

# cAMP Analogs and Their Metabolites Enhance TREK-1 mRNA and K<sup>+</sup> Current Expression in Adrenocortical Cells<sup>S</sup>

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## ABSTRACT

bTREK-1 K<sup>+</sup> channels set the resting membrane potential of bovine adrenal zona fasciculata (AZF) cells and function pivotally in the physiology of cortisol secretion. Adrenocorticotrophic hormone controls the function and expression of bTREK-1 channels through signaling mechanisms that may involve cAMP and downstream effectors including protein kinase A (PKA) and exchange protein 2 directly activated by cAMP (Epac2). Using patch-clamp and Northern blot analysis, we explored the regulation of bTREK-1 mRNA and K<sup>+</sup> current expression by cAMP analogs and several of their putative metabolites in bovine AZF cells. At concentrations sufficient to activate both PKA and Epac2, 8-bromoadenosine-cAMP enhanced the expression of both bTREK-1 mRNA and K<sup>+</sup> current. N<sup>6</sup>-Benzoyl-adenosine-cAMP, which activates PKA but not Epac, also enhanced the expression of bTREK-1 mRNA and K<sup>+</sup> current measured at times from 24 to 96 h. An Epac-selective cAMP analog, 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8CPT-2'-OMe-cAMP), potentially stimulated bTREK-1 mRNA and K<sup>+</sup> current expression, whereas the nonhydrolyzable Epac activator 8-(4-chlorophenylthio)-2'-O-methyl-cAMP, Sp-isomer was ineffective. Me-

tabolites of 8CPT-2'-OMe-cAMP, including 8-(4-chlorophenylthio)-2'-O-methyladenosine-5'-O-monophosphate and 8CPT-2'-OMe-adenosine, promoted the expression of bTREK-1 transcripts and ion current with a temporal pattern, potency, and effectiveness resembling that of the parent compound. Likewise, at low concentrations, 8-(4-chlorophenylthio)-cAMP (8CPT-cAMP; 30 μM) but not its nonhydrolyzable analog 8-(4-chlorophenylthio)-cAMP, Sp-isomer, enhanced the expression of bTREK-1 mRNA and current. 8CPT-cAMP metabolites, including 8CPT-adenosine and 8CPT-adenine, also increased bTREK-1 expression. These results indicate that cAMP increases the expression of bTREK-1 mRNA and K<sup>+</sup> current through a cAMP-dependent but Epac2-independent mechanism. They further demonstrate that one or more metabolites of 8-(4-chlorophenylthio)-cAMP analogs potentially stimulate bTREK-1 expression by activation of a novel cAMP-independent mechanism. These findings raise significant questions regarding the specificity of 8-(4-chlorophenylthio)-cAMP analogs as cAMP mimetics.

Bovine adrenal zona fasciculata (AZF) cells express bTREK-1 (or KCNK2) K<sup>+</sup> leak-type channels that set the resting membrane potential and function pivotally in the physiology of cortisol secretion (Enyeart et al., 1993, 2002;

Mlinar et al., 1993). Cortisol synthesis is stimulated by the pituitary peptide adrenocorticotropin (Simpson and Watterman, 1988). Adrenocorticotropin exerts rapid and long-term control over the electrical and secretory properties of AZF cells by regulating both the activity of pre-existing ion channels and the expression of genes coding for these same channel proteins (Mlinar et al., 1993; Enyeart et al., 1996, 2000, 2003; Liu et al., 2008). In particular, in whole-cell recordings, adrenocorticotropin rapidly (within seconds to minutes) inhibits the activity of bTREK-1 K<sup>+</sup> channels by a cAMP-dependent mechanism (Mlinar et al., 1993; En-

