MULTIPLE TYPES OF VOLTAGE-DEPENDENT Ca^{2+}-ACTIVATED K^+ CHANNELS OF LARGE CONDUCTANCE IN RAT BRAIN SYNAPTOSOMAL MEMBRANES

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ABSTRACT K^+-selective ion channels from a mammalian brain synaptosomal membrane preparation were inserted into planar phospholipid bilayers on the tips of patch-clamp pipettes, and single-channel currents were measured. Multiple distinct classes of K^+ channels were observed. We have characterized and described the properties of several types of voltage-dependent, Ca^{2+}-activated K^+ channels of large single-channel conductance (>50 pS in symmetrical KCl solutions). One class of channels (Type I) has a 200–250-pS single-channel conductance. It is activated by internal calcium concentrations $>10^{-7}$ M, and its probability of opening is increased by membrane depolarization. This channel is blocked by 1–3 mM internal concentrations of tetraethylammonium (TEA). These channels are similar to the BK channel described in a variety of tissues. A second novel group of voltage-dependent, Ca^{2+}-activated K^+ channels was also studied. These channels were more sensitive to internal calcium, but less sensitive to voltage than the large (Type I) channel. These channels were minimally affected by internal TEA concentrations of 10 mM, but were blocked by a 50 mM concentration. In this class of channels we found a wide range of relatively large unitary channel conductances (65–140 pS). Within this group we have characterized two types (75–80 pS and 120–125 pS) that also differ in gating kinetics. The various types of voltage-dependent, Ca^{2+}-activated K^+ channels described here were blocked by charybdotoxin added to the external side of the channel. The activity of these channels was increased by exposure to nanomolar concentrations of the catalytic subunit of cAMP-dependent protein kinase. These results indicate that voltage-dependent, charybdotoxin-sensitive Ca^{2+}-activated K^+ channels comprise a class of related, but distinguishable channel types. Although the Ca^{2+}-activated (Type I and II) K^+ channels can be distinguished by their single-channel properties, both could contribute to the voltage-dependent Ca^{2+}-activated macroscopic K^+ current (I_C) that has been observed in several neuronal somata preparations, as well as in other cells. Some of the properties reported here may serve to distinguish which type contributes in each case. A third class of smaller (40–50 pS) channels was also studied. These channels were independent of calcium over the concentration range examined (10^{-7}–10^{-3} M), and were also independent of voltage over the range of pipette potentials of −60 to +60 mV. Type III channels were unaffected by internal TEA concentrations ≤50 mM. Our results also indicate that the study of K^+ channels in lipid bilayers may allow the identification and characterization of novel K^+ channels from brain regions otherwise inaccessible to conventional recording techniques.

INTRODUCTION

Many various distinct K^+ channels have been described and characterized in diverse cell types (e.g., Thompson and Aldrich, 1980; Dubois, 1983; Latorre and Miller, 1983; Hille, 1984; Adams and Galvan, 1986; Rudy, 1988). Potassium channels are both ubiquitous and diverse in their gating, conductance, and functional role. Potassium conductances are involved in such physiological processes as determination of resting potentials, action potential repolarization, after potential generation, and the control of repetitive firing.

Potassium conductances also appear to play key roles in many examples of neuronal modulation (Kaczmarek and Levitan, 1987). They may be involved in the modulation of transmitter release from presynaptic nerve terminals (Siegelbaum et al., 1982) as well as in persistent excitability changes of the somatic membranes of central neurons (Kaczmarek and Levitan, 1987; Bartfai et al., 1985; Ewald et al., 1985; Farley and Auerbach, 1986). Many interesting examples of neural modulation in which K^+ channels have been implicated occur within the mammalian brain (see examples in Adams and Galvan, 1986; Kaczmarek and Levitan, 1987; Rudy, 1988), often in regions inaccessi-

ble for conventional voltage- or patch-clamp studies of ion conductances. Thus, techniques that would permit the study of channels from these regions are of obvious interest. Here, we describe the detection and characterization of several distinct varieties of K^+ channels, obtained from a rat brain membrane preparation, by the incorporation of synaptosomal membrane vesicles into artificial membranes made on the tips of patch-clamp electrodes (Coronado and Latorre, 1983).

Two channel groups described here are Ca^{2+}-activated. The calcium-activated K^+ channels are distributed in many cell types and play important functional roles in the control of membrane excitability (Meech, 1978; Lew and Ferreira, 1978; Latorre and Miller, 1983; Peterson and Murayama, 1984; Dubinsky and Oxford, 1985; Adams and Galvan, 1986; Madison and Nicoll, 1986). Two classes of Ca^{2+}-activated K^+ channels have been well characterized and are considered to be typical of various excitable cells including neurons from mammalian brain. One is a voltage-dependent channel of characteristic large unitary conductance (BK channel; Marty, 1981). This channel is blocked by nanomolar concentrations of charybdotoxin (CTX), a toxin found in the venom of the scorpion *Leiurus quinquestriatus* (Miller et al., 1985), as well as by low (0.1–1 mM) concentrations of tetraethylammonium (TEA) applied to the exterior membrane surface (Latorre et al., 1982; Blatz and Magleby, 1984). The second channel shows little or no voltage-dependence, is of small (10–14 pS) unitary conductance (Blatz and Magleby, 1986), and is blocked by the bee venom toxin apamin (Romey and Lazdunski, 1984; Pennefather et al., 1985; Lazdunski, 1983). This apamin-sensitive channel is resistant to block by 20–25 mM concentrations of external TEA (Romey and Lazdunski, 1984). In addition to these two channels, other calcium-activated K^+ channels with conductances ranging between 4 and 60 pS have also been described in skeletal muscle (Blatz and Magleby, 1986), molluscan neurons (Lux et al., 1981; Ewald et al., 1985; Farley, 1988), red blood cells (Hamill, 1983; Grygorczyk et al., 1984), and HeLa cells (Sauve et al., 1986).

One of the channels that we found in rat brain membranes has the properties of the BK channel. We also found other voltage-dependent, CTX-inhibited Ca^{2+}-activated K^+ channels of relatively large conductance which can be distinguished from the BK channel by their size and sensitivities to Ca^{2+}, voltage, and internal TEA. Our results suggest the presence of a family of large-conductance, calcium-dependent, CTX-inhibited K^+ channels in mammalian brain whose individual subclasses may play different functional roles in the determination of membrane excitability. Activity of these Ca^{2+}-activated K^+ channels was increased by exposure to nanomolar concentrations of the catalytic subunit of cAMP-dependent protein kinase. Although the various types of large conductance channels can be distinguished by their single-channel properties, they may be difficult to distinguish in macroscopic voltage-clamp or current-clamp studies, and both may contribute to I_C, the macroscopic current usually associated with the BK channel. That all of these channels were often observed in the same type of membrane in our studies suggests that their distinctive properties are intrinsic to the channel rather than due to the membrane environment.

Preliminary reports of these studies have appeared (Farley and Rudy, 1985; Reeves et al., 1986).

MATERIALS AND METHODS

Glass and Pipettes

Patch-clamp electrodes were made from Boralex capillaries (100-μl microcapillaries; Fisher Scientific Co., Pittsburgh, PA) or soda-lime class II glass (Sherwood Medical Industries, Inc., St. Louis, MO). Pipettes were pulled with a vertical pipette puller (No. 700; David Kopf Instruments, Tujunga, CA) using the standard two-pull method (Hamill et al., 1981). Heater current was adjusted to produce electrodes with "bubble-numbers" of 2–4 (Corey and Stevens, 1983). We estimate these electrodes to have tip diameters of 1–2 μm. In early experiments, all electrodes were fire-polished by bringing the electrode tip close to the tip of a 10-μm platinum wire coated with soft glass. We later determined that fire polishing was not critical to the success of the experiments. The electrode tip was washed in methanol just before use. Tip resistances of electrodes were measured in a symmetrical solution of 200 mM KCl, 10 mM Tris-Cl (pH 7.0), and only electrodes with resistances between 5 and 15 MΩ were used.

Solutions

All solutions were prepared on the day of use from reagent-grade solid salts (Fisher Scientific Co.) or stock solutions in early experiments. Millipore Super Q purified water was used throughout (18 MΩ cm^{-1}; Millipore Corp., Bedford, MA). Bilayer chambers were, in early experiments, made of Teflon. These chambers were rinsed with chloroform, HCl, NaOH, and filtered water before each experiment. In later experiments, sterile disposable 35-mm polystyrene tissue culture dishes (Corning Glass Works, Corning, NY) were used once and then discarded. Most experiments were done in 0.2 M KCl + X M Ca^{2+} Cl_2 + 10 mM Tris Cl solution, pH 7.0. Free calcium concentrations in the bath depended upon the experiment and were adjusted between 10^{-8} and 10^{-3} M, using Ca/EGTA buffers and assuming a dissociation constant of 10^{-7} M (Caldwell, 1970). Membranes were initially formed with a free bath calcium concentration of 10^{-5} M. Pipette solutions always contained 10^{-5} M calcium. Pipette and bath solutions were otherwise always the same, except when TEA (Aldrich Chemical Co., Milwaukee, WI), 4-aminopyridine (4-AP; Sigma Chemical Co., St. Louis, MO), or CTX (gift of Dr. C. Miller, Brandeis University) were added as blocking agents. These were prepared on the day of use. In experiments with the catalytic subunit (CS) of AMP-dependent protein kinase (cAMP PK), 2–10 mM Mg/ATP was also present in the bath and pipette. The millimolar concentrations of Mg^{2+} allowed us to maintain stable bilayers with lower than usual (1 μM) *cis* free calcium concentrations.

Preparation of Rat Brain Homogenate

Synaptosomal membrane preparations were prepared from whole rat brains using variations of methods described by Krueger et al. (1979). Similar results were obtained with sucrose-gradient fractionated synaptosomes or with cruder synaptosomal membranes. Membrane preparations were kept frozen at −80°C, thawed on the day of use, and successfully used over a 5–6-mo period after the initial preparation.

