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Caffeic Acid Esters Activate TREK-1 Potassium Channels and Inhibit Depolarization-Dependent Secretion

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ABSTRACT

In whole-cell and single-channel patch-clamp recordings from bovine adrenal fasciculata cells, it was discovered that selected caffeic acid derivatives dramatically enhanced the activity of background TREK-1 K^+ channels. Cinnamyl 1–3,4-dihydroxy-α-cyanocinnamate (CDC), activated TREK-1 when this agent was applied externally to cells or outside-out patches at concentrations of 5 to 10 μM. Structure/activity studies showed that native bTREK-1 channels were also activated by other caffeic acid esters, including caffeic acid phenethyl ester (CAPE), which contain a benzene or furan ring in the ester side chain. The activation of bTREK-1 by caffeic acid derivatives did not occur through inhibition of lipoxygenases because other potent lipoxygenase inhibitors failed to activate bTREK-1. In bovine adrenal zona fasciculata (AZF) cells, bTREK-1 K^+ channels set the resting membrane potential. Inhibition of these channels by corticotropin leads to depolarization-dependent Ca^{2+} entry and cortisol secretion. CDC, which activates up to thousands of dormant bTREK-1 channels in AZF cells, was found to overwhelm the inhibition of bTREK-1 by corticotropin, reverse the membrane depolarization, and inhibit corticotropin-stimulated cortisol secretion. These results identify selected caffeic acid derivatives as novel K^+ channel openers that activate TREK-1 background K^+ channels. Because of their ability to stabilize the resting membrane potential and oppose electrical activity and depolarization-dependent Ca^{2+} entry, these compounds may have therapeutic potential as neuroprotective or cardioprotective agents.

K^+-selective ion channels comprise several large families of membrane proteins that as a group set the resting membrane potential and regulate action potential frequency and duration (Hille, 2001). Mammalian K^+ channels can be divided into three main structural classes that contain two, four, or six transmembrane segments (Lesage and Lazdunski, 2000; Goldstein et al., 2001; Hille, 2001; Chloe, 2002).

Leak-type K^+ channels that contain two pore domains and four transmembrane segments (2P/4TMS) comprise the most recently discovered superfamily of K^+ channels. 2P/4TMS channels are emerging as a widely distributed family of voltage-insensitive, noninactivating background channels that are expressed by neurons, muscle cells, and endocrine cells (Lesage and Lazdunski, 2000; Goldstein et al., 2001; Patel and Honore, 2001). At least 15 members of the 2P/4TMS family have been identified by molecular cloning and patch clamp techniques (Lesage and Lazdunski, 2000; Goldstein et al., 2001; Patel and Honore, 2001). Because the open probability of these channels is largely voltage independent, they set the resting potential at negative values near the predicted Nernst Equilibrium potential for K^+.

The properties of background K^+ channels allow them to act as a "brake" on electrical activity and thereby to function pivotally in regulating cell functions including secretion of neurotransmitters and hormones, as well as the contraction of muscle cells (Mlinar et al., 1993; Goldstein et al., 2001; Patel and Honore, 2001; Enyeart et al., 2002). Agents that modulate the activity of background K^+ channels would be of considerable value as research tools and, potentially, as therapeutic agents.

In this regard, the 2P/4TMS channels possess distinctive pharmacological properties that are currently being explored. Before these background channels were identified with molecular cloning techniques, long-chain *cis*-polyunsaturated fatty acids (PUFAs), such as arachidonic acid and linoleic acid, were found to activate unidentified leak K^+ channels in myocytes and neurons (Kim and Clapham, 1989; Kim et al., 1995).

These same PUFAs were later shown to activate a partic-

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ABBREVIATIONS: 2P/4TMS, two pore/four transmembrane segment; PUFA, polyunsaturated fatty acid; AZF, adrenal zona fasciculata; PBS, phosphate-buffered saline; CDC, cinnamyl 1–3,4-dihydroxy-α-cyanocinnamate; CAPE, caffeic acid phenethyl ester; EDHBCA, ethyl 3,4-dihydroxybenzylidenecyanoacetate; TEDHBCA, 2-(1-thienyl)ethyl 3,4,-dihydroxy-benzylidenecyanoacetate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tyrphostin B46, (*E*)-2-cyano-3-(3,4-dihydroxyphenyl)-*N*-(3-phenyl-propyl)-2-propenamide; DMEM, Dulbecco's modified Eagle's medium; PPP_i, inorganic polytriphospate; AA, arachidonic acid; CHO, Chinese hamster ovary.

ular subgroup of cloned 2P/4TMS K^+ channels, including TREK-1 (KCNK2), TREK-2 (KCNK10), and TRAAK (KCNK4) (Fink et al., 1998; Maingret et al., 2000; Patel and Honore, 2001). These three channels are also known collectively as the mechanogated subgroup because each is activated by membrane stretch (Patel et al., 1998; Maingret et al., 1999; Patel and Honore, 2001).

Soon after the effects of the PUFAs on background K^+ channels were discovered, they were shown to be neuroprotective. Specifically, PUFAs suppressed seizure activity and cell death in an in vivo model of epilepsy and prevented hippocampal cell death in a model of transient global ischemia (Lauritzen et al., 2000).

Before they were identified as neuroprotective agents, PUFAs were shown to be cardioprotective and were particularly effective in preventing fatal ventricular arrhythmias and sudden cardiac death (Billman et al., 1997; Kris-Etherton et al., 2003). Accordingly, PUFA-activated K^+ channels, such as TREK-1, are also expressed in cardiac myocytes (Aimond et al., 2000).

These results suggest that activators of background K^+ channels may have great potential in therapeutics as neuro- and cardioprotective agents. However, in contrast to the relative abundance of K^+ channel antagonists, agents that effectively open K^+ channels have been difficult to identify.

In this regard, bovine adrenal zona fasciculata (AZF) cells express TREK-1 channels that set the resting potential and couple corticotropin receptor activation to depolarization-dependent Ca^{2+} entry and cortisol secretion (Enyeart et al., 1993; Mlinar et al., 1993; Enyeart et al., 1996; Enyeart et al., 2002). AZF cells provide an ideal system for exploring the modulation of native TREK-1 channels by pharmacologic agents. We have identified a group of caffeic acid derivatives that dramatically increase the activity of bTREK-1 K^+ channels in whole-cell and single-channel patch-clamp recordings.

Materials and Methods

Materials. Tissue culture media, antibiotics, fibronectin, and fetal calf sera were obtained from Invitrogen (Carlsbad, CA). Coverslips were from Bellco (Vineland, NJ). Phosphate-buffered saline (PBS), enzymes, BAPTA, ATP, adenyl-5′-yl imidodiphosphate, and corticotropin (1–24) were from Sigma (St. Louis, MO). Cinnamyl 1–3.4-dihydroxy-α-cyanocinnamate (CDC), 3,4-dihydroxycinnamic acid (caffeic acid), caffeic acid phenyl ester (CAPE), and 5,6,7-trihydroxyflavone (baicalein) were obtained from BIOMOL (Plymouth Meeting, PA). Ethyl 3,4-dihydroxybenzylidenecyanoacetate (EDHBCA), 2-(1,-thienyl)ethyl 3,4,-dihydroxy-benzylidenecyanoacetate (TEDHBCA), and (*E*)-2-cyano-3-(3,4-dihydroxyphenyl)-*N*-(3-phenylpropyl)-2-propenamide (Tyrphostin B46) were obtained from Tocris (Ellisville, MO).

Methods. Isolation and Culture of AZF cells. Bovine adrenal glands were obtained from steers (aged 2 to 3 years) within 1 h of slaughter at a local slaughterhouse. Fatty tissue was removed immediately, and the glands were transported to the laboratory in ice-cold PBS containing 0.2% dextrose. Isolated AZF cells were obtained and prepared as described previously (Enyeart et al., 1997). After isolation, cells were either resuspended in DMEM/Ham's F12 (1:1) with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and the antioxidants 1 μM tocopherol, 20 nM selenite, and 100 μM ascorbic acid (DMEM/Ham's F12+) and plated for immediate use or resuspended in fetal bovine serum/5% dimethyl sulfoxide, divided into 1-ml aliquots, each containing $\sim 4 \times 10^6$ cells, and stored in liquid nitrogen for future use. Approximately 1.20×10^8 cells were obtained by enzymatic dissociation of six bovine adrenal glands. Cells were plated in either 60-mm dishes or 35-mm dishes containing 9-mm^2 glass coverslips. Dishes or coverslips were treated with fibronectin (10 μg/ml) at 37°C for 30 min then rinsed with warm, sterile PBS immediately before adding cells. Cells were plated in DMEM/Ham's F12+ and were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Transient Transfection and Visual Identification of CHO-K1 Cells Expressing bTREK-1. For whole-cell patch-clamp recording of cloned bTREK-1 K^+ currents, CHO-K1 cells were cotransfected with a mixture of pCR3.1 Uni-bTREK-1 and an expression plasmid (p3-CD8) for the α-subunit of the human CD8 lymphocyte surface antigen at a 5:1 ratio using LipofectAMINE (Invitrogen). Cells were visualized 1 to 2 days after transfection after a 15-min incubation with anti-CD8 antibody-coated beads (Dynal Biotech Inc., Lake Success, NY) as described previously (Jurman et al., 1994).