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**ABBREVIATIONS:** AZF, adrenal zona fasciculata; Epac, exchange protein directly activated by cAMP; ESCA, exchange protein directly activated by cAMP-selective cAMP analog; PKA, cyclic AMP-dependent protein kinase; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; BAPTA, 1,2 bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; kb, kilobase; 8-Br-cAMP, 8-bromoadenosine-cAMP; 6-Bnz-cAMP, N<sup>6</sup>-benzoyl-adenosine-cAMP; 8CPT-2'-OMe-cAMP, 8-(4-chlorophenylthio)-2'-O-methyl-cAMP; Sp-8CPT-2'-OMe-cAMP, hydrolysis-resistant 8-(4-chlorophenylthio)-2'-O-methyl-cAMP; 8CPT-2'-OMe-5'AMP, 8-(4-chlorophenylthio)-2'-O-methyladenosine-5'-O-monophosphate; 8CPT-2'-OMe-Ado, 8-(4-chlorophenylthio)-2'-O-methyladenosine; 8CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; Sp-8CPT-cAMP, hydrolysis-resistant 8-(4-chlorophenylthio)-cAMP; 8CPT-Ado, 8-(4-chlorophenylthio)-adenosine; 8CPT-Ade, 8-(4-chlorophenylthio)-adenine; H-89, N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline.

yeart et al., 1996, 2000, 2003). Adrenocorticotropin also induces, with a delay of several hours, an increase in bTREK-1 mRNA and maintains the expression of the associated K<sup>+</sup> current (Enyeart et al., 2003).

The signaling pathways by which adrenocorticotropin regulates the expression of bTREK-1 K<sup>+</sup> channel mRNA and current are only partially understood. Early studies of cortisol synthesis established cAMP as the principal intracellular messenger for adrenocorticotropin in AZF cells (Haynes and Berthet, 1957; Grahame-Smith et al., 1967; Richardson and Schulster, 1973; Sala et al., 1979). Accordingly, bovine AZF cells express a high-affinity MC2R melanocortin receptor coupled to adenylate cyclase through Gs (Penhoat et al., 1989; Raikhin et al., 1994). Until recently, all of the cAMP-dependent actions of adrenocorticotropin were believed to be mediated by PKA. However, alternative signaling pathways for cAMP-mediated responses are present in these cells. Specifically, two cAMP-activated guanine nucleotide exchange factors Epac1 and Epac2 (also known as cAMP-GEFI and cAMP-GEFII) have been identified and implicated in the regulation of cellular processes, including gene expression (Kawasaki et al., 1998; de Rooij et al., 1998; Holz et al., 2006). Although Epac1 is expressed in many tissues, Epac2 is robustly expressed in selected areas of the brain and the adrenal glands of rats and humans (de Rooij et al., 1998). We discovered recently that Epac2 is strongly expressed in bovine AZF cells, raising the possibility that cAMP could produce responses through this protein and PKA (Liu et al., 2008).

Differentiating between PKA- and Epac-dependent signaling pathways in cells has been hampered by the absence of specific agents that selectively activate each of these two proteins. Exploiting differences in the cAMP-binding domains of these proteins, rational drug design has been used to synthesize cAMP derivatives that, at appropriate concentrations, specifically activate Epacs or PKA (Enserink et al., 2002; Christensen et al., 2003; Holz et al., 2008). In a patch-clamp study, we found that in addition to the well documented PKA-dependent inhibition of bTREK-1 channel activity, the Epac-selective cAMP analog (ESCA) 8CPT-2'-OMe-cAMP potently inhibited these channels (Liu et al., 2008). Thus, cAMP seems to inhibit bTREK-1 channel function by the activation of both PKA and Epac2.

In a more recent study, we found that 8CPT-2'-OMe-cAMP stimulated a delayed increase in cortisol synthesis by inducing the expression of genes coding for steroidogenic proteins, including several steroid hydroxylases (Enyeart and Enyeart, 2009). However, the 8CPT-2'-OMe-cAMP-stimulated increases in cortisol synthesis were not mediated through the activation of Epac2. Rather, the effect was produced by one or more metabolites of this 8-(4-chlorophenylthio)-cAMP derivative.

With this knowledge in hand, the present study was done to characterize the signaling pathways by which adrenocorticotropin and cAMP regulate the expression of bTREK-1 mRNA and corresponding ion current. It was discovered that several cAMP analogs, including those that selectively activate PKA or Epac2, and those that activate both of these proteins, enhanced the expression of bTREK-1 mRNA and membrane current. As a consequence, treatment of AZF cells

with any of these cAMP analogs suppresses the time-dependent disappearance of bTREK-1 that typically occurs in culture. However, the increases in bTREK-1 transcripts and current induced by low concentrations of 8CPT-2'-OMe-cAMP and 8CPT-cAMP were mediated indirectly through one or more metabolites of these compounds by activation of an unknown signaling pathway.