Enzyme

The catalytic subunit of cAMP-dependent PK was purified to homogeneity according to methods outlined by Reimann and Beham (1983). The enzyme ran as a single 41,000-mol-wt band using SDS-PAGE. Specific activity was 9.8×10^5 U/mg protein, where 1 U = 1 pmol of phosphate transferred/min, assayed according to Corbin and Reimann (1974). Purified enzyme was stored in a buffer containing 45 mM KH_2PO_4, pH 7.0 at −4°C, and thawed 1–2 h before use. Final bath concentrations spanning the range of 10^{-9}–10^{-7} M were achieved by addition of 2–200 μl aliquots to the bath volume (2 ml).

Lipids, Bilayers, and Electronics

All lipids were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and were stored in chloroform, under nitrogen at −80°C for not more than 1 mo. The majority of experiments used a 70:30 molar ratio of bovine brain phosphatidylethanolamine (PE) and phosphatidylserine (PS). The final lipid mixture concentration was 10 mg ml^{-1} and was made in pentane (Aldrich Gold Label). Membranes were formed at neutral pH (7.0 ± 0.2). The channels described here were also observed in membranes made with the synthetic lipid di-oleolylphosphatidylethanolamine (DOPE; Avanti cat. No. 850725) at pH 6.0 (Coronado, 1985). DOPE membranes are not charged at this value. Several other lipid combinations were also explored, but the 70:30 PE/PS mixture yielded the most stable and reliable membranes.

High resistance bilayers were formed on the tips of patch-clamp electrodes using minor variations of methods first described by Coronado and Latorre (1983). To form bilayers, the pipette was first introduced into the bath in the absence of lipids with positive pressure applied to the pipette. Seals (1–20 GΩ) were formed by adding 1–2 μl of the lipid mixture to the surface, waiting several minutes for the solvent to evaporate, and then withdrawing the pipette into the air after releasing the pressure and quickly dipping it back a second time. If, after several passes, a seal of at least one gigaohm (GΩ) had not formed, the initial bath calcium concentraion of 10 μM was raised. If, after several additional passes, no membrane was formed, the electrode was discarded and the process begun again. In the majority of our studies using mixed membranes, we found that bath free calcium concentrations of at least 10^{-5} M were necessary to form gigaohm seals reliably (~90% of the time) and stably (membranes lasting 10 min or more), in agreement with results reported by Coronado (1985) for pure PS membranes. We also observed that the stabilizing effects of Ca^{2+} generally could not be mimicked by equimolar substitution of other divalent cations (Ba^{2+} and Mg^{2+}). Channel incorporation into bilayers was started by adding synaptosomal membranes to the bath (Miller, 1978). In a few experiments, the synaptosomal preparation was added to the pipette in a 0.2 M KCl solution containing 10^{-5} M Ca. In these experiments the bath concentration of Ca^{2+} was raised to 1 mM.

In early experiments, before the incorporation and detection of K^+ channels from rat brain, we periodically checked that the seals formed were actually bilayers by the recording of alamethicin and gramicidin channels.

The pipette holder and head stage amplified were mounted on a coarse micromanipulator (Narashige M0-103N; Narashige Scientific Instrument Lab., Tokyo, Japan). The head stage amplifier (10-GΩ feedback resistance) was in turn connected to a patch-clamp amplifier (model 8900; DAGAN Corp. Minneapolis, MN), which was used in the voltage-clamp mode to amplify current and to control voltage across the bilayer. The convention adopted hereafter is to refer all potentials to the bath: ($V_{bath} - V_{pipette}$). Thus, a negative potential change in the pipette results in a depolarization sensed by channels whose "intracellular" side faced the bath. Small voltage drops (0 to −10 mV) were present between the pipette and ground at the beginning of the experiment. Reported potential values are absolute and were not corrected for these voltage drops. Membrane resistance was measured using the internal pulses available from the amplifier (either 0.2 or 20 mV from an adjustable holding potential). Ag/AgCl electrodes served to connect the aqueous solutions to the electronics. For all examples reported in this paper, *cis* refers to the side of the membrane to which vesicles were added (usually the bath). The bath was connected through a 0.2 M KCl agar bridge to ground.

Amplifier output was low-pass filtered (8-pole Bessel filter; Frequency Devices, Beverly, MA, No. 902LPF) between 300 and 1,500 Hz, depending upon the experiment. In early experiments, patch-clamp currents were recorded on magnetic tape (model No. 760; Ampex Corp., Redwood City, CA) at tape speed 7½ ips, and played back for display on either a storage oscilloscope or a model 220 brush recorder (Gould Inc., Cleveland, OH), for data analysis by hand. In later experiments, patch-clamp currents were recorded on video cassette tapes using a Neuro Data Instruments, New York, NY, PCM digitizing recorder (44 kHz; model No. 384). Recordings were further digitized at 0.1-ms time intervals and stored in an IBM-XT computer. The digitized record was used to measure the amplitude and duration of individual current pulses, as well as the interval between successive pulses, by an interactive semi-automatic procedure ("p clamp" software; Axon Instruments, Inc., Burlingame, CA). The data from the idealized record were used for construction of single-channel current amplitude, open-, and closed-time histograms. Probability of channel opening (P_{open}, equal to the ratio of the total time spent in the open state to the total length of record) was also determined from the records. All reported measurements of open probabilities and open/shut intervals were taken after the membrane had been held at the indicated potential for a minimum of 30 s, unless otherwise indicated.

RESULTS

After formation of a planar lipid bilayer on the tip of the patch electrode, and before the addition of the synapto-

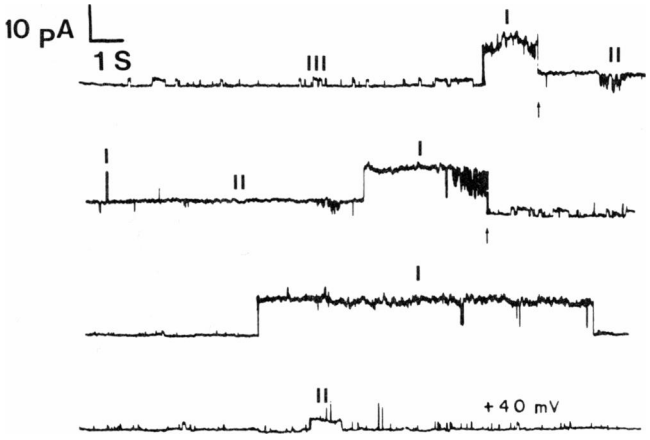

FIGURE 1 Three distinct types of K^+-selective ion channels recorded from the same bilayer on a patch-clamp electrode. Membrane was clamped at +40 mV throughout. Free calcium concentration was 10^{-5} M and records are continuous with respect to time. The largest channel (Type I) had a unitary conductance of 218 pS in this experiment, and often exhibited rapid flickering. An intermediate-sized channel (Type II) had a unitary conductance of 83 pS in this experiment and was characterized by extremely long open times. For example, an intermediate-sized Type II channel first opened during the time that a large (Type I) channel was open in trace 1 at top (*arrow*) and remained open for ~9 s before closing in the second trace (*arrow*). A briefer opening of this channel also occurs in the fourth trace. The third channel (Type III) had a unitary conductance of 45 pS in this experiment and is clearly resolved during the first several seconds of the top trace and during the final several seconds of the second and last traces. In general, all three channel types were seen in the same membrane, and often several of the same channel types were present. Occasionally, however, single channels of each one of the three classes could be recorded. Records filtered at 300 Hz.

somal membrane vesicles to the bath, voltage jumps applied to the pipette gave rise to a brief capacitative transient followed by a steady-state current which was linearly related to the change in holding potential (between −30 and +50 mV). After the addition of the synaptosomal membrane preparation, the same voltage jumps gave rise to the appearance of discrete step-increases in current, which represent the opening of single ion channels. A variety of channel "types" were observed in different membranes. We report here on those classes of channel that were conspicuous, because of their large size and the high frequency with which they were encountered.

Fig. 1 illustrates three classes of K^+ channels seen in many experiments. More than one channel type was often observed in the same membrane, as in these records. On a few occasions, channels of a single type were incorporated and studied. These channel types were clearly distinguishable on the basis of their (*a*) unitary conductance, (*b*) calcium sensitivity, (*c*) voltage-dependence, (*d*) kinetics, and (*e*) pharmacology. All these channels are K^+-selective. Single-channel current was positive (cation flow into the pipette) at negative pipette potentials. Channels reversed polarity at 0 mV (plus the uncorrected potential that appeared after membrane formation, as described earlier) in symmetric 200 mM KCl solutions. Addition of 100 mM NaCl to the bath, or substitution of K-methanesulfonate for KCl did not change the magnitude of the single-channel currents at any given potential or the reversal potential.