CHO-K1 cells were used for patch clamping 24 to 72 h after transfection with bTREK-1. Transfected cells were plated on 9-mm glass coverslips as described above. Fifteen minutes before initiating a patch-clamp experiment, anti-CD8 antibody–coated beads were added to the culture dish. Upon transferring coverslips to the recording chamber, transfected cells were identified based on decoration with the beads. Whole-cell bTREK-1 currents were recorded from transfected cells as described below for AZF cells.

Patch Clamp Experiments. Patch clamp recordings of K^+ channel currents were made in the whole-cell and outside-out patch configuration. For whole-cell recordings, the standard pipette solution consisted of 120 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM BAPTA, 10 mM HEPES, 1 mM ATP, and 200 μM GTP, with pH titrated to 7.1 using KOH. Pipette solution of this composition yielded a free Ca^{2+} concentration of 2.2×10^{-8} M, as determined by the Bound and Determined software program (Brooks and Storey, 1992). In some experiments, MgATP was raised to 5 mM or inorganic polytriphosphate (PPP_i) was added to the pipette as noted in the text. The external solution consisted of 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, with pH adjusted to 7.4 using NaOH.

The standard external and pipette solutions used for single-channel recording from outside-out patches were identical to those used for whole-cell recordings. All solutions were filtered through 0.22-μm cellulose acetate filters.

Recording Conditions and Electronics. AZF cells were used for patch-clamp experiments 2 to 12 h after they were plated. Typically, cells with diameters <15 μm and capacitances of 10 to 15 pF were selected. Coverslips were transferred from 35-mm culture dishes to the recording chamber (volume, 1.5 ml), which was continuously gravity-perfused at a rate of 3 to 5 ml/min. For whole-cell recordings, patch electrodes with resistances of 1.0 to 2.0 MΩ were fabricated from Corning 0010 glass (World Precision Instruments, Sarasota, FL). These electrodes routinely yielded access resistances of 1.5 to 4.0 MΩ and voltage-clamp time constants of <100 μs. For single-channel recordings, patch electrodes with higher resistances of 3 to 5 MΩ were used. K^+ currents were recorded at room temperature (22–25°C) according to the procedure of Hamill et al. (1981) using an Axopatch 1-D (Axon Instruments, Union City, CA) or EPC-7 (List Electronics, Darmstadt, Germany) patch-clamp amplifier.

Pulse generation and data acquisition were done using a personal computer and pCLAMP software with TL-1 interface (Axon Instruments). Currents were digitized at 2 to 10 KHz after filtering with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using summed, scaled hyperpolarizing steps of 1/2 to 1/4 pulse amplitude. Data were analyzed using pCLAMP 6.04 (CLAMPAN and CLAMPFIT) and SigmaPlot (ver 8.0; SPSS Inc., Chicago, IL) software. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

Secretion Experiments. AZF cells were cultured on fibronectin-coated 35-mm plates at a density of 4×10^5 cells/dish in DMEM/

Ham's F12 (1:1) containing 10% fetal calf sera, 100 U/ml penicillin, 0.1 mg streptomycin, and the antioxidants tocopherol (1 μM), selenite (20 nM), and ascorbic acid (100 μM). After 24 h, the media was aspirated and changed to defined media consisting of DMEM/Ham's F12 (1:1), 50 μg/ml BSA, 100 μM ascorbic acid, 1 μM tocopherol, 10 nM insulin, and 10 μg/ml transferrin. Drugs were added directly to media from concentrated stock. Samples of media (200 μl) were collected at selected times and frozen at −20°C for later assay. Cortisol concentration was determined using a solid phase radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). Experiments were performed in triplicate and assayed for cortisol in duplicate.

Measurement of bTREK-1 K^+ Currents. The absence of time- and voltage-dependent inactivation of the bTREK-1 K^+ current allowed it to be easily isolated for measurement in whole-cell recordings from AZF cells using either of two voltage-clamp protocols. When voltage steps of 300-ms duration were applied from a holding potential of −80 mV to a test potential of +20 mV, bTREK-1 could be selectively measured near the end of the voltage step, where the rapidly inactivating bKv1.4 K^+ current had completely inactivated. Otherwise, bTREK-1 was selectively activated with an identical voltage step, after a 10-s prepulse to −20 mV had fully inactivated bKv1.4 K^+ current (see Fig. 1A).

Results

CDC Opens Native bTREK-1 K^+ Channels. In whole-cell patch-clamp recordings from AZF cells, it was discovered that CDC induced a pronounced, concentration-dependent, and reversible increase in the noninactivating component of K^+ current. In the experiment illustrated in Fig. 1A, K^+ currents were recorded with pipette solutions that limit the activity of bTREK-1 K^+ channels (pH 7.1, 1 mM MgATP). Voltage clamp protocols that allow recording of both Kv1.4 and bTREK-1 (Fig. 1A, left traces) or bTREK-1 alone (Fig. 1A, right traces) were applied as described under *Materials and Methods*.

During 10 min of recording in control saline, the noninactivating K^+ current did not increase in amplitude (traces 1 and 2). Upon superfusing the cell with 10 μM CDC, the noninactivating K^+ current increased more than 20-fold within 8 min (traces 3 and 4). This increase was completely reversed upon washing (traces 5 and 6). The K^+ current activated by CDC was indistinguishable from bTREK-1 and included a large instantaneous and smaller time-dependent component as described previously (Fig. 1A, right traces) (Mlinar et al., 1993; Enyeart et al., 1996).

CDC increased the noninactivating K^+ current over a narrow range of concentrations (Fig. 1B). Whereas 5 μM CDC triggered no significant increase in K^+ current, 10 μM CDC increased the current density by an average of more than 6-fold. The 8-fold increase in K^+ current produced by 20 μM CDC provides a minimum estimate of its effectiveness at this concentration, because gigaohm seals were typically lost before a stable maximum was achieved.

In addition to markedly increasing the noninactivating component of K^+ current, CDC partially inhibited the Kv1.4 K^+ current at similar concentrations. Specifically, when pipettes contained 200 μM cAMP to inhibit expression of bTREK-1 (Enyeart et al., 2002), CDC inhibited Kv1.4 current by 49.5 ± 9.0% (n = 3) (data not shown).

In whole-cell recordings from AZF cells, bTREK-1 channels display very weak voltage dependence (Enyeart et al., 1997). Increases in current amplitude at successively more positive test potentials occur largely through increases in driving force, rather than channel open probability. With pipette solutions containing 1 mM ATP at pH 7.1, current-voltage relationships showed that bTREK-1 could not be significantly increased, even at very positive test voltages (Fig. 2). In contrast, superfusing the cell with CDC (20 μM) increased the noninactivating K^+ current amplitude by 6.5- to 8-fold at potentials between −10 and +40 mV, a range over which accurate measurement was possible.

Identification of the CDC-activated Current as bTREK-1. Although the current activated by CDC was indistinguishable from the native bTREK-1 K^+ current with respect to kinetic properties, additional proof was required to establish its identity. When recorded in physiological saline, native bTREK-1 appears as an outwardly rectifying K^+-selective current. If the current activated by CDC is TREK-1, it should display similar voltage-dependent rectification.

The activity of native bTREK-1 channels in AZF cells is enhanced by acidification of the pipette solution and by PPP_i (Xu and Enyeart, 2001; Enyeart et al., 2002). The rectifying properties of bTREK-1, activated by acidifying the pH of the pipette solution to 6.4 and adding 5 mM PPP_i, were compared with those of the CDC-activated current using ramp voltage protocols. In Fig. 3A, currents are shown that were recorded in response to 2-s linear voltage ramps, applied between +60 and −140 mV before (control) and after superfusing the cell with CDC (10 μM). Both currents showed similar strong outward rectification, crossing the voltage axis near the Nernst equilibrium potential for K^+.

Scaling of the CDC-activated current to the control current [Fig. 3A, 10 μM CDC (scaled)] resulted in nearly superimposed current traces. These results indicate that CDC activated a K^+-selective current with rectifying properties that are indistinguishable from native TREK-1.

If the K^+ current activated by CDC is bTREK-1, then this current should be inhibited by corticotropin (Mlinar et al., 1993). In the experiment illustrated in Fig. 3B, superfusion of the cell with 20 μM CDC triggered a large increase in the K^+ current from its initial value of nearly zero (trace 1) to 2830 pA after an 11-min exposure to the drug (trace 2). At this time, the K^+ current had not reached a stable maximum value. Superfusion with saline containing corticotropin (200 pM), in addition to CDC, transiently reduced the current to a minimum of less than 800 pA after 3.5 min (trace 3), at which time the K^+ current resumed a slow monotonic increase over the next 6 min (trace 4).

These results indicate that the CDC-activated K^+ current is inhibited by corticotropin but eventually this inhibition can be overwhelmed by the agonist properties of CDC at this concentration. In three other experiments in which bTREK-1 was activated by 10 μM CDC and an apparent maximum increase was obtained, corticotropin (200–500 pM) inhibited the CDC-induced current by 86.7 ± 11.2%.

When the open probability of native bTREK-1 channels is increased in whole-cell recordings by acidified pipette solution, pH 6.4, containing 5 mM MgATP, subsequent application of CDC still produces a pronounced increase in bTREK-1 amplitude. However, the -fold increase relative to the control current amplitude is markedly reduced. In the experiment illustrated in Fig. 4A, K^+ currents were recorded from an AZF cell with pipette solution, pH 6.4, containing 5 mM MgATP. Under these conditions, bTREK-1 increased to a stable maximum value near

1000 pA within 10 min (traces 1 and 2). Superfusion of the cell with 10 μM CDC tripled the amplitude of bTREK-1 within 8 min (traces 3 and 4). Overall, when bTREK-1 was preactivated by acidified pipette solution and 5 mM MgATP, CDC increased this current to 287 $\pm$ 31% (n = 3) of its maximum pretreatment value. By comparison, using standard pipette solution (pH 7.1, 1 mM MgATP), CDC (10 M) increased TREK-1 to 604 $\pm$ 106% (n = 11) of its control value.