## Materials and Methods

**Materials.** Tissue culture media, antibiotics, fibronectin, and fetal bovine sera (FBS) were obtained from Invitrogen (Carlsbad, CA). Phosphate-buffered saline, BAPTA, MgATP, collagenase, DNase, H-89, and adrenocorticotropin (1–24) were obtained from Sigma-Aldrich (St. Louis, MO). 8-Bromoadenosine-cAMP (8-Br-cAMP), N<sup>6</sup>-benzoyladenosine-cAMP (6-Bnz-cAMP), 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8CPT-2'-OMe-cAMP), hydrolysis-resistant 8-(4-chlorophenylthio)-2'-O-methyl-cAMP, Sp-isomer (Sp-8CPT-2'-OMe-cAMP), 8-(4-chlorophenylthio)-2'-O-methyladenosine-5'-O-monophosphate (8CPT-2'-OMe-5'AMP), 8-(4-chlorophenylthio)-2'-O-methyladenosine (8CPT-2'-OMe-Ado), 8-(4-chlorophenylthio)-cAMP (8CPT-cAMP), hydrolysis-resistant 8-(4-chlorophenylthio)-cAMP, Sp-isomer (Sp-8CPT-cAMP), 8-(4-chlorophenylthio)-adenosine (8CPT-Ado), and 8-(4-chlorophenylthio)adenine (8CPT-Ade) were purchased from Biolog (distributed by Axxora, LLC, San Diego, CA). [ $\alpha$ -<sup>32</sup>P]dCTP was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Ultra-hyb was purchased from Ambion (Austin, TX). RNeasy columns for total RNA isolation were obtained from QIAGEN (Valencia, CA). bTREK-1 probe was labeled with [<sup>32</sup>P]dCTP by random priming (Prime-It II kit; Stratagene, La Jolla, CA). Full-length bTREK-1 cDNA (1414 base pairs) was obtained as described previously (Enyeart et al., 2002).

**Isolation and Culture of AZF Cells.** Bovine adrenal glands were obtained from steers (aged 2–3 years) at a local slaughterhouse. 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% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and the antioxidants  $\alpha$ -tocopherol (1  $\mu$ M), 20 nM selenite, and 100  $\mu$ M ascorbic acid (DMEM/Ham's F12) and plated for immediate use, or resuspended in FBS/5% dimethyl sulfoxide, divided into 1-ml aliquots, and stored in liquid nitrogen for future use. To ensure cell attachment, dishes were treated with fibronectin (10  $\mu$ g/ml) at 37°C for 30 min and then rinsed with warm, sterile phosphate-buffered saline immediately before adding cells. For patch-clamp experiments, cells were plated in DMEM/Ham's F12 in 35-mm dishes containing 9-mm<sup>2</sup> glass coverslips (Bellco, Vineland NJ). Coverslips were treated with fibronectin (10  $\mu$ g/ml) as described above. Cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub>.

**Measurement of bTREK-1 mRNA.** RNeasy columns treated with RNase-free DNase (both from Qiagen) to remove genomic contamination were used to extract total RNA from AZF cells. Total mRNA (10  $\mu$ g/lane) was separated on denaturing 8% formaldehyde, 1.0% agarose gels, and transferred to nylon membranes (GeneScreen Plus; PerkinElmer Life and Analytical Sciences). RNA was fixed to the membrane by UV cross-linking (Stratalinker; Stratagene), prehybridized for 2 h at 42°C in ULTRAhyb (Ambion), and then hybridized with a [ $\alpha$ -<sup>32</sup>P]dCTP-labeled bTREK-1 full-length cDNA as described previously (Enyeart et al., 2003). Northern autoradiograms were imaged using a Typhoon 9200 variable-mode PhosphorImager and quantitated using ImageQuant TL v2003.3 software (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). mRNA values are presented as mean  $\pm$  S.E.M. of at least three independent determinations. For the figures, a representative Northern blot of at least three independent experiments is shown. Statisti-