Type I: Large Ca^{2+}-activated K^+ Channels

Channel Type I (Figs. 1 and 2), seen clearly in 108 of 117 experiments, had the largest (mean ± SEM; 221 ± 12.3

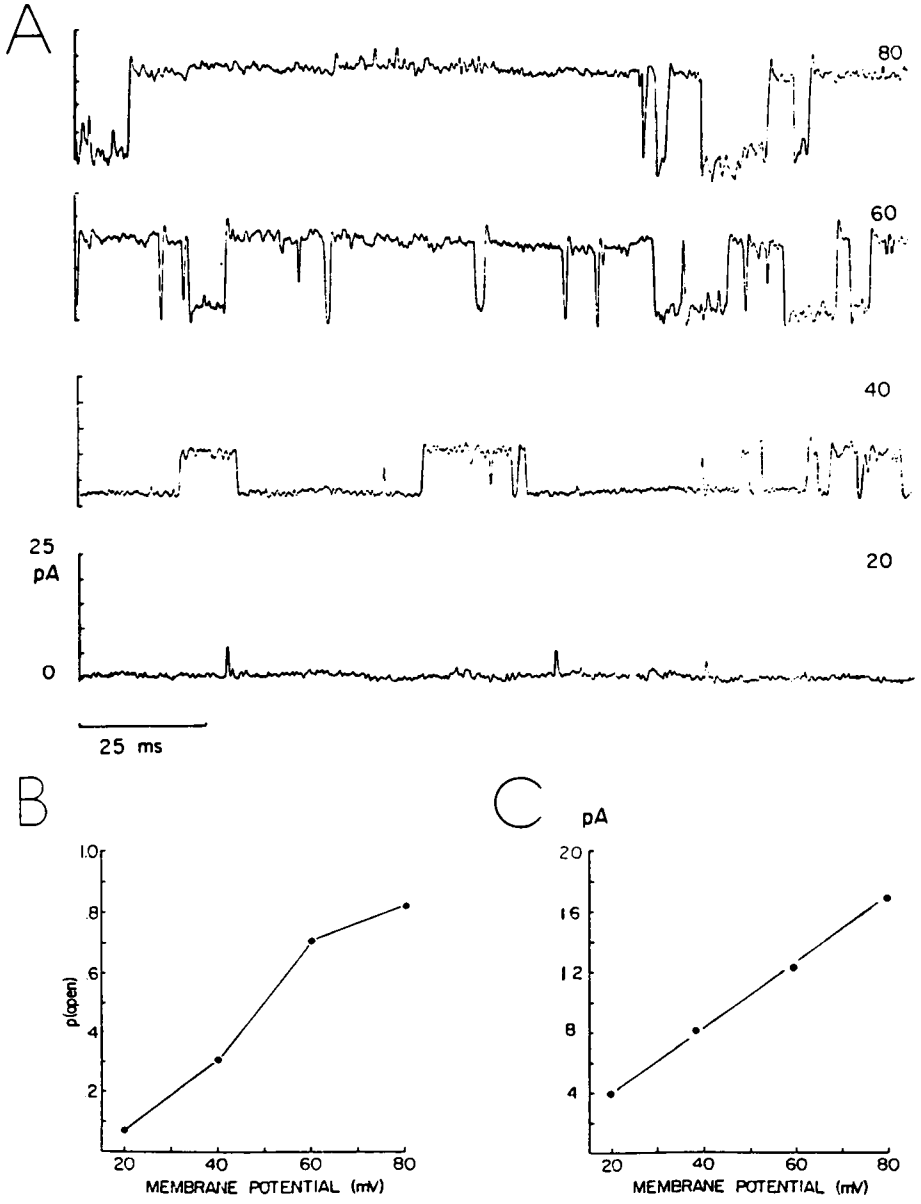

FIGURE 2 Voltage-dependence of large calcium-dependent K^+ channel (Type I). (*A*) Samples (150 ms) of data used to calculate voltage-dependence. PE/PS bilayer was voltage-clamped to indicated membrane potential, spanning the range between +20 and +80 mV. Free calcium concentration was 10^{-5} M. A single Type I channel was present in the membrane. (*B*) Summary of data from 12.5-s records, like those depicted in *A*, at indicated membrane potential. Over the steepest portion of the curve (20–60 mV), the probability of the channel being in the open state increased *e*-fold per 11.0 mV. Similar voltage-dependency was observed at a lower free calcium concentration (10^{-6} M), but the open-time probabilities were less at equivalent membrane potentials. (*C*) Single-channel, current-voltage relation for records in *A*. Slope conductance (200 pS) of channel is approximately linear.

pS) unitary conductance. At *cis* free Ca^{2+} concentrations of 10^{-5} M, the value used in the majority of our experiments, the single-channel current amplitude was proportional to voltage (Fig. 2 *c*). In general, single-channel current amplitudes were linearly related to membrane potential over the range of −30 to +50 mV (not shown). At Ca^{2+} concentrations of 10^{-5} M, the large conductance channel showed complicated gating kinetics with long periods of fast fluctuations between a closed and open state separated by long periods in which the channel is shut (Fig. 3 *e*). The effect of steady-state voltages on large channel gating behavior during periods of activity was analyzed from single-channel records in which the holding potential was maintained for 20–30 s. Channel behavior was studied in membranes in which no more than one large channel was open during the period of observation. As the pipette voltage was made more positive, the channel remained longer in the open state (Fig. 2 *a*), with approximately *e*-fold increases in the probability of being in the open state per 11 mV increases in voltage over the range of steepest voltage-dependency (20–60 mV; Fig. 2 *b*). At lower Ca^{2+} concentrations, the voltage dependency was shifted towards more positive potentials without pronounced changes in the slope of the function relating membrane potential to open-time probability. At Ca^{2+} concentrations higher than 10^{-5} M, the channels were opened a large fraction of the time at the negative membrane potentials tested. These effects of Ca^{2+} upon the channel's voltage-dependence are qualitatively similar to those reported by other investigators concerning large calcium-activated K^+ channels seen in cultured bovine chromaffin cells (Marty, 1981), skeletal muscle (Barrett et al., 1982; Methfessel and Boheim, 1982), and t-tubule membranes from mammalian muscle (Moczydlowski and Latorre, 1983; Latorre et al., 1983). In several cases a decrease in unitary conductance and channel block was also seen at high

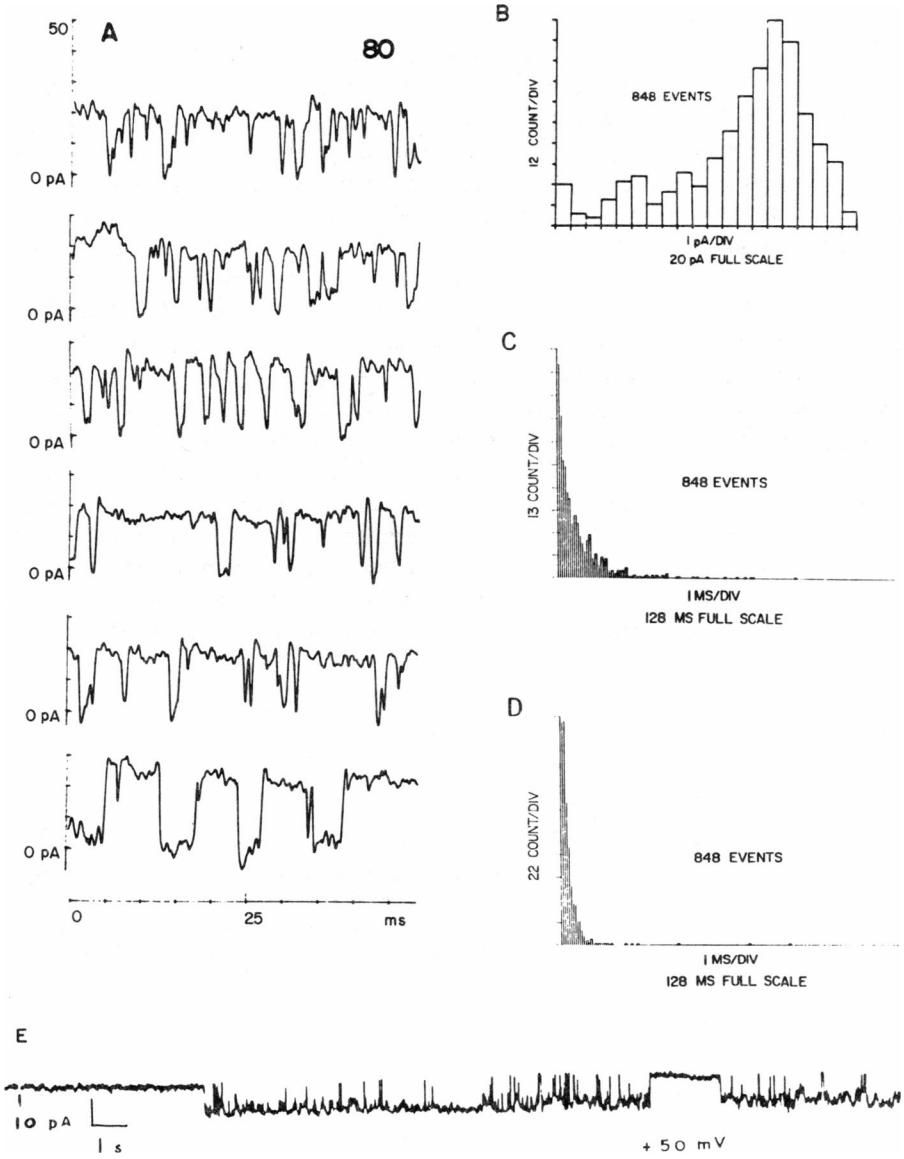

FIGURE 3 (*A*) Representative 50-ms samples of activity of Type I channel, during a burst, at 80 mV in presence of 10^{-5} M Ca^{2+}. Note presence of short open times and rapid flickering in first three samples, and longer open times in the last three samples. (*B*) Single-channel current amplitude histogram constructed from 848 instances of channels, as depicted in *A*. The mode of the distribution occurs between 15 and 16 pA, yielding a modal unitary conductance of 185–200 pS. (*C*) Open-time histogram constructed from 256 consecutive 50-ms samples of activity. This histogram is not well-fit by a single exponential decay function. (*D*) Closed-time histogram for channel in *A*. (*E*) Low-speed records of Type I channel activity illustrating presence of long closed and open states. Channel opening is upward. Free calcium concentration was 10^{-5} M. Records filtered at 300 Hz.