CDC also enhanced the activity of cloned bTREK-1 channels, expressed in CHO-K1 cells (see *Materials and Methods*). Under these conditions of high basal activity, CDC produced significant but relatively smaller increases in bTREK-1 amplitude. In the experiment illustrated in Fig. 4B, bTREK-1 grew to a maximum of 1100 pA (trace 2) before superfusing CDC (10 μM) which increased bTREK-1 to 1920 pA (trace 3). At a concentration of 5 μM, CDC also induced small, but significant, increases in bTREK-1 current in transfected cells (Fig. 4B). Gigaohm seals for transfected CHO-K1 cells could not be maintained at CDC concentrations above 10 μM.

CDC Acts Externally to Activate bTREK-1: Whole-Cell and Single-Channel Recording. In whole-cell patch-clamp experiments, small molecules of molecular weight $\approx$1000 can be applied intracellularly through the patch electrode. For small spherical cells such as AZF cells, these molecules reach a maximum intracellular concentration in the cytoplasm within seconds (Pusch and Neher, 1988). In previous studies, we have shown that bTREK-1 channels are effectively activated by nucleotides and inorganic polytriphosphates, applied through the patch pipette (Enyeart et al., 1997; Xu and Enyeart, 2001).

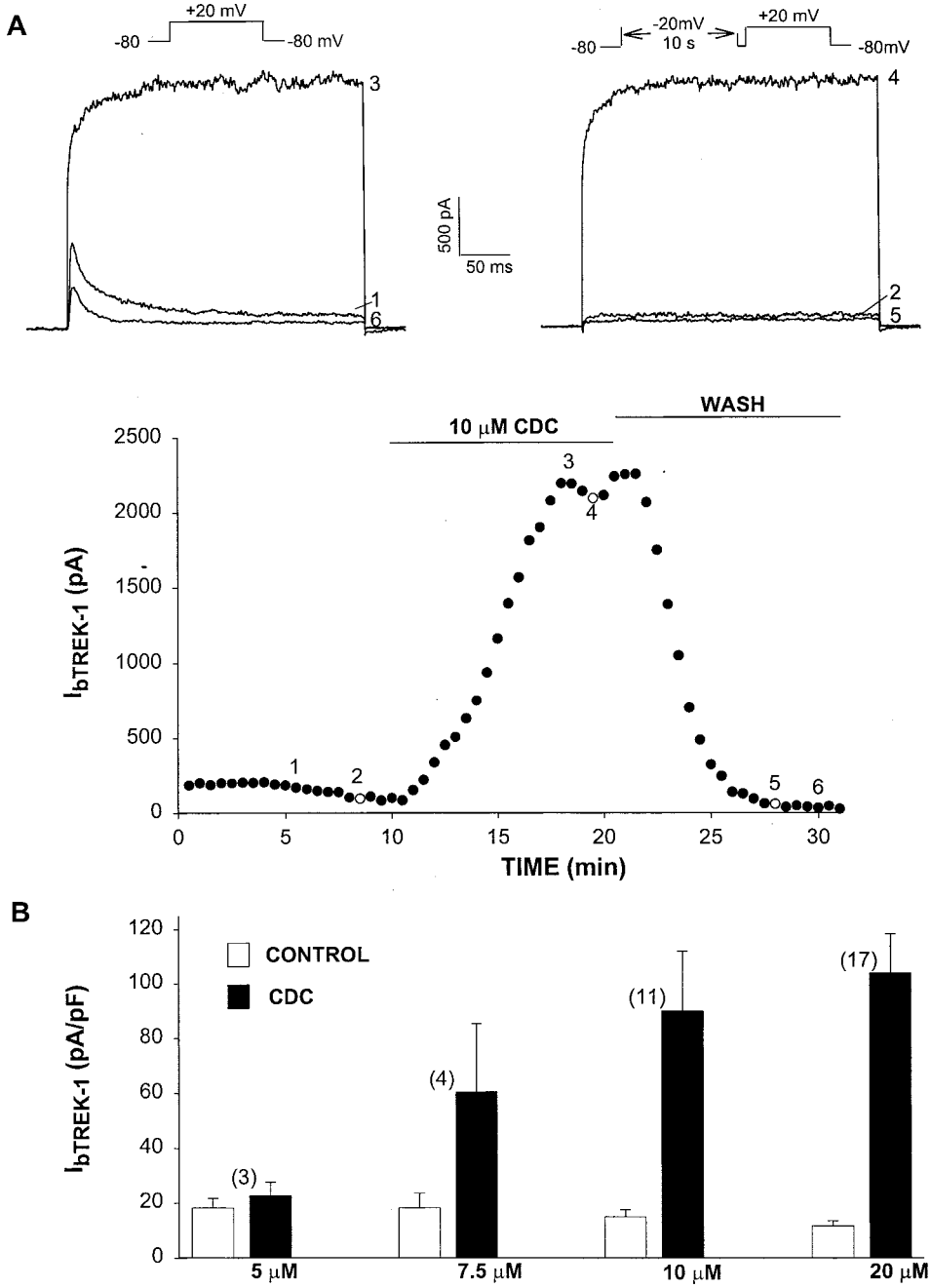

Fig. 1. Activation of native bTREK-1 K^+ channels by CDC. Whole-cell K^+ currents were recorded from AZF cells with or without depolarizing prepulses as indicated and described under *Materials and Methods*. After recording currents in control saline, cells were superfused with CDC at concentrations ranging from 5 to 20 μM. A, effect of 10 μM CDC. K^+ currents were recorded in standard saline, with (○) or without (●) depolarizing prepulses, before superfusing the cell with 10 μM CDC for 10 min. Numbers on current traces correspond to those on plot of bTREK-1 amplitudes. B, summary data from experiments as in A. bTREK-1 current densities (in picoamperes per picofarad) were determined from current amplitudes measured immediately before and after the maximum increase in bTREK-1 was obtained in the presence of CDC. Values are mean $\pm$ S.E.M. of indicated number of determinations at each concentration.

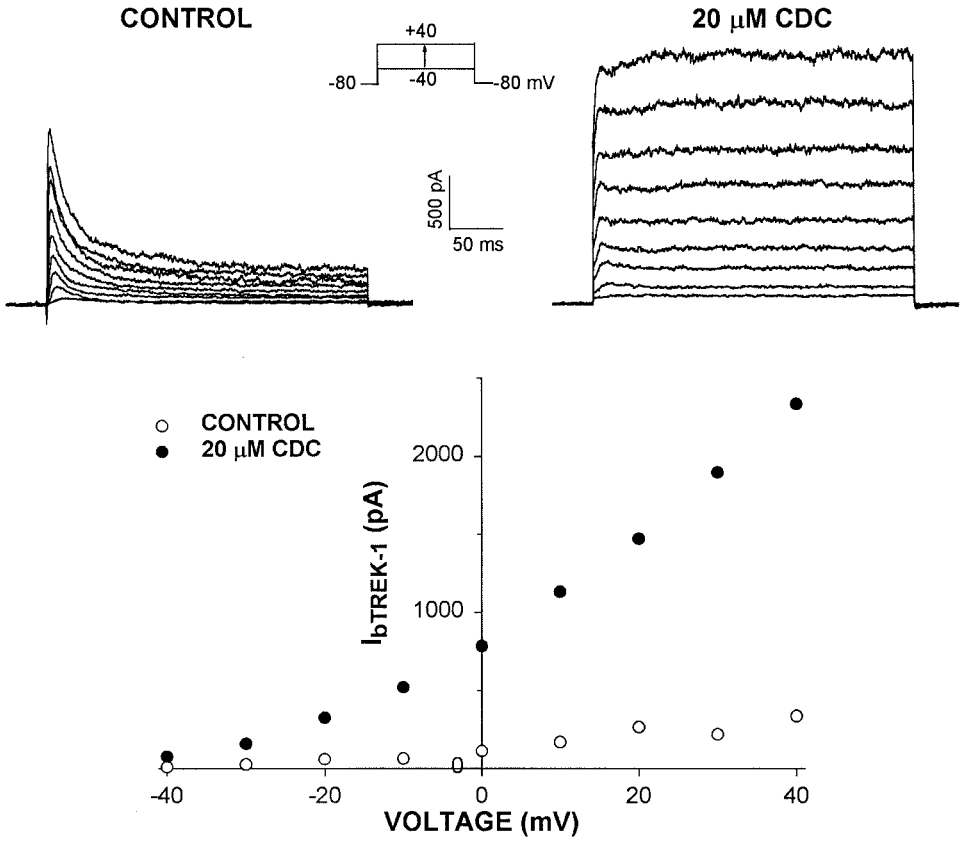

Fig. 2. Effect of CDC on bTREK-1 current-voltage relationship. Whole-cell K^+ currents were recorded from AZF cells at 30-s intervals in response to test potentials between −40 and +40 mV, applied from a holding potential of −80 mV before and after superfusing the cell with 20 μM CDC. Amplitudes of the noninactivating K^+ current (bTREK-1) shown at top are plotted against test voltage.