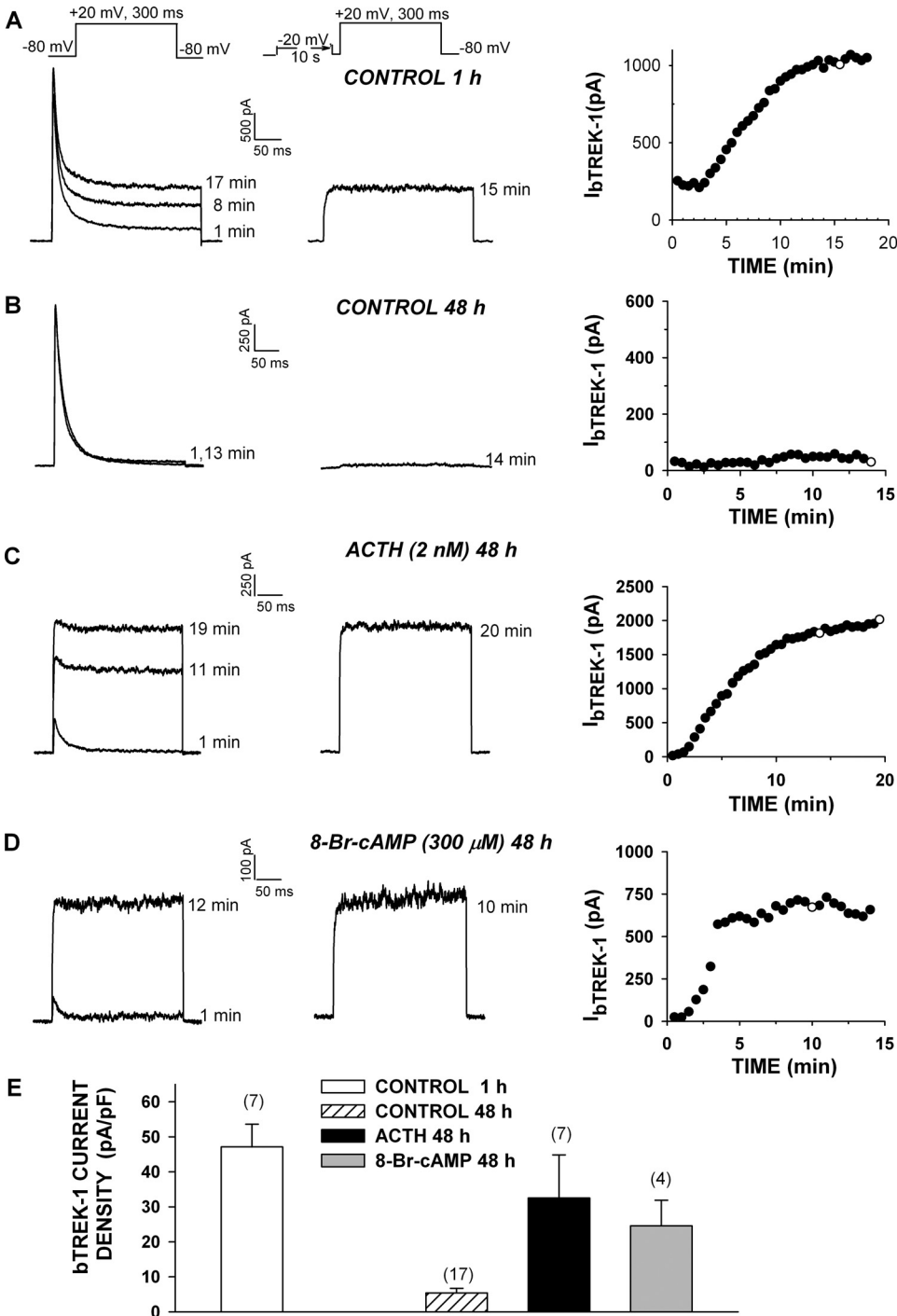
cally significant differences were determined by unpaired *t* test analysis (GraphPad Software, Inc., San Diego, CA). *P* values <0.05 were considered statistically significant.