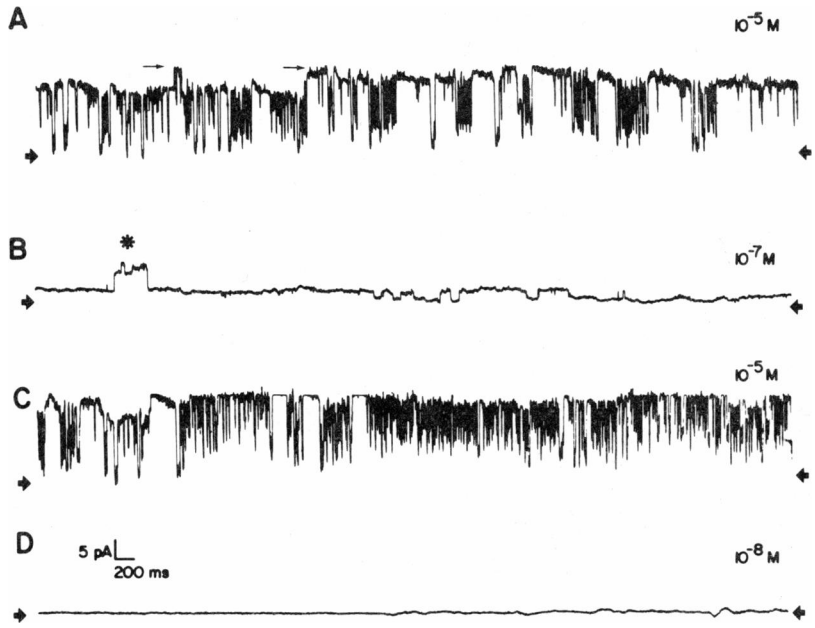

FIGURE 4. Calcium-sensitivity of the large (Type I) calcium-activated K^+ channel. (*A*) Single channel activity recorded at 10^{-5} M Ca^{2+}. Membrane was held at 80 mV throughout. Note that an intermediate-sized channel opened during the recording (long arrows at top of trace). (*B*) Channel activity ~2 min after reduction of free calcium to $<10^{-7}$ M by EGTA addition. The large (Type I) channel has closed, but both the larger (*asterisk*) and smaller Type II channels are still active. (*C*) Reappearance of Type I channel when calcium was again raised to 10^{-5} M. (*D*) Disappearance of all channel activity when calcium was reduced to $\sim 10^{-8}$ M. Record obtained ~2 min after EGTA addition. Zero current level indicated by small arrows at both left and right of each trace.

(millimolar) calcium concentrations (cf. Marty, 1981; Moczydlowski and Latorre, 1983).

Open-time histograms (Fig 3 *c*) for the large (Type I) channel during periods of "bursting" were not well-fit by single exponential decay functions, suggesting the presence of multiple open states. Within periods of "bursting" activity, closed-time histograms seem well-approximated by single-exponential decay (Fig. 3 *d*).

The Type I channels were calcium-dependent. Reduction of free Ca^{2+} in the bath (*cis* side) to $\sim 10^{-7}$ M (Fig. 4), by the addition of an EGTA buffer, resulted in closure of large channels, while other channels of intermediate (65–140 pS) unitary conductance (Type II) remained active. Further reduction of Ca^{2+} to $\leq 10^{-8}$ M resulted in closure of Type II channels as well. In general, these effects of Ca^{2+} were reversible. The activity of Type I and Type II channels was restored by raising free Ca^{2+} to 10^{-6} M. Raising free Ca^{2+} to 10^{-7} M restored the activity of Type II, but not Type I channels (not shown).

The large (Type I) channel was blocked by 1–3 mM concentrations of TEA, applied to the *cis* (interior surface) side of the membrane (Fig. 5). TEA block was voltage-dependent, being reduced at high ($\geq +100$ mV) depolarization. These concentrations are less than the K_d value of 45 mM reported for the t-tubule channel (Latorre et al., 1982; Vergara et al., 1984). Several other recent studies of the same channel have reported blocking constants for internal TEA in the range of 27–60 mM (Blatz and Magleby, 1984; Iwatsuki and Petersen, 1985). The 1–3 mM value observed in the present study compares favorably, however, with more recent work on the large K^+ channels from pituitary cells (Wong and Adler, 1986). Other differences in TEA sensitivity have been observed in mouse astrocytes in culture (Nowak et al., 1987). Here, internal TEA concentrations greatly reduced the apparent single-channel conductance (Fig. 5) in a concentration-dependent manner. A half-blocking concentration of 0.8 mM was estimated in several experiments at +50 mV. In

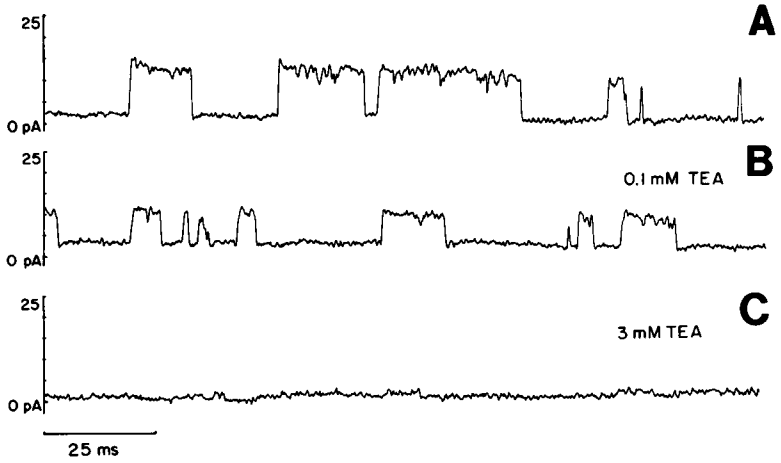

FIGURE 5. TEA block of Type I channel. (*A*) Channel activity recorded at 50 mV in presence of 10^{-5} M Ca^{2+} and no TEA. (*B*) 0.1 mM *cis* applied TEA reduced the conductance of the large Type I channel by ~20%. (*C*) 3 mM TEA blocked all Type I channel activity.

contrast to the sensitivity of this channel to TEA, it was relatively unaffected by 4-AP (up to 5 mM, *cis* applied, not shown).

Type II: Intermediate-sized Ca^{2+}-activated K^+ Channels

A group of channels with unitary conductance ranging from 65 to 140 pS was observed. One reason for considering these channels of diverse size as a group is that in all cases in which free calcium concentrations were varied over a sufficiently large range, these channels were found to have a similar calcium-dependency. At 10^{-7} M concentrations, these channels were still open a large fraction of the time while activity of the large (Type I) channel was very infrequently seen (Fig. 4). Reducing free calcium further (to $<10^{-7}$ M) resulted in large reductions in activity of the intermediate-sized channels. Thus, a lower

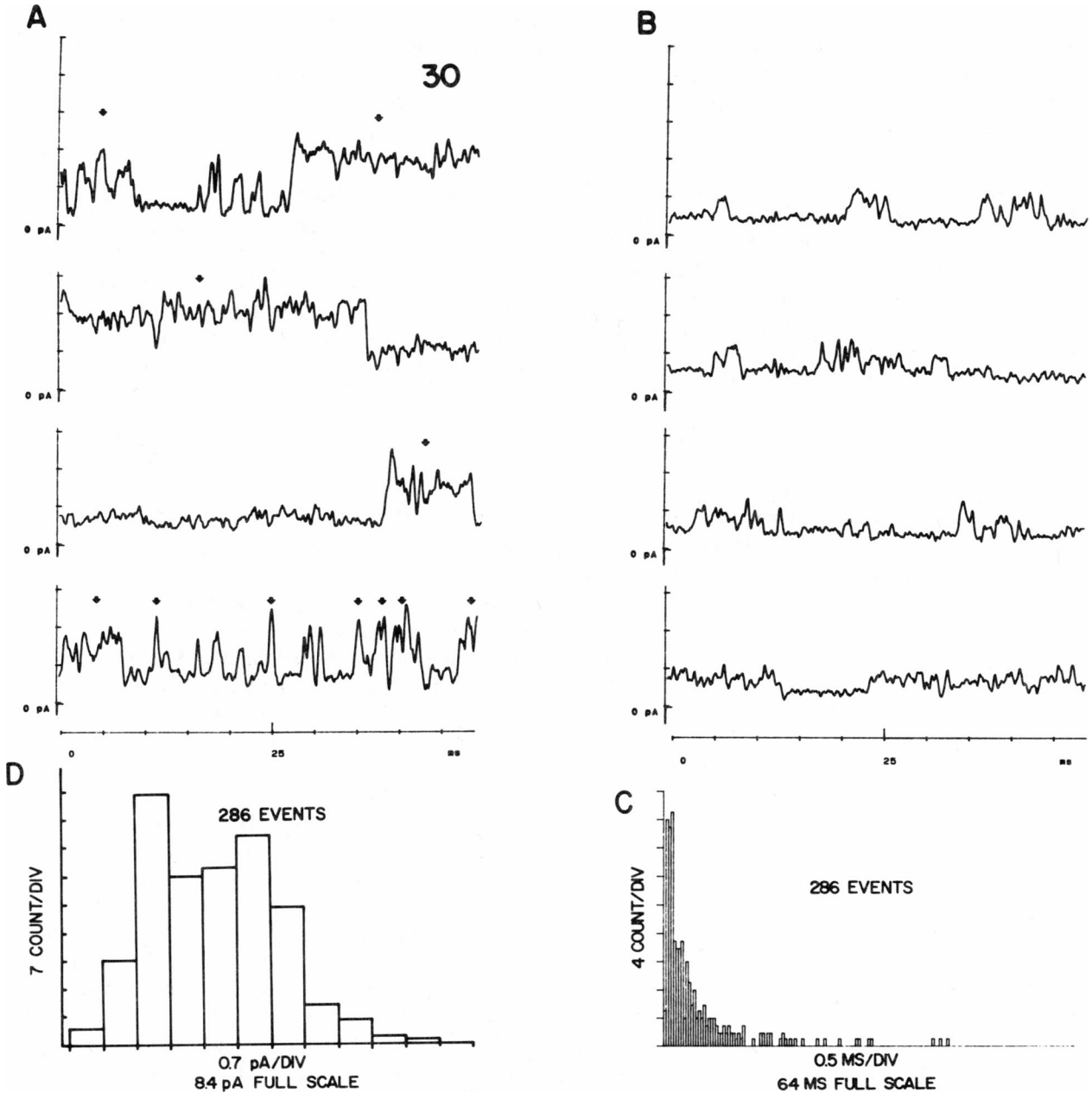

FIGURE 6 (*A*) Representative 50-ms samples of activity from a membrane in which both the smaller and larger Type II channels were present. Records obtained at 30 mV in 10^{-5} M Ca^{2+}. The larger channels are indicated by arrows. Smaller channels are also present. (*B*) Representative samples of activity of the smaller Type II channel in isolation. Each division on the vertical, 2.5 pA in *A* and *B*. (*C*) Composite open-time histogram for both Type II channels. Histogram obtained from channel openings during 256 consecutive 50-ms records, as in *A* and *B*. (*D*) Single-channel current amplitude histogram constructed from 286 instances of the channels depicted in *A* and *B*. The histogram has one mode at 2.1 pA and another at 4.2 pA. The corresponding single channel conductances are 70 and 140 pS, respectively.

free calcium concentration was sufficient to ensure activity of the intermediate vs. the large channels.