In whole-cell recordings, it was discovered that bTREK-1 channels were activated only by externally applied CDC. In the experiment illustrated in Fig. 5A, the AZF cell was voltage-clamped in the whole-cell configuration with a pipette containing standard solution (1 mM MgATP, pH 7.1), supplemented with CDC (10 μM). Under these conditions,

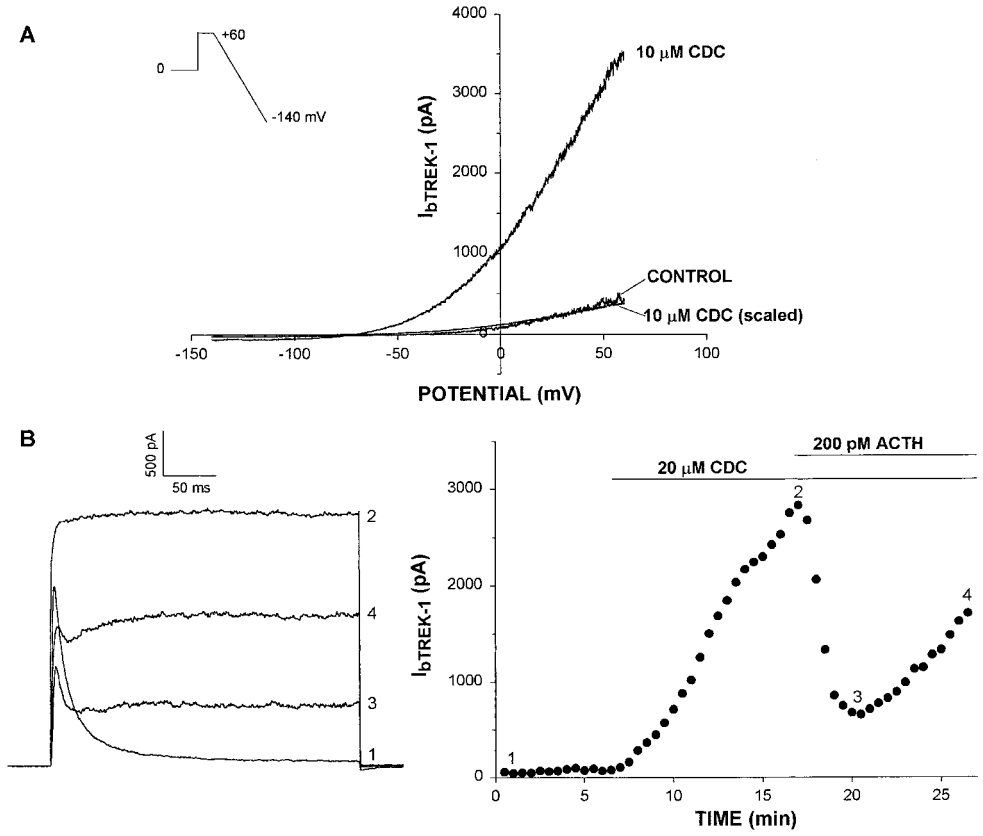

Fig. 3. Voltage-dependent rectification and inhibition of the CDC-activated current by corticotropin. A, rectification of CDC-activated current. Linear voltage ramps of 100 mV/s were applied from a holding potential of 0 mV to potentials between +60 and −140 mV in whole-cell recordings from AZF cells after bTREK-1 reached a maximum in standard saline (control) and after a maximum increase was reached in response to CDC (10 μM). The scaled trace was produced by dividing the CDC trace by a scaling factor determined from the maximum values of the CDC and control traces measured at + 60 mV. B, inhibition of CDC-activated current by corticotropin (ACTH). Whole-cell K^+ currents were activated from −80 mV in response to voltage steps to +20 mV applied at 30-s intervals. After recording currents in standard saline, the cell was sequentially superfused with saline containing CDC (20 μM) and CDC plus corticotropin (200 pM). Numbered traces at left correspond to those on plot of bTREK-1 amplitude at right.

bTREK-1 reached a maximum value of 125 pA. Superfusion of the cell with saline containing 10 μM CDC increased bTREK-1 more than 6-fold. Overall, with 10 μM CDC in the pipette, bTREK-1 reached a maximum density of 9.2 ± 3.4 pA/pF (n = 3). Superfusion of the same cells with 10 μM CDC increased bTREK-1 density to 73.7 ± 7.2 pA/pF.

Externally applied CDC also activated bTREK-1 K^+ channels in excised outside-out patches from AZF cells. In the experiment illustrated in Fig. 5B, unitary bTREK-1 activity was recorded at +30 mV with pipettes containing standard solution before superfusing the patch with 10 μM CDC. With standard external saline, unitary currents with a mean amplitude of 3.80 ± 0.7 pA were present, but openings were infrequent, suggesting the presence of a single active channel in the patch (Fig. 5B, left traces). A 3-min exposure to externally applied CDC (10 μM) induced a pronounced increase in the activity of several channels of the same unitary current amplitude (Fig. 5B, right traces). In contrast to its effectiveness in outside-out patches, CDC failed to activate bTREK-1 channels when applied to the cytoplasmic surface of excised inside-out patches (n = 4, data not shown).

Activation of bTREK-1 by Caffeic Acid Derivatives: Structure/Activity Relationships. The discovery that bTREK-1 K^+ channels are dramatically activated by CDC was unexpected. It suggested that other caffeic acid derivatives might also behave as TREK-1 K^+ channel openers. To learn more about the structural requirements for bTREK-1 openers, CDC was compared with caffeic acid and several derivatives with respect to their potency and effectiveness as activators of bTREK-1 channels (Fig. 6A).

Caffeic acid, the parent compound of CDC, at a concentration of 40 μM failed to produce a significant increase in native bTREK-1 current (Fig. 6C). A second compound EDHBCA shares structural and functional properties with CDC. Both are esters of caffeic acid containing a cyano group, and each potently inhibits 12-lipoxygenase (Cho et al., 1991).

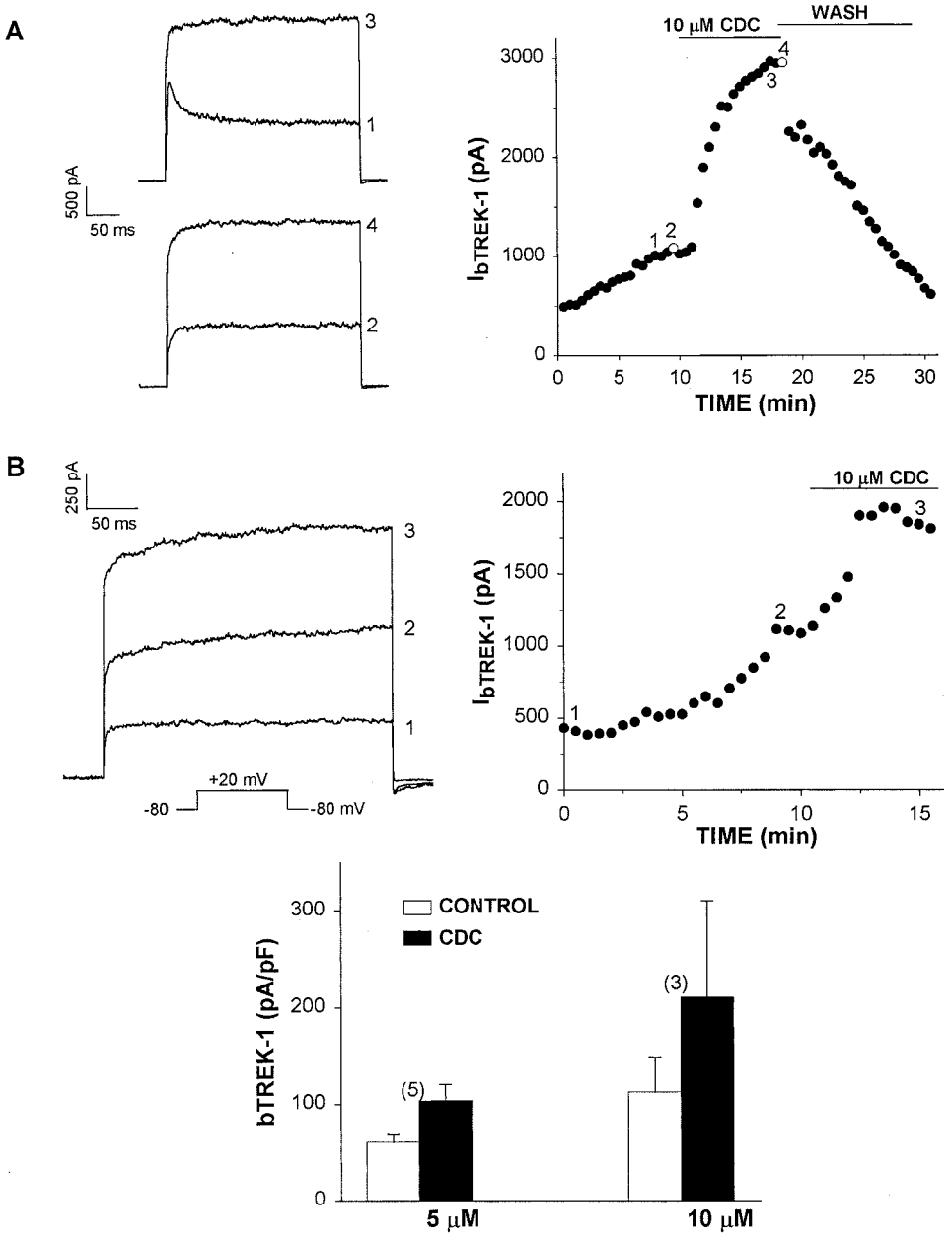

Fig. 4. Effect of CDC on preactivated native and cloned bTREK-1 K^+ channels. A, native bTREK-1. K^+ currents were recorded from AZF using the voltage protocols described in the legend to Fig. 1. Pipette solutions contained 5 mM MgATP at pH 6.4. After bTREK-1 reached a maximum amplitude in saline, cell was superfused with CDC (10 μM) as indicated. Numbers on traces at left correspond to those on plot of bTREK-1 amplitude at right. B, CDC on transfected bTREK-1 channels expressed in CHO-K1 cells. Whole-cell K^+ currents were recorded from CHO-K1 cells that had been transiently cotransfected with pCR3.1-uni-bTREK-1 and P3-CD8 cDNAs (see *Materials and Methods*), using voltage steps to +20 mV from a holding potential of −80 mV. After bTREK-1 reached a stable amplitude, cells were superfused with CDC. Numbers on traces correspond to those on plot of current amplitudes at right. Bar graphs show summary data from similar experiments. Values are expressed as mean ± S.E.M. of bTREK-1 current densities measured in picoamperes per picofarad before and after superfusing cells with CDC.