**Patch-Clamp Experiments.** Patch-clamp recordings of K<sup>+</sup> channel currents were made in the whole-cell configuration from bovine AZF cells. The standard external solution consisted of 140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM HEPES, and 5 mM glucose, with pH adjusted to 7.3 using NaOH. The standard pipette solution consisted of 120 mM KCl, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 11 mM BAPTA, 10 mM HEPES, 5 mM ATP, and 200 μM GTP, with pH titrated to 6.8 using KOH.

**Recording Conditions and Electronics.** AZF cells were used for patch-clamp experiments 2 to 12 h after plating. In general, cells with diameters <15 μm and capacitances of 10 to 15 pF were se-

lected. Coverslips were transferred from 35-mm culture dishes to the recording chamber (volume, 1.5 ml) that was continuously perfused by gravity 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. K<sup>+</sup> currents were recorded at room temperature (22–25°C) according to the procedure of Hamill et al. (1981) using a List EPC-7 patch-clamp amplifier.

Pulse generation and data acquisition were done using a personal computer and pCLAMP software with a Digidata 1200 interface (Molecular Devices, Sunnyvale, CA). Currents were digitized at 2 to 10 KHz after filtering with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity



**Fig. 1.** Long-term effect of adrenocorticotropin and 8-Br-cAMP on the expression of bTREK-1 current. AZF cells were used for patch-clamp experiments 1 to 48 h after plating. AZF cells were plated in media containing no further addition (control; A and B), 2 nM adrenocorticotropin (ACTH) (C), or 8-Br-cAMP (D). Whole-cell K<sup>+</sup> currents were recorded in response to voltage steps to +20 mV applied from –80 mV at 30-s intervals with or without depolarizing prepulses to –20 mV. Pipettes contained standard solution (see *Materials and Methods*). A to D, representative K<sup>+</sup> current traces recorded with (right traces) and without (left traces) depolarizing prepulses, and corresponding plot of bTREK-1 amplitudes with (○) and without (●) depolarizing prepulses. Times indicated on traces correspond to those on the graph at right. E, summary of experiments as in A to D. Bars represent bTREK-1 current density expressed as mean ± S.E.M. of indicated number of determinations at 1 and 48 h in control media and after 48-h exposure to adrenocorticotropin (2 nM) or 8-Br-cAMP (300 μM), as indicated.