Often, several intermediate-sized channels of different apparent unitary size were observed in the same membrane, as in Fig. 6. For 90 channels, randomly chosen from various experiments, the distribution of unitary conductances was bimodal, with one mode at 75–80 pS and another at 120–125 pS (Fig. 7). The fact that channels with a unitary conductance lying between these two modes were infrequently encountered suggests that there may be a small number (perhaps two) of distinct channel subclasses.

Both subclasses of Type II channels were voltage-dependent. The analysis of their voltage-dependence was more difficult than that of Type I channels because they seldom appeared alone. Their gating behavior was also characteristic for each channel subclass and distinct from Type I channels. Often, at 10 μM Ca^{2+}, membrane potentials more positive than 30–40 mV were required to produce significant opening of the large Type II channel. Depolarization seems to have two effects on these channels: it increases the probability of opening and it produces a dramatic increase in open-time duration. At membrane potentials where the probability of opening is high, the 110–125-pS channels were characterized by open-time histograms suggesting at least two distinct open states, while the closed-time distribution could be fit by a single exponential decay function (Fig. 8).

As illustrated in Figs. 1 and 9, the 75–85-pS channels were characterized by extremely long open times at free calcium concentrations of 10^{-5} M or greater. The threshold for voltage activation at 10 μM Ca^{2+} was much more negative than for the 110–125-pS channel. For the 75–85-pS channel, the probability of opening is ~0.5 at 30 mV. At higher voltages the channel is open most of the time (Fig. 9) with individual openings often exceeding several hundred milliseconds. During these long open states, the channel may briefly shut. Thus open- and closed-time histograms for long stretches of records indicate the presence of at least two open and two closed states (Fig. 10).

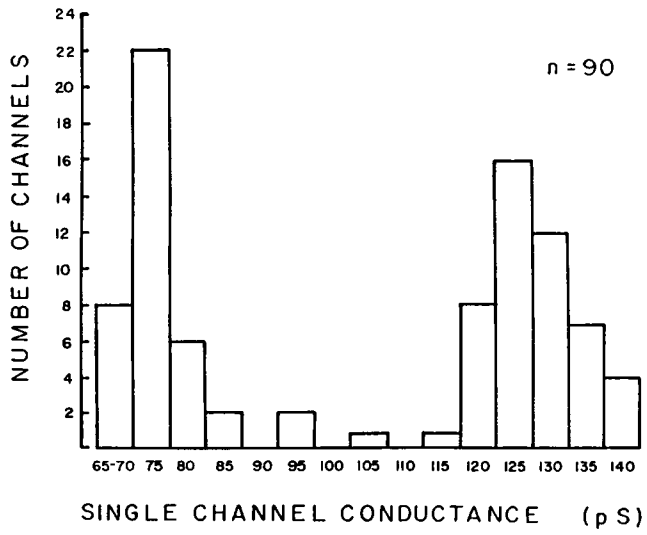

FIGURE 7 The intermediate-sized class of Ca^{2+}-dependent K^+ channels may consist of subclasses. Bimodal distribution of average single-channel conductances for intermediate-sized (Type II) calcium-dependent K^+ channels observed in 90 channels selected randomly from various experiments. The majority (85%) of channels measured were from experiments in which free calcium concentrations ranged between 10^{-6} and 10^{-5} M.

Unlike the large calcium-activated channels, the intermediate-sized channels were generally unaffected by a *cis* TEA concentration of 10 mM (not shown). The channel could be blocked by higher concentrations, however (50 mM; not shown). Like the Type I channel, the intermediate-sized channels were unaffected by a *cis* 4-AP concentration of 5 mM.

CTX Block of Ca^{2+}-activated K Channels

When membrane vesicles were added to the pipette (instead of the bath), channels were incorporated with the opposite polarity. The outer mouth of the channel was not exposed to the bath, while the "intracellular" side of the channel was exposed to the pipette solution. We observed both large (Type I) and intermediate-sized (Type II) channels under these conditions. CTX at 10 nM blocked both the large and intermediate-sized groups of channels (Fig. 11).

cAMP-dependent Protein Kinase Effects upon K^+ Channels

The various classes of voltage-dependent, calcium-activated K^+ channels may be targets for modulation by neurotransmitters and second messengers. Addition of 10^{-9}–10^{-8} M concentrations of cAMP PK CS to the bath, in the presence of 2–10 mM Mg-ATP (10^{-6} M Ca^{2+}), resulted in increases in the open time of both the large (Type I) and one of the intermediate (Type II) sized Ca^{2+}-dependent K^+ channels. Fig. 12 illustrates the effect of CS upon the large Ca^{2+}-dependent K^+ channel. Within seconds after CS addition, the frequency of channel bursting dramatically increased. Before CS addition, activity was stable with the channel open only infrequently. After several minutes (not shown), the channel remained virtually exclusively in the open state. We observed similar results in (*a*) six other membranes in which the large channel was the sole channel present and (*b*) 30 other membranes in which multiple channels were present, either as multiple instances of the large channel or as a mixture of Type I and II channels. In contrast to these effects of active CS, heat-inactivated CS (by boiling for 2 h) had no effect upon Type I channel activity in (*a*) three membranes in which it was the sole channel present or (*b*) 11 membranes in which multiple channel types were apparent.

The increases in open-time probability of the large (Type I channel) produced by cAMP PK CS were

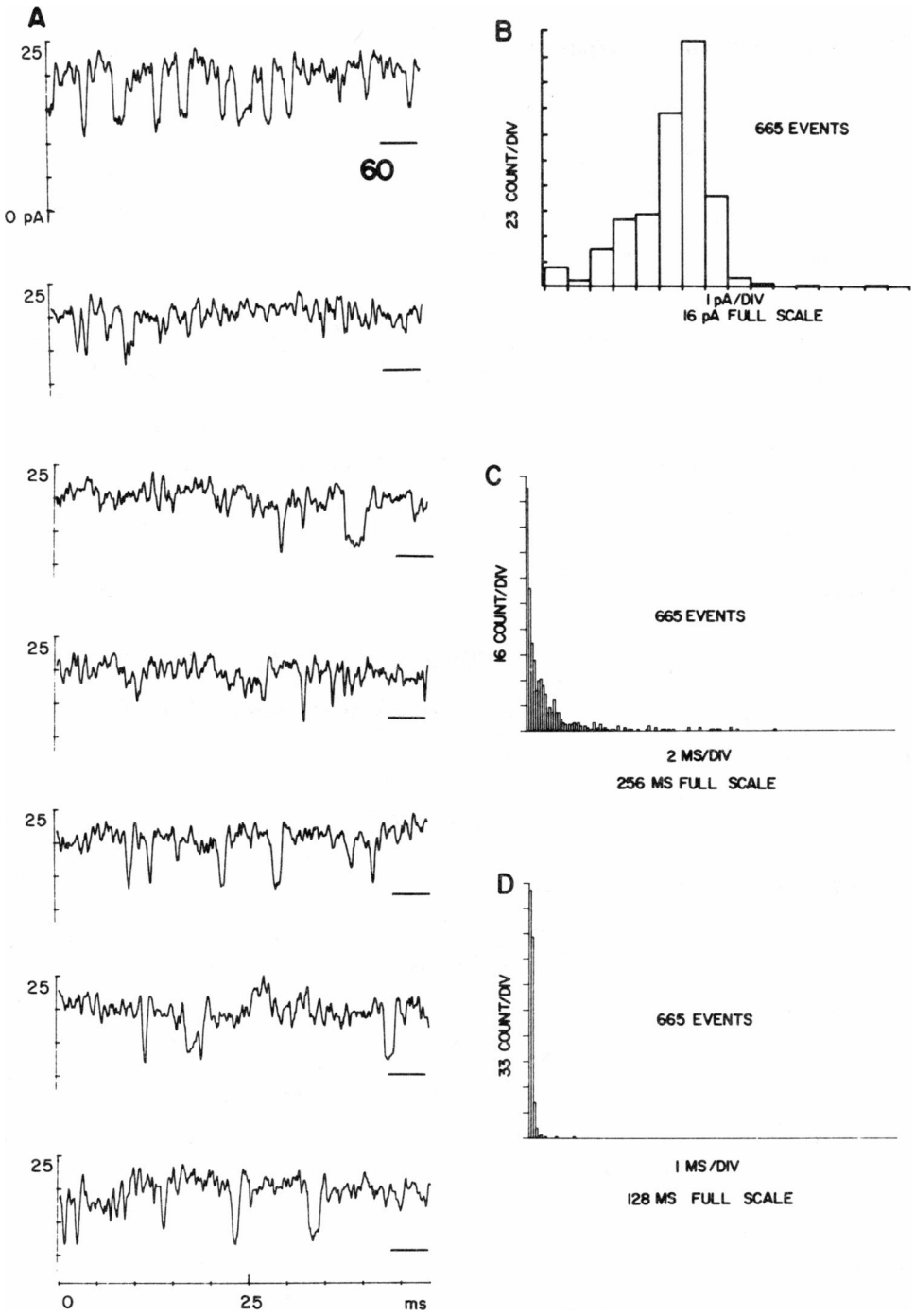

FIGURE 8 (*A*) Consecutive 50-ms samples of activity of a larger Type II channel, recorded at 60 mV in 10^{-5} M Ca. Note the rapid flickering of the channel. Current level corresponding to the closed state is indicated at right of each sample. (*B*) Single-channel current amplitude histogram constructed from 665 instances of the channel depicted in *A*. The histogram has a single mode with the majority of channel occurrences having amplitudes ranging between 6 and 8 pA, and thus a unitary conductance of 120 pS. (*C*) Open-time histogram constructed from 256 consecutive 50-ms samples of activity for the channel, as depicted in *A*. This histogram is not well fit by a single exponential decay function, and instead requires at least two exponentials. (*D*) Closed-time histogram for channel in *A* can be well fit by a single-exponential and suggests a single shut state.

observed to result from increases in the frequency of channel bursting, as well as in consistent shifts in the open-time distribution from shorter to longer modal open times during these bursts (Fig. 13).