However, unlike CDC, EDHBCA failed to activate bTREK-1 at concentrations of 20 or 40 μM (Fig. 6C).

TEDHBCA is a cyano group-containing caffeic acid ester that includes a furan ring in the ester side chain. This agent effectively increased bTREK-1 current but was less potent than CDC (Fig. 6, B and C). At a concentration of 20 μM, TEDHBCA produced no significant increase in bTREK-1 current density. However, 40 μM TEDHBCA increased bTREK-1 from 17.8 $\pm$ 3.1 pA/pF to 115.2 $\pm$ 42.7 pA/pF (n = 3) (Fig. 6C).

Activation of native bTREK-1 by TEDHBCA suggested that a ring structure in the R group of the caffeic acid ester is essential for activity as a bTREK-1 opener. Caffeic acid phenethyl ester (CAPE) is a naturally occurring plant-derived caffeic acid derivative with a benzene ring in the side chain (Natarajan et al., 1996). However, unlike CDC and TEDHBCA, CAPE lacks a cyano group and is therefore a far less potent lipoxygenase inhibitor (Fig. 7A). Although less potent than CDC, CAPE effectively activated bTREK-1 (Fig. 7, B and C). At a concentration of 40 μM, CAPE increased bTREK-1 current density from a control value of 9.7 $\pm$ 2.3 pA/pF to 63.6 $\pm$ 7.8 pA/pF (Fig. 7C). CAPE also inhibited the rapidly inactivating Kv1.4 current, but this effect was not studied systematically.

Tyrphostin B46 is structurally similar to CDC and TEDHBCA but includes an amide rather than an ester bond (Fig. 7A). Tyrphostin B46 (40 μM) failed to produce a significant increase in bTREK-1, demonstrating the importance of the ester bond in bTREK-1 activation (Fig. 7C).

Although a benzene or furan ring structure in the ester side chain was necessary for TREK-1 activation, benzene itself did not activate bTREK-1 channels at concentrations of 10 and 20 μM in any of four cells (data not shown). At higher concentrations, benzene was toxic to the cells.

Baicalein is a potent lipoxygenase inhibitor but is not a caffeic acid derivative (Fig. 7A). Baicalein failed to increase bTREK-1 amplitude at a concentration of 20 μM, which is greater than 1000 times the IC_{50} for inhibition of 12-lipoxygenase (Fig. 7C) (Cho et al., 1991).

Comparative Effects of $CHCl_3$ and CDC on bTREK-1 K^+ Currents. In addition to PUFAs, volatile anesthetics activate K^+-selective channels of the 2P/4TMS family. In a study on cloned 2P/4TMs channels, $CHCl_3$ was distinctive in activating only TREK-type K^+ channels (Patel et al., 1999). We compared $CHCl_3$ to CDC with respect to its potency and effectiveness as an activator of native bTREK-1 channels in AZF cells.

Although $CHCl_3$ did significantly increase bTREK-1 current in AZF cells, compared with CDC, it was far less potent and effective (Fig. 8). Furthermore, the $CHCl_3$-induced increase in bTREK-1 was transient and was followed within several minutes by a decrease toward control value, even in the continued presence of $CHCl_3$ (Fig. 8A). Overall, $CHCl_3$ (5 mM) increased bTREK-1 current density from a control value of 5.90 $\pm$ 1.67 pA/pF to 18.30 $\pm$ 6.67 pA/pF (n = 5). By comparison, CDC (10 μM) at a 500-fold lower concentration, increased bTREK-1 from a control value of 14.9 $\pm$ 2.7 pA/pF to 90.0 $\pm$ 21.8 pA/pF (n = 11) (Fig. 8B).

Effect of CDC on AZF Cell Membrane Potential and Cortisol Secretion. In previous studies, we demonstrated that TREK-1 inhibition by corticotropin was coupled to depolarization-dependent Ca^{2+} entry through T-type Ca^{2+} channels and cortisol secretion (Enyeart et al., 1993). Within the framework of this model, CDC would be expected to oppose AZF cell depolarization and cortisol secretion by activation of bTREK-1 K^+ channels.

At concentrations that activate bTREK-1 and oppose corticotropin-mediated inhibition of these K^+ channels, CDC reversed corticotropin-stimulated depolarization of AZF cells, and suppressed corticotropin-stimulated cortisol secre-

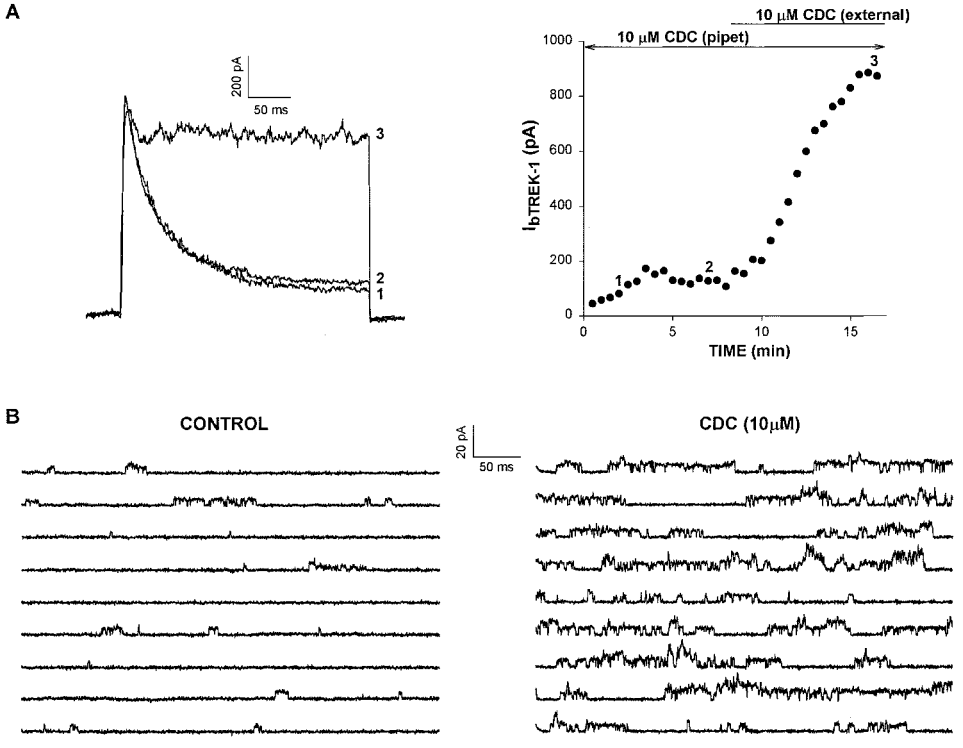

Fig. 5. CDC activates whole-cell and single-channel bTREK-1 currents through an external site. A, whole cell. K^+ currents were recorded from AZF cells in response to voltage steps to +20 mV applied at 30-s intervals from a holding potential of −80 mV. The patch pipette contained standard solution (1 mM MgATP and 200 μM GTP at pH 7.1) supplemented with 10 μM CDC. The cell was superfused with CDC (10 μM) as indicated. Numbers on traces at left correspond to those on plot of bTREK-1 amplitudes at right. B, outside-out patch recordings. Unitary bTREK-1 currents were recorded from excised outside-out AZF cell patches with a patch pipette containing standard solution (1 mM MgATP and 200 μM GTP, pH 7.1). Voltage steps of 300-ms duration to +30 mV were applied at 10 s intervals from a holding potential of −40 mV. After recording unitary channel activity in standard external saline (control), the cell was superfused with CDC (10 μM). Traces show unitary TREK-1 activity after 4-min exposure to CDC.

tion. In the experiment illustrated in Fig. 9A, whole-cell currents were recorded from AZF cells with acidified pipette solution, pH 6.4, containing 5 mM MgATP. After $I_{bTREK-1}$ reached a maximum value, the membrane potential was recorded upon switching to current clamp. Superfusion of 200 pM corticotropin rapidly depolarized the cell by approximately 45 mV, from its resting value of approximately −63 mV to −18 mV. Superfusing the cell with saline containing 200 pM corticotropin and 20 μm CDC repolarized the membrane potential to its original value within 10 min.

