurons in neocortical slices. The properties of these two channels and a third, K_{dr}, that was activated by depolarizing steps, are summarized in Table 1. Fast and transient opening of K_f channels with a threshold of −22 mV suggests that they contribute to AP repolarization but not to sculpting interspike voltage trajectories. Openings were brief (Fig. 1*Ab*, postspike duration: 6.1 ± 0.6 ms), suggesting that they did not have a role in setting interspike intervals. The fast-transient K^+ current previously reported in whole cell recordings (Spain et al. 1991) likely results from K_f channel activities because both

TABLE 1. *Properties of K_f, K_A, and K_{dr} channels*

	Conductance, pS	Opening Threshold, mV	Activation	Postspike Duration, ms	Steady-State Inactivation, mV	TEA (1 mM)	4-AP (5 mM)
K_f	37 ± 1.5	−22 ± 2.5	Fast	6.1 ± 0.6	0 ~ −20	Sensitive	—
K_A	19 and 36	−45 ± 2.9	Fast	32 ± 6	−44 ~ −64	Insensitive	Sensitive
K_{dr}	17.5 ± 0.7	−23 ± 2.4	Slow	—	—	Insensitive	Insensitive

The values of conductance (except for K_A), threshold, and postspike duration are mean ± SE for 5–13 patches. Numbers for steady-state inactivation are voltage ranges for 7 and 10 patches, respectively. —, not tested. 4-AP, 4-amino pyridine.

events are similar in terms of voltage-dependent activation and sensitivity to 1 mM TEA. Although K_f channels are very sensitive to TEA, they are distinct from BK channels in terms of their conductance of 37 pS (Fig. 2*C*), voltage-gated activation without requirement for AP-induced Ca^{2+} influx (Fig. 3*A*) and insensitivity to ChTX.

K_A channels also were activated by action potentials. Action-potential-activated K_A channels might have been misinterpreted as Ca^{2+}-activated channels because K_A channel activities were attenuated by perfusion of cadmium in previous studies (Alger et al. 1994; Kang et al. 1995). However, results of BAPTA experiments (Fig. 5) showed that K_A channels are not Ca^{2+} dependent. Voltage-dependent activation of K_A channels at a low threshold of −45 mV suggests that these channels influence AP generation. Postspike duration of K_A channel activities was 32 ± 6 ms at −64 mV, indicating that these channels contribute to both AP repolarization and regulation of repetitive firing rate. When patches were depolarized, postspike duration of channel activities was much longer than that at rest (Fig. 7, *B* and *C*), suggesting voltage-dependent inactivation from the opening state of K_A channels. This property suggests that K_A channels play even larger roles when neurons are transiently depolarized, such as during repetitive firing. However, if neurons are depolarized for a prolonged time, K_A channels will be inactivated because of steady-state inactivation that occurs from closed state of channels (Fig. 8*B*). Steady-state inactivation is a well-known property of I_A and explains why spike frequency was higher when neurons were held at depolarized potentials (data not shown).

Although spike frequency adaptation was suppressed by chelation of intracellular Ca^{2+} in BAPTA diffusion experiments (Fig. 5), when using pipettes filled with control solution, we could not record activities of single Ca^{2+}-activated K^+ channels in cell-attached patches during the spike frequency adaptation or slow AHPs after repetitive spikes. A possible explanation is that Ca^{2+}-activated K^+ channels responsible for the spike adaptation and the slow AHP have too small a conductance (Sah and Isaacson 1995) to allow their detection from the background noise of our recording system. Another possibility is that SK channels are primarily dendritic in location.

Although BK channels have been hypothesized to contribute to AP repolarization and the fast AHP in muscle (Pallota et al. 1981; Walsh and Singer 1983), chromaffin cells (Marty 1981), GH_3 anterior pituitary cells (Lang and Ritchie 1987), and hippocampal neurons (Lancaster and Nicoll 1987; Storm 1987; Yoshida et al. 1991), direct evidence in this study demonstrates that somatic BK channels do not open during AP repolarization and the fast AHP in intact neocortical pyramidal neurons. We did not observe any BK channel activities in cell-attached patches from "healthy" neurons during normal neuronal firing. The absence of BK channel activities did not result from dilution of intracellular Ca^{2+} by whole cell electrodes because the same results were observed in cell-attached patch recordings combined with extracellular stimulation-induced APs, where the neuronal membrane was intact. BK channels were not activated even when neurons were firing at a high rate (>20 spikes per train). At this spiking rate, intracellular Ca^{2+} should be higher than the threshold concentration of Ca^{2+} necessary for activation of BK channels (Kang et al. 1996; Ross 1993). Absence of BK channel openings was not due to lack of local Ca^{2+} entry because BK channel activities were not observed during APs when the cell-attached patch contained $CaCl_2$. Under such conditions, APs should have depolarized the patch membrane intracellularly and induced Ca^{2+} influx through Ca^{2+} channels in the patch. The results in the present study are in accordance with the previous observations that spike repolarization was not Ca^{2+} dependent in neocortical pyramidal neurons (Lorenzon and Foehring 1995; Pineda et al. 1998; Schwindt et al. 1988). BK channels were observed only as neurons deteriorated. At the time BK channels started to open, neuronal spikes had always become smaller or absent (Fig. 7, 40 min) and morphological alterations were often seen. Therefore BK channels may function in pathological conditions but do not contribute to AP repolarization and the fast AHP in these cells.

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