We also observed that CS produced consistent, reliable increases in open-time probability of the smaller (70–85 pS) Type II channel. As depicted in Fig. 14, before CS addition, a single Ca^{2+}-dependent K^+ channel was open ~70% of the time. CS addition (10^{-9} M) resulted in the channel remaining in the open state virtually all of the time, with only occasional brief transitions to the closed state. Similar observations were made in four other membranes in which a single channel was present.

Since in the majority of our experiments multiple channels (Type I and II) were present, we quantitated our results for multiple-channel membranes by analysis of the effects of CS upon average current level, during successive 25-s time samples, without regard to the precise identities of the channels through which current was carried. In 32 of 37 experiments, we observed that cAMP PK CS increased the average outward current by 50% or more. These levels were reached within 0.5–1.5 min after enzyme addition. In

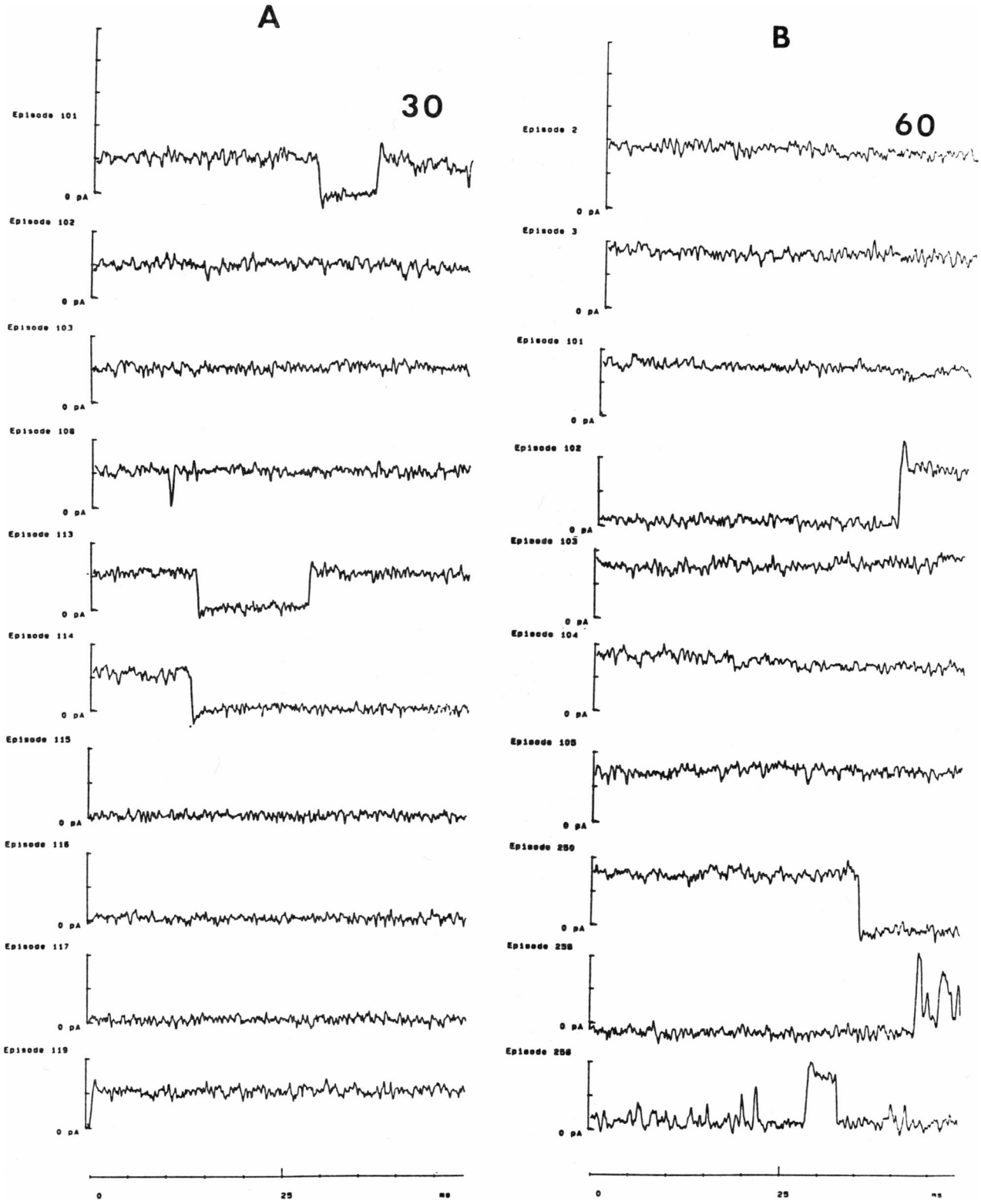

FIGURE 9 Illustrative 50-ms samples of small (~83 pS) Type II channel activity during 12.5-s recording periods at indicated membrane potentials. (*A*) At 30 mV, the channel is open a large fraction of the time and is characterized by very long open and closed states. Long open state is illustrated by channel opening at the end of episode 101, a very brief shutting in epsiode 108, followed by a more prolonged closures in episodes 113 and 114. The closure in episode 114 persisted for ~240 ms (episodes 114–119) before another long opening was observed. (*B*) At 60 mV, the channel was open virtually the entire time. It was open during episode 2 and remained open, without evidence of closure, until the end of episode 101 (~550 ms). The 40-ms closure in episode 102 preceeded another extremely long open state (episodes 103–250) lasting 7.4 s. Briefer openings are depicted in episodes 255 and 256. Free calcium concentration was 10^{-5} M. Record filtered at 1 kHz. Amplitude gain is 2.5 pA per division.

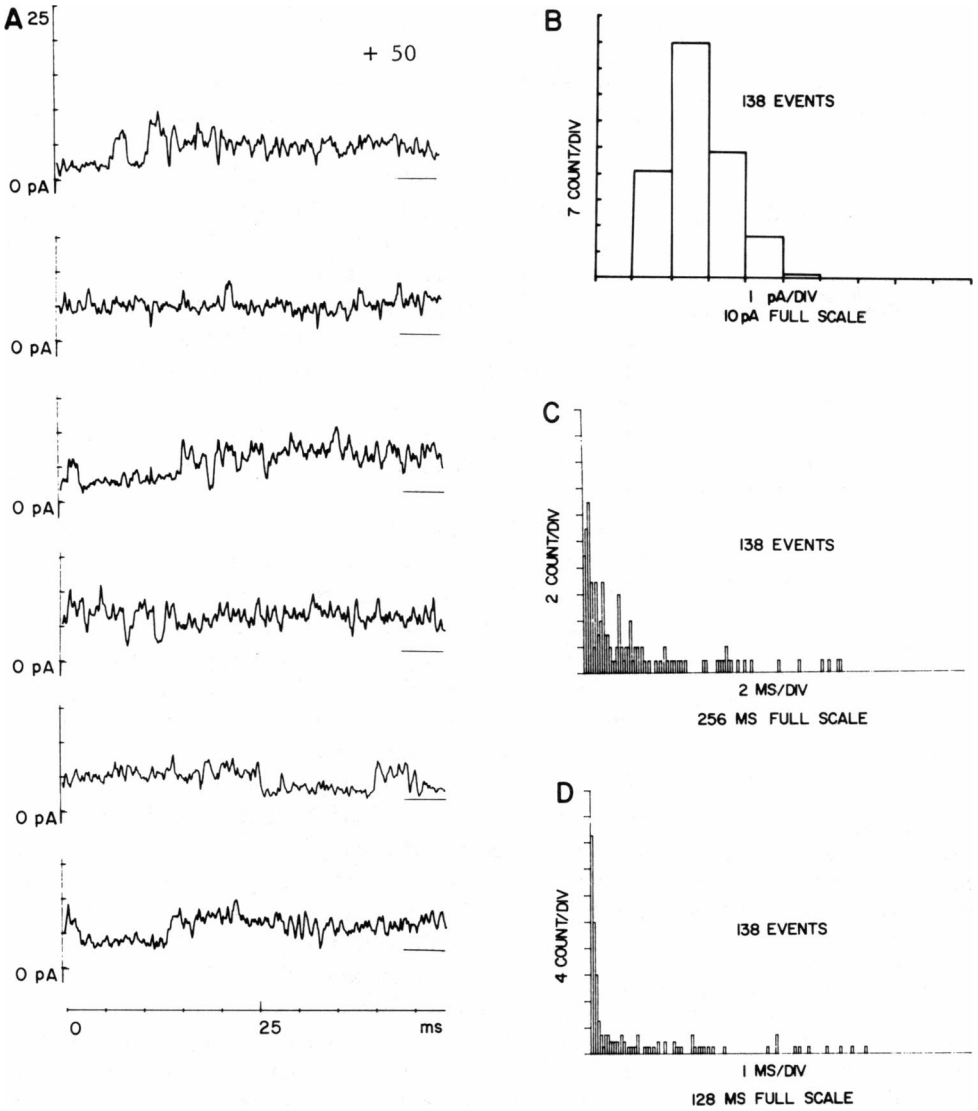

FIGURE 10 (*a*) Consecutive 50-ms samples of activity of a smaller Type II channel, recorded at 50 mV in 10^{-5} M Ca^{2+}. Note the presence of long open times. (*B*) Single-channel current amplitude histogram constructed from 138 instances of the channel depicted in *A*. The histogram has a single mode with the majority of channel occurrences having amplitudes ranging between 3.0 and 4.0 pA, and thus a unitary conductance of 60–80 pS. (*C*) Open-time histogram constructed from 256 consecutive 50-ms samples of activity for the channel as in *A*. Although the small number of discrete channel openings (138) obviates any detailed conclusions as to the exact shape of the distribution, it is obvious that open times are much longer than the larger Type II channel. (*D*) Closed-time histogram for channel in *A*. It cannot be well fit by a single exponential decay function.

the other five experiments, CS also clearly increased the average outward current, but here the increase was <50% being limited by the fact that the channels were open most of the time before the CS addition. Heat-inactivated kinase failed to produce consistent, reliable increases in activity in any of 14 membranes studied in which multiple channels were present.