At concentrations identical to those that open native bTREK-1 channels, CDC was found to inhibit corticotropin-stimulated cortisol secretion. In the experiment illustrated in Fig. 9B, cultured AZF cells were exposed to corticotropin (50 pM) in the absence or presence of CDC at several concentrations. At a concentration of 4 μM, CDC failed to significantly inhibit the quantity of cortisol secreted during a 90-min incubation period. In contrast, 20 and 40 μM CDC inhibited cortisol secretion in this experiment by 91.3 ± 0.8% and 99.1 ± 0.9%, respectively. The CDC-induced inhibition of cortisol secretion was not mediated through inhibition of lipoxygenases, because baicalein (20 μM) failed to alter corticotropin-stimulated cortisol secretion.

CDC was equally effective at inhibiting cortisol secretion stimulated by several concentrations of corticotropin from 2.5 to 250 pM. In the experiment illustrated in Fig. 9C, CDC (20 μM) inhibited cortisol secretion stimulated by 2.5, 25, and 250 pM corticotropin by 82.8 ± 0.7, 82.0 ± 6.9, and 78.1 ± 0.2%, respectively.

Discussion

In this study, CDC and selected caffeic acid esters were identified as novel bTREK-1 K^+ channel openers. When ap-

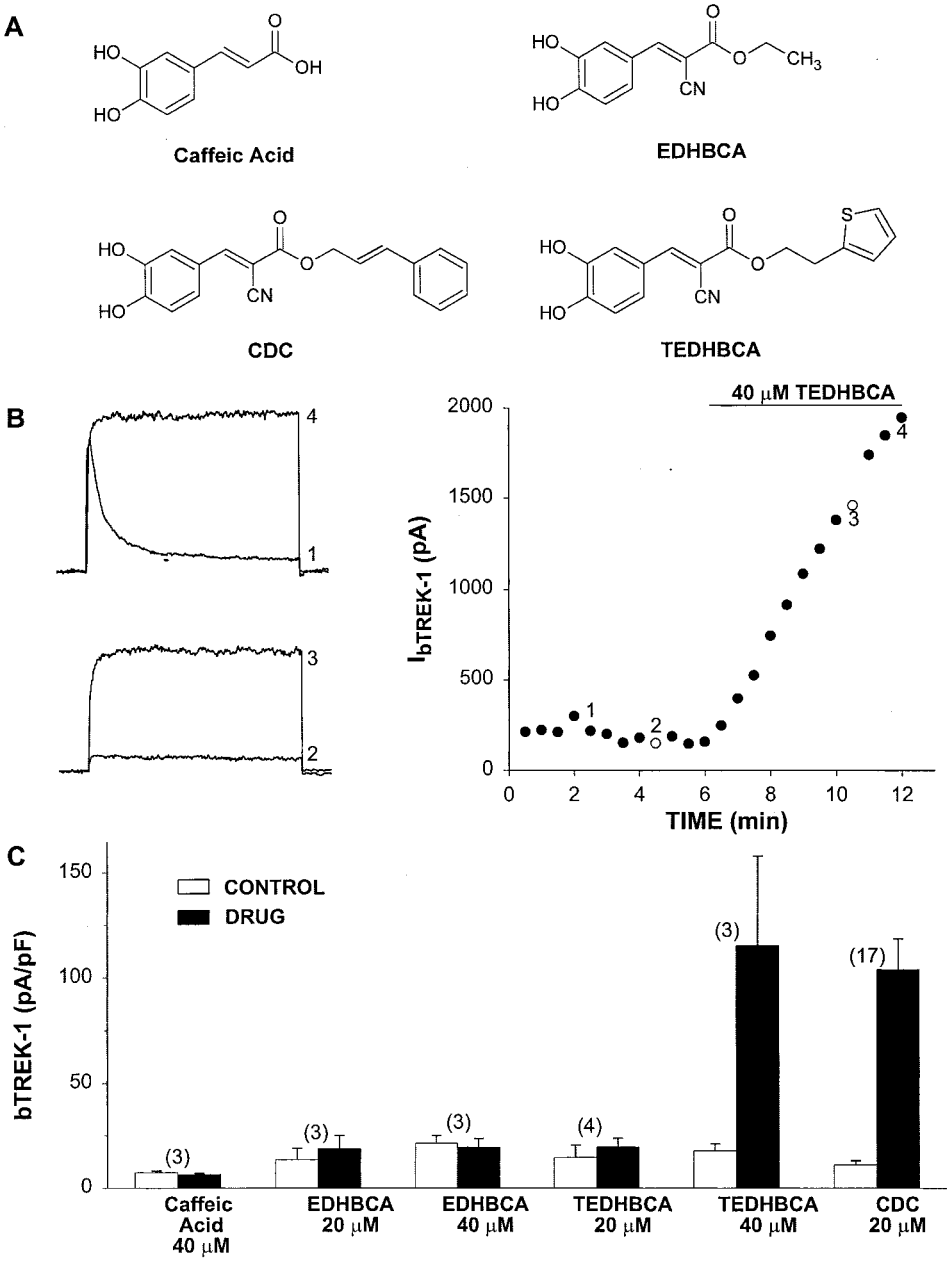

Fig. 6. Activation of bTREK-1 by caffeic acid derivatives. Caffeic acid and two caffeic acid esters were compared with CDC with respect to activation of bTREK-1 K^+ channels in whole-cell patch-clamp recordings from AZF cells. A, chemical structures of CDC, caffeic acid, EDHBCA, and TEDHBCA. B, activation of bTREK-1 by TEDHBCA. Whole-cell K^+ currents were recorded in response to voltage steps applied from −80 mV to +20 mV with (bottom traces) or without (top traces) depolarizing prepulses. After recording currents in standard saline, cell was superfused with 40 μM TEDHBCA. Numbers on traces at left correspond to those on plot of current amplitudes at right. C, summary of data from experiments as in B. bTREK-1 current densities (in picoamperes per picofarad) were determined from current amplitudes measured immediately before and after superfusing the cell with one of the drugs. Values are expressed as mean ± S.E.M. of indicated number of determinations.

plied externally to bovine AZF cells, these agents effectively activated up to thousands of dormant TREK-1 channels. Activation of bTREK-1 channels by caffeic acid derivatives demonstrated a structural requirement for an ester group containing a ring. CDC effectively opposed corticotropin-mediated inhibition of bTREK-1 channels, reversed the membrane depolarization, and suppressed corticotropin-stimulated cortisol secretion.

Potency and Effectiveness of Caffeic Acid Esters. The activation of native bTREK-1 channels by CDC was characterized by a steep concentration-response relationship. Although 5 μM produced no significant increase in bTREK-1, 10 μM CDC increased bTREK-1 by 6-fold. Although the mechanism that underlies this steep relationship is not clear, a similar pattern has been observed for activation of native and cloned TREK-1 channels by arachidonic acid, lysophospholipids, and volatile anesthetics (Patel et al., 1999; Maingret et al., 2000; Danthi et al., 2003).

For all of these agents, determination of a maximally effective concentration was complicated by the loss of gigaohm seals that typically occur at high concentrations (i.e., 20–40 μM). The loss of gigaohm seals observed with CDC was not caused by toxicity because cultured AZF cells could be incubated for hours with this agent at the same concentrations with no effect on cell viability (data not shown).

Although CDC increased the amplitude of bTREK-1 in transiently-transfected CHO-K1 cells, the increase relative to the control current was typically far less than that observed in AZF cells under similar conditions. TREK-1 channels in transfected CHO-K1 cells are quite active even in the absence of stimulation with ATP or acidified pipette solution (Enyeart et al., 2002). Therefore, the maximum possible CDC-induced increase in the transfected cells seems to be limited by the high open probability (P_o) of the channels previous to CDC exposure.

By comparison, in AZF cells, the P_o of bTREK-1 channels

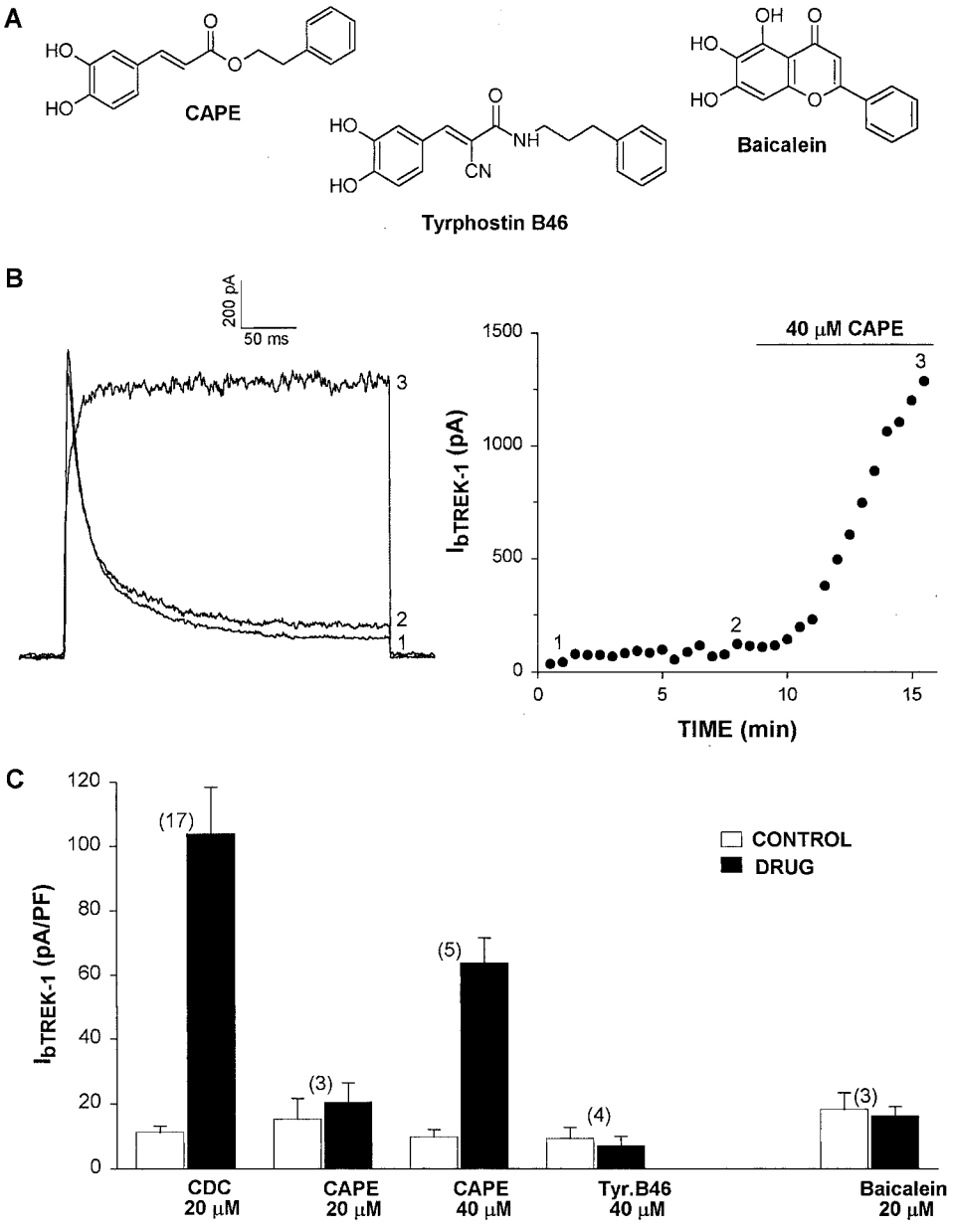

Fig. 7. Activation of bTREK-1 by CAPE. The effects of CAPE, Tyrphostin B46, and baicalein on bTREK-1 were determined in whole-cell patch-clamp recordings from AZF cells. A, chemical structures of CAPE, Tyrphostin B46, and baicalein. B, activation of bTREK-1 by CAPE. Whole-cell K^+ currents were recorded in response to voltage steps applied from −80 mV to a test potential of +20 mV. After recording currents in standard saline, the cell was superfused with 40 μM CAPE. Numbers on traces on left correspond to those on plot of current amplitudes at right. C, summary of data from experiments as in B. bTREK-1 current densities (in picoamperes per picofarad) were determined from current amplitudes measured immediately before and after superfusing the cell with the indicated drug. Values are expressed as mean ± S.E.M. of indicated number of determinations. Tyr.B46, Tyrphostin B46.

was typically quite low with standard pipette solution (1 mM ATP, pH 7.1). Because CDC can apparently open otherwise inactive channels where P_0 must approach zero, it produced marked increases in TREK-1 amplitude relative to control under these conditions. When native TREK-1 channels were preactivated with pipette solutions containing 5 mM ATP at acidified pHs, the relative increase in TREK-1 amplitude produced by CDC was, as expected, far smaller.

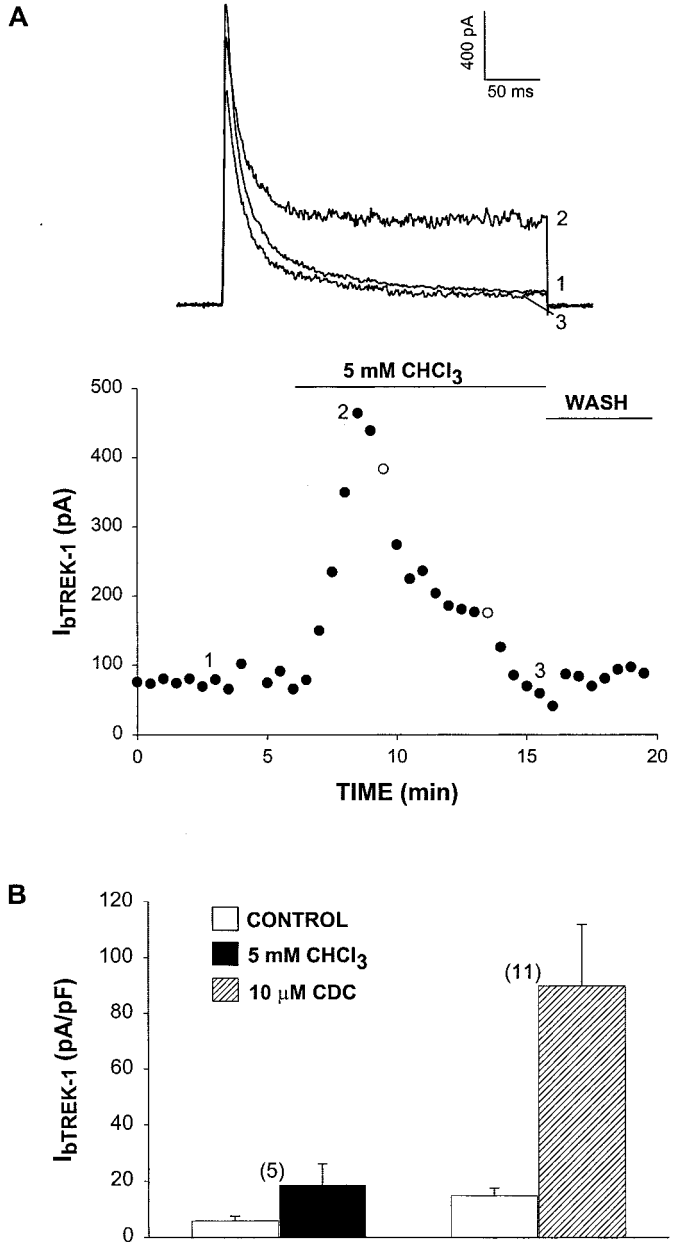

Fig. 8. Activation of native bTREK-1 by chloroform. K^+ currents were activated by voltage steps to +20 mV from a holding potential of −80 mV with (○) or without (●) depolarizing prepulses. After recording K^+ currents in standard saline, the cell was superfused with 5 mM $CHCl_3$. Numbers on current traces (recorded without depolarizing prepulses) correspond to numbers on plot of current amplitudes. B, comparative effects of $CHCl_3$ and CDC on bTREK-1. Summary data from experiments as in A above and similar experiments with CDC. bTREK-1 current densities (in picoamperes per picofarad) were determined from current amplitudes determined immediately before and after the maximum increase in bTREK-1 was obtained in the presence of $CHCl_3$ (5 mM) or CDC (10 μM). Values are expressed as mean ± S.E.M. of indicated number of determinations.

The effectiveness of CDC in activating bTREK-1 K^+ channels demonstrates that AZF cells express a large number of dormant TREK-1 channels that, when activated, strongly hyperpolarize the cell toward the K^+ equilibrium potential. Calculations based on maximum current densities observed in the presence of 20 μM CDC and unitary TREK-1 amplitudes indicate that a typical 20-pF AZF cell expresses at least 550 TREK-1 channels. Why the great majority of these seem to be inactive in unstimulated cells is unclear.

Comparison of CDC with Other 2P/4TMS K^+ Channel Openers. In both whole-cell and single-channel patch-clamp recordings, CDC activated bTREK-1 channels only when applied to the external membrane surface. CDC was ineffective when applied intracellularly in whole-cell and inside-out patch recordings. These results indicate that CDC acts externally through an action on the membrane or a site associated with the channel protein.

The activation of bTREK-1 by CDC resembles, in some

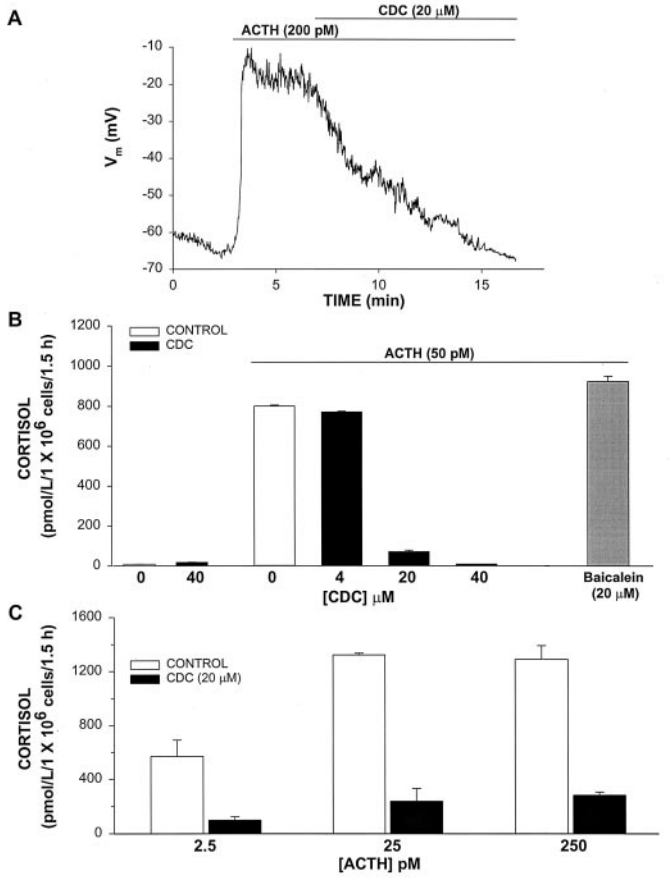

Fig. 9. CDC reverses corticotropin-mediated depolarization of AZF cells and inhibits corticotropin-stimulated cortisol secretion. A, the membrane potential of an AZF cell was recorded in whole-cell recording under current clamp with a patch electrode containing acidified pipette solution, pH 6.4, supplemented with 5 mM MgATP and 200 μM GTP. After recording resting membrane potential in standard external solution, the cell was sequentially superfused with saline containing 200 pM corticotropin (ACTH), followed by one containing corticotropin and 20 μM CDC, as indicated. Membrane potential was measured at 100 ms intervals. B and C, cultured AZF cells were incubated in serum-free defined media (see *Materials and Methods*) or the same media containing corticotropin (ACTH) alone or in combination with CDC or baicalein at the indicated concentrations. After a 90-min incubation, media samples were collected and assayed for cortisol as described under *Materials and Methods*. Values are expressed as mean ± S.E.M. of triplicate determinations assayed in duplicate.

respects, activation of these same channels by PUFAs including arachidonic acid (AA). Specifically, activation of bTREK-1 channels by AA is slow in onset and occurs over a narrow range of concentrations (Danthi et al., 2003). Anionic amphipaths such as AA preferentially insert in the external leaflet of the bilayer inducing a positive (convex) curvature that may transmit a mechanical force directly to the channel, leading to channel opening (Sheetz and Singer, 1974; Patel et al., 1998; Maingret et al., 2000).