Type III: Small, Voltage-independent K^+ Channels

In addition to the big and intermediate calcium-dependent K^+ channels, a smaller (50 ± 5 pS) channel was also often seen (Fig. 1). The open-time probability of this channel was virtually independent of membrane potential (±60 mV) and Ca^{2+} concentrations 10^{-3}–10^{-6} M that we could reliably manipulate. Like the intermediate-sized channels, the Type III channels were insensitive to *cis* applied TEA concentrations ≤50mM.

DISCUSSION

The small size of dendrites, presynaptic nerve terminals, and the relative inaccessibility of many areas of the mammalian brain precludes the use of conventional electrophysiological techniques as methods for directly exploring the ionic channels present in these regions. We have used the method of incorporation of channels into artificial membranes to bypass these problems, and have studied some of the physiological and pharmacological properties of several distinct K^+ channels from mammalian brain. With the vesicle preparation methods used here, spatial information as to the localization of channels was necesarily lost. Thus, it was not possible to determine which brain regions the observed channel types were preferentially localized in. Nor was it possible to say for certain from which regions of neurons (soma membranes, dendrites, pre- or postsynaptic membrane specializations, etc.) these channels were obtained. However, it is highly likely that the channels were from presynaptic nerve

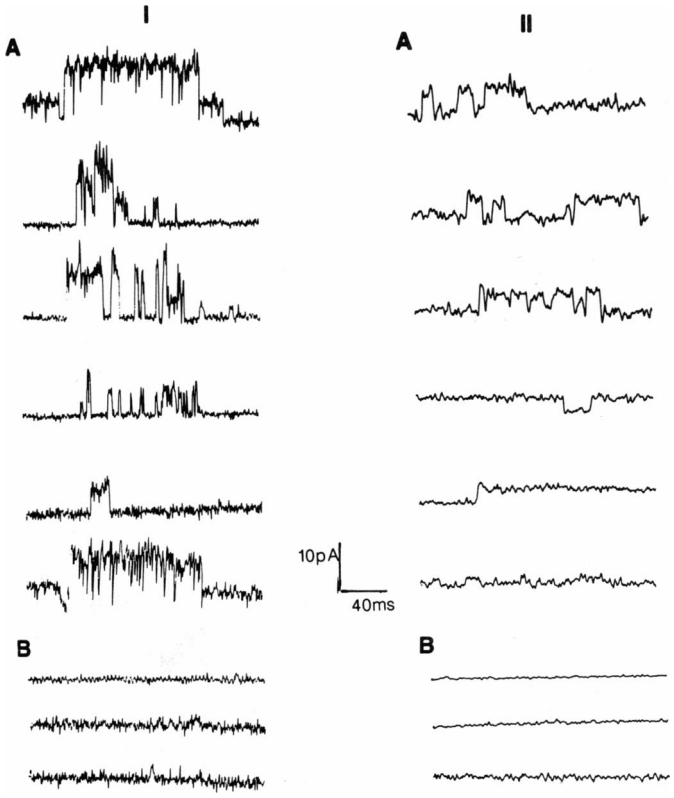

FIGURE 11 CTX block of Type I and II channels. Synaptosomal vesicles were present in the pipette. Ca^{2+} was 10 μM in the pipette and 1 mM in the bath. Pipette and bath solutions were 200 mM KCl, 10 mM Hepes-Tris pH 7.0. Selected current traces from a series of 200-ms voltage-steps to -50 mV (using the same convention as before: $V_{bath} - V$ pipette) for two different membranes (I and II). The records for the membrane shown in I were filtered at 1 kHz. The records for the membrane shown in II were filtered at 300 Hz. (*A*) Before the addition of CTX. (*B*) 1.5 min after the addition of 10 nM CTX to the bath. The membrane shown in I contained the large Type I channel (g = 200 pS) and at least one of the Type II channels (g = 118 pS). The membrane shown in II contained the two classes of Type II channels (g = 120 pS and 81 pS).

terminals since we observed the same types of channels regardless of whether crude synaptosomal preparations or sucrose gradient purified synaptosomes (Krueger et al., 1979) were used. More definitive answers to questions concerning channel localization might be provided by the use of more selectively purified membrane preparations and more circumscribed microdissected brain regions.

We have characterized several types of K^+ channels on the basis of their conductances and gating properties, their sensitivity to calcium and voltage, and to some degree their pharmacology. Channel Type I has a large unitary conductance, was both voltage- and calcium-dependent, and was blocked by 1–3 mM concentrations of TEA applied to the "intracellular" side of the channel and 10 nM CTX applied to the extracellular side of the channel. This channel appears to be similar, if not identical, to the "BK" channel previously described in other tissues (Marty, 1981; Pallotta et al., 1981; Latorre et al., 1982) with the exception of its sensitivity to internal TEA, which is similar to that of the BK channel in pituitary cells (Wong and Adler, 1986).

We also found a group of intermediate-sized channels that were both calcium-and voltage-dependent, and were also blocked by CTX. Size and open-state characteristics suggest that there may be distinct subclasses of these channels. We have characterized two apparent subclasses. These differ in single-channel conductance and gating behavior. The calcium sensitivity of both subclasses of intermediate-sized channels was greater than the Type I channel, but their voltage-dependence was smaller. Unlike our Type I channels, both smaller and larger intermediate-sized channels were relatively insensitive to intracellularly applied TEA. A 40–60-pS Ca^{2+}-activated K channel observed in molluscan neurons is also more sensitive to Ca^{2+} and less sensitive to voltage than the BK channel (Ewald et al., 1985; Hermann, 1985). The smaller subclass of Type II channels which we observed may correspond to the molluscan channel. To our knowledge, these intermediate-sized channels have not yet been described in cell-attached recordings from mammalian brain cells. This may reflect the fact that relatively few cell types have been studied to date. It is also possible that these channels are preferentially located in nonsomatic membrane areas. In contrast, the BK channel has been described both in neuronal somata (Gruol, 1984; Lancaster et al., 1987) and in glial cells (Nowak et al., 1987) from mammalian brain. These results indicate the existence of a family of voltage-dependent, CTX-sensitive, Ca^{2+}-activated K^+ channels.

FIGURE 12 Catalytic subunit of cAMP-dependent protein kinase opens Type I channel. (*A*) 20-s sample of activity at +50 mV before kinase addition. Channel was infrequently open. (*B*) 20-s sample of activity ~1 min after addition of 10^{-8} M kinase concentration. Channel openings are more frequent. Free calcium concentration was 10^{-6} M.

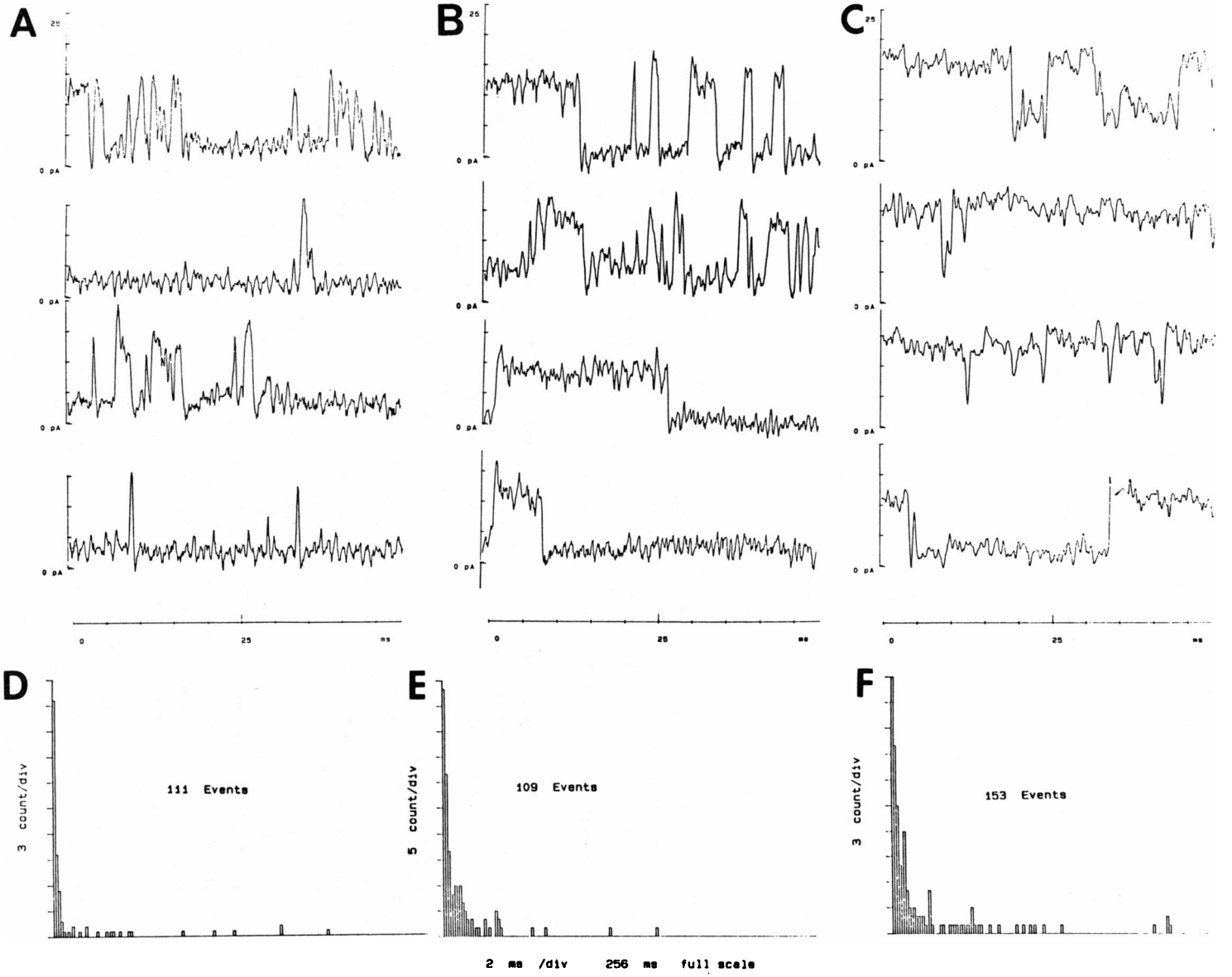

FIGURE 13 Catalytic subunit of cAMP-dependent protein kinase increases the average open-time duration as well as the frequency of channel opening. (*A–C*) Representative samples of Type I channel activity before (*A*), 1 min (*B*), and 5 min (*C*) after 10^{-8} M CS addition. cAMP PK increased the average open time. (*D–F*) Open-time histograms for channel activity before (*D*), 1 min (*E*), and 5 min (*F*) after 10^{-8} M CS addition. Each histogram derived from 12.5-s records. Note the increased frequency of opening 5 min after kinase addition, and the shift towards longer open times. Membrane potential was +40 mV throughout. Free calcium concentration was 10^{-6} M.