However, in contrast to CDC, AA has been reported to activate TREK-1 K^+ channels when it is applied to the cytoplasmic surface of inside-out patches (Maingret et al., 2000). Although this result does not eliminate the possibility that AA again acts through interaction with the external leaflet of the bilayer, it suggests that CDC acts through a different mechanism. In this regard, whether CDC activates TREK-1 channels by a membrane action or a direct effect on the channel remains to be determined.

A number of volatile anesthetics activate a variety of 2P/4TMS K^+ channels (Patel et al., 1999). In general, these agents are less potent, less specific, and less effective as activators of TREK-1 channels. Of the volatile anesthetics, $CHCl_3$ is distinctive in that it seems to preferentially activate TREK channels (Patel et al., 1999). Compared with CDC, $CHCl_3$ was less effective and potent in activating native bTREK-1 in AZF cells. In addition, in whole-cell recordings, the $CHCl_3$-induced increase in bTREK-1 was transient and followed by inhibition, an effect not previously reported.

The neuroprotective agent riluzole transiently enhances the activity of native and cloned TREK-1 channels (Duprat et al., 2000; Enyeart et al., 2002). However, in contrast to CDC, riluzole is ineffective unless TREK-1 channels have been preactivated by acidic pH, ATP, or other mechanism (Enyeart et al., 2002). Riluzole is also significantly less potent than CDC.

Structure/Activity Relationships. Studies with caffeic acid and several of its derivatives indicated that the structural requirements for TREK-1 activation are relatively stringent. Specifically, we discovered that, of the agents tested, only esters of caffeic acid with furan or benzene rings activated TREK-1 channels. EDHBCA, a caffeic acid ester lacking a ring in the side chain, did not activate bTREK-1 channels.

In this regard, the cyano group present in EDHBCA that is required for 12-lipoxygenase inhibition was neither necessary nor sufficient for bTREK-1 activation (Cho et al., 1991). CAPE lacks this cyano group yet effectively activates bTREK-1 channels. Furthermore, the potency of a drug as a lipoxygenase inhibitor was not correlated with its ability to activate TREK-1 channels. In addition to EDHBCA, the potent lipoxygenase inhibitor baicalein failed to increase bTREK-1 activity in AZF cells.

The ester bond found in caffeic acid derivatives that activate bTREK-1 seems to be essential for this effect. Tyrphostin B46, which failed to open TREK-1 channels, is nearly identical to CDC in structure except for the substitution of an amide for the ester bond in the molecule.

The specific structural requirements for activation of bTREK-1 K^+ channels imply interaction with a specific binding site. The site of interaction of these K^+ channel openers on bTREK-1 channels or on the membrane has not yet been determined. If these agents interact with an external site on the channel protein, the extracellular M_1P_1 loop of the TREK-1 channel is a likely location for the binding site. In this regard, the M_1P_1 loop is a common structural feature of 2P/4TMS K^+ channels (Lesage and Lazdunski, 2000). It remains to be seen whether other background K^+ channels of this family are activated by CDC and other caffeic acid derivatives. Other agents that activate 2P/4TMS K^+ channels typically activate more than a single subtype. Arachidonic acid and riluzole preferentially activate the mechanogated subgroup of background K^+ channels (Duprat et al., 2000; Patel and Honore, 2001). It is possible that the caffeic acid derivatives activate only a subset of related 2P/4TMS K^+ channels, such as the mechanogated channels. Alternatively, these agents could activate a diverse group of these background channels, as do the volatile anesthetics. However, this seems unlikely because, in contrast to CDC, anesthetics activate these channels at millimolar concentrations through a less specific membrane effect.

CDC and Cortisol Secretion. CDC inhibited corticotropin-stimulated cortisol secretion and reversed membrane depolarization at concentrations identical to those that markedly activate bTREK-1 K^+ channels. This result is consistent with our previously presented model, in which corticotropin-mediated inhibition of bTREK-1 channels triggers depolarization-dependent Ca^{2+} entry and cortisol secretion (Enyeart et al., 1993, 1996; Mlinar et al., 1993). By activating bTREK-1 channels, CDC opposes membrane depolarization and Ca^{2+} entry through T-type Ca^{2+} channels. It thus acts as a brake on the corticotropin-induced ionic events that stimulate cortisol secretion.

At higher concentrations, CDC was shown to overwhelm the inhibitory effects of corticotropin on bTREK-1 channels. Whether CDC can reactivate bTREK-1 channels that have been inhibited by corticotropin and cAMP remains unknown. On the other hand, results from the present study and a previous one show that bovine AZF cells express up to several thousand bTREK-1 K^+ channels, the great majority of which remain dormant unless activated by chemical or metabolic factors (Danthi et al., 2003). By activating a small fraction of the large reserve pool of TREK-1 channels that remains inhibited in the presence of corticotropin, CDC may effectively clamp the membrane potential near the K^+ equilibrium potential, preventing depolarization-dependent Ca^{2+} entry required for cortisol secretion.

The inhibition of corticotropin-stimulated cortisol secretion by CDC illustrates its potential value as an experimental tool in assessing the role of TREK-1 channels in cell physiology. For example, TREK-1 K^+ channels are widely distributed in the peripheral and central nervous systems (Hervieu et al., 2001; Patel and Honore, 2001; Talley et al., 2001). Caffeic acid derivatives such as CDC could be used to determine the importance of TREK-1 channels in regulating membrane potential, Ca^{2+} entry, and transmitter release from peripheral and CNS neurons.

Caffeic Acid Derivatives as Neuroprotective Agents. Several lines of evidence suggest that CDC and other caffeic acid derivatives may display significant neuroprotective properties. First, with regard to mechanism, the excitotoxicity that accompanies short-term neurological insults including seizures, ischemia, and hypoglycemia involves excessive Ca^{2+} entry in response to repetitive or sustained depolarization (Abele et al., 1990; Garcia-Guzman et al., 1994; Lee et al., 1999). Because 2P/4TMS K^+ channels including TREK-1

stabilize the resting membrane potential and oppose electrical activity and depolarization-dependent Ca^{2+} entry, agents that open these channels would be expected to prevent or reduce the excitotoxicity that accompanies the short-term neurological insult.

In addition to the PUFAs, which open selected 2P/4TMS K^+ channels and display neuroprotective protective properties, other agents that activate background channels are also neuroprotective (Lauritzen et al., 2000). Riluzole, a clinically used neuroprotective agent, was later shown to activate TREK and TRAAK K^+ channels (Malgouris et al., 1989; Duprat et al., 2000). Accordingly, other agents, such as volatile anesthetics that activate 2P/4TMS channels, are also neuroprotective (Patel et al., 1999).

Overall, a remarkable correlation has been established for a number of agents linking their neuroprotective properties to their effectiveness as activators of background K^+ channels. Although no cause-and-effect relationship has been established, it is quite likely that activation of tandem pore K^+ channels would underlie or contribute to their therapeutic actions.

The cardioprotective effects of PUFAs, particularly the ω-3 fatty acids, may also be mediated through activation of 2P/4TMS channels, particularly TREK-1 (Billman et al., 1997; Aimond et al., 2000; Lauritzen et al., 2000; Kris-Etherton et al., 2003). However, the modulation of voltage-gated Na^+ and Ca^{2+} channels by these agents may also contribute to their protective actions (Vgeugdenhil et al., 1996; Leaf et al., 2003).

It will be interesting to determine whether CDC and other caffeic acid derivatives activate 2P/4TMS channels in addition to bTREK-1 and to determine whether these agents display neuro- or cardioprotective properties. These experiments will also help to determine whether activation of 2P/4TMS K^+ channels are uniformly neuroprotective.

Because of their potential as therapeutic agents, it would be of great interest to identity new agents that selectively activate tandem pore K^+ channels. Caffeic acid derivatives such as CDC and CAPE may be important lead compounds from which more effective neuro- and cardioprotective agents are developed.

Finally, CDC and CAPE are used experimentally as inhibitors of lipoxygenases and the nuclear transcription factor nuclear factor-κB (Cho et al., 1991; Natarajan et al., 1996). In view of the findings presented in this study, their effect on background K^+ channels should be considered when interpreting their actions in cells, particularly excitable cells.

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