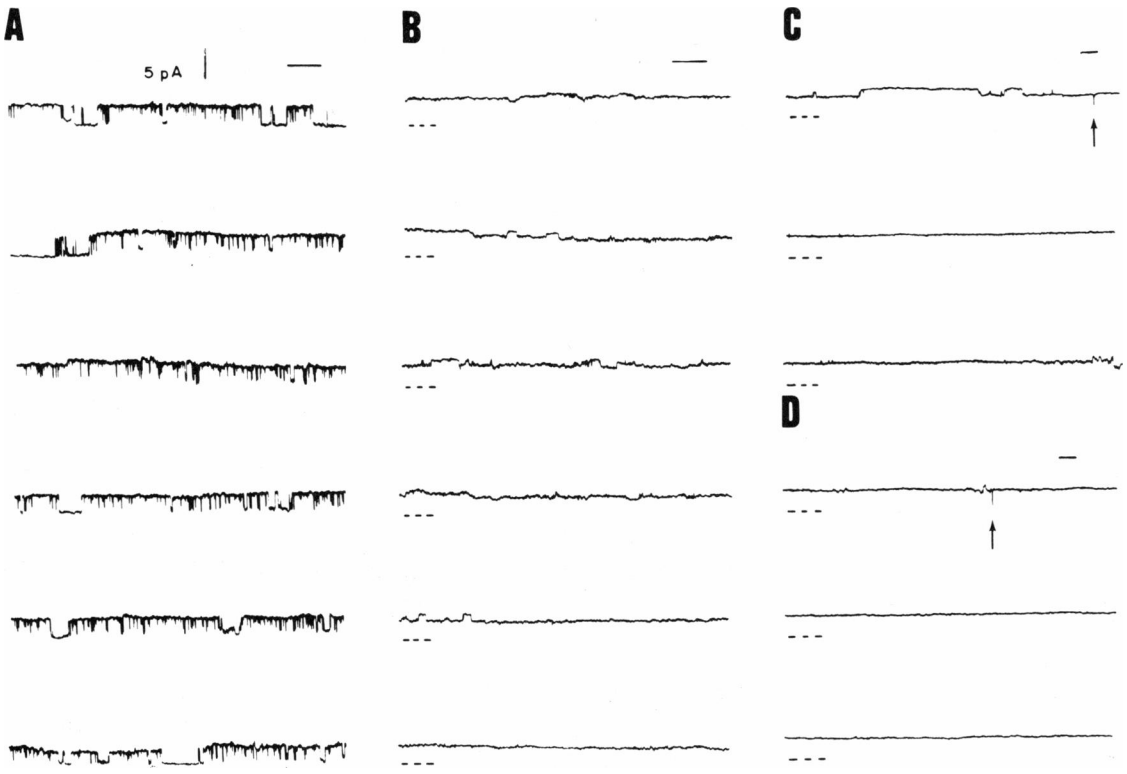

FIGURE 14 Catalytic subunit of cAMP PK opens a smaller Type II calcium-dependent K^+ channel. (*A*) (*top* to *bottom*). Consecutive samples of single channel activity during a 60-s baseline period immediately before addition of CS. A single channel is open ~70% of the time. (*B–D*) Consecutive 60-s samples of activity immediately (*B*), 2 min (*C*), and 10 min (*D*) after nanomolar bath-applied concentration of cAMP PK CS. Channel was opened by CS, with only occasional, brief transitions to the closed state (*dotted line*, left of each record) occurring at the indicated times (*arrows*). Free calcium concentration was 10^{-6} M throughout. Membrane potential was +50 mV. Time scales, 1 s; note change to slower scales in *C* and *D*.

Two types of effects of cAMP and cAMP-dependent protein kinase on Ca^{2+}-activated K^+ channels have previously been described. The first involves an increase in Ca^{2+}-activated K^+ channel activity. In *Helix* neurons, intracellular perfusion of the catalytic subunit of cAMP PK selectively enhances a voltage-dependent, Ca^{2+}-activated K^+ current (DePeyer et al., 1982), which single-channel studies reveal results from prolonged opening of a 40–60-pS channel (Ewald et al., 1985). These channels appear to belong to the family of Ca^{2+}-activated K^+ channels described here, since they are voltage-dependent and may be blocked by CTX (Hermann, 1985). As mentioned previously, they may correspond to our smaller Type II channel. Although we are unaware of any unequivocal report of increased activity of calcium-activated, voltage-dependent K^+ channels in mammalian neurons by cAMP, such effects would not be entirely without precedent. For example Krnjevic and Van Meter (1976) have reported that cAMP increases the fast after hyperpolarization (AHP) and also accelerates spike frequency accommodation in cat spinal motoneurons. Both of these phenomena may depend in part upon increases in a Ca^{2+}-dependent K^+ current (Krnjevic and Lisiewicz, 1972).

On the other hand, an inhibition by cAMP of a slow AHP has been extensively documented in hippocampal neurons. Intracellular application of cAMP (Madison and Nicoll, 1986) has been reported to reduce the AHP and spike frequency accommodation which are characteristic of these cells, thus mimicing the effect of catecholamines. Similar effects of cAMP upon the AHP of murine dorsal root ganglion cells have recently been reported (Grega et al., 1987). It should be appreciated, however, that the calcium-activated K^+ channels mediating the slow AHPs of hippocampal cells are believed to be distinct from the family of channels described here. Although they are calcium-activated, they seem to be voltage-independent. They may correspond to the apamin-inhibited channel (Pennefather et al., 1985; Lancaster et al., 1987; Lancaster and Adams, 1986) described in muscle (Blatz and Magleby, 1986; Romey and Lazdunski, 1984) or may instead represent yet another group of Ca^{2+}-activated K^+ channels.

Although the three classes of channels described here were the predominant types observed, other smaller channels were also seen and are currently the subject of further analysis. Slight modifications of present techniques may allow the study of other channels as well. It should be emphasized that we have concentrated only upon those channels that are active during steady-state depolarizations of the bilayer.

Several distinct varieties of K^+ channels from rat brain have also been previously described in a study that incorporated synaptosomal membrane vesicles into large (0.5–0.7 mM) planar lipid bilayers (Nelson et al., 1983). With the exception of a large 230-pS voltage-dependent channel seen in that study, which appears similar if not identical to the Type I channel described here, three of the four channels seen in the earlier study appear to be different from those we have characterized. Their unitary conductances ranged between 10 and 40 pS, one showed outward rectification, and in general appeared to have kinetic properties quite different from those of the channels we have studied. An exception is the Type IV (40 pS) channel described by Nelson et al. (1983), which may be similar to one of our Type II or III channels. Since calcium was not systematically varied by Nelson et al. (1983), we do not know how the calcium-dependence of their channels compares to ours.

Several different types of calcium-activated and voltage dependent K^+ channels in mammalian brain presynaptic nerve terminals have also been inferred from $^{86}Rb^+$ efflux tracer studies (Bartschat and Blaustein, 1985*a*, *b*). These channels are characterized by distinct pharmacological profiles as demonstrated by the differential susceptibility of kinetically different flux components to known K^+ channel blockers. At present, no direct comparison is possible between those channels studied by tracer flux methods and those that we have studied. Nonetheless, the pharmacological profiles provided by Bartschat and Blaustein (1985*a*, *b*) should prove valuable in subsequent studies of single K^+ channels using the present methods.

Reconstitution in lipid bilayers offers several distinct advantages for the study of brain K^+ channels. First, it permits a direct analysis of the conductance, permeation, and gating characteristics of conductances at the single-channel level. It is thus superior to tracer-flux methods in these respects. Second, channels usually become incorporated with the same orientation, from experiment-to-experiment, offering the possibility of having controlled and independent access to both faces of the channel. In the case of the method used here, this was achieved by the addition of vesicles to either the bath or pipette fluid. Third, relative to other methods of channel incorporation (such as the use of large black lipid membranes [BLM]), the patch pipette method allows, in principle, greater time resolution and lower noise, due to the decrease in capacitance. However, it does not have the resolution of the patch-technique applied to cells because bilayers made with pipettes treated with Sylgard to reduce pipette capacitance contain artifactual currents and are unstable. An improvement on the resolution we have obtained should be possible by using harder glass for the patch pipettes. Finally, we note that we have also observed the same types of channels shown here in BLMs. These latter bilayers may allow a characterization of these channels over a wider range of Ca^{2+} concentrations. However, since our goal was to explore the diversity of K^+ channels that may be found in brain membranes and BLMs are more restricted in the time resolution permitted, we preferred to carry out all our studies in the same bilayer type.

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