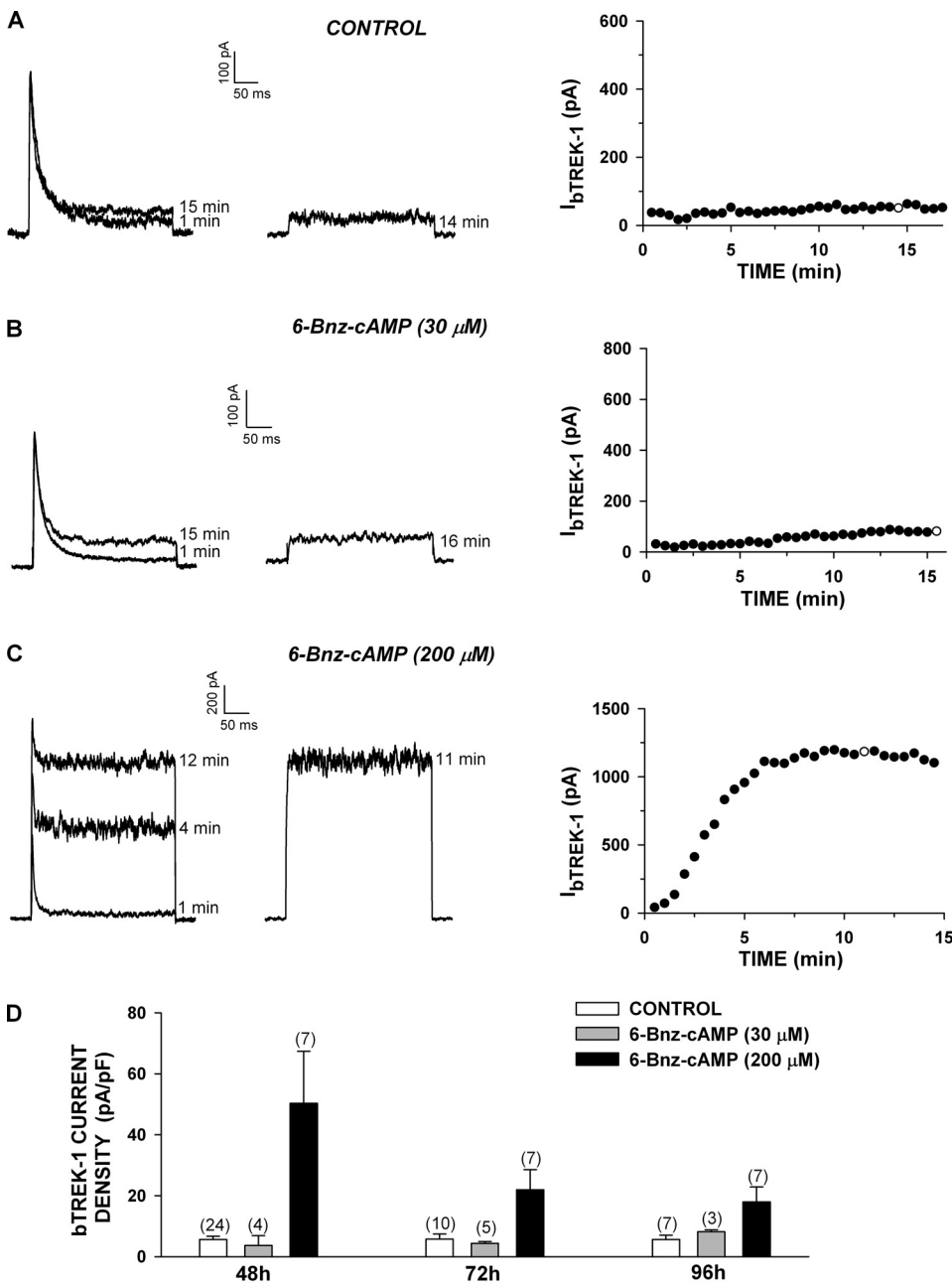
currents were subtracted from current records using summed scaled hyperpolarizing steps of 1/2 to 1/4 pulse amplitude. Data were analyzed using Clampfit 9.2 (Molecular Devices) and SigmaPlot (version 10.0; Systat Software, San Jose, CA) software. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

## Results

**Adrenocorticotropin and 8-Br-cAMP Promote the Expression of bTREK-1 K<sup>+</sup> Current.** Bovine AZF cells express two types of K<sup>+</sup> channels: a voltage-gated, rapidly inactivating Kv1.4 (or KCNA4) channel; and a two-pore domain, four transmembrane-spanning segment bTREK-1 (or KNCK2) background K<sup>+</sup> channel (Mlinar and Enyeart, 1993; Mlinar et al., 1993; Enyeart et al., 2002). In whole-cell patch-clamp recordings, bTREK-1 amplitude sponta-

neously increases over a period of 10 to 20 min to a stable maximum. The absence of time- and voltage-dependent inactivation allows bTREK-1 K<sup>+</sup> currents to be isolated in whole-cell recordings using either of two voltage-clamp protocols. When voltage steps of several hundred milliseconds' duration are applied from a holding potential of -80 mV, bTREK-1 current can be measured near the end of a voltage step when the Kv1.4 current has completely inactivated (Fig. 1, A-D, left traces). Alternatively, bTREK-1 current can be selectively activated by an identical voltage step, applied immediately after a 10-s prepulse to -20 mV has fully inactivated Kv1.4 channels (Fig. 1A-D, right traces).

When bovine AZF cells were cultured in serum-supplemented media for periods of 24 h or more, the bTREK-1 K<sup>+</sup> current was markedly diminished compared with currents re-



**Fig. 2.** 6-Bnz-cAMP induces the expression of bTREK-1 current. AZF cells were cultured in media containing no further addition (control) (A), 30 μM 6-Bnz-cAMP (B), or 200 μM 6-Bnz-cAMP (C). Whole-cell K<sup>+</sup> currents were recorded from AZF cells in response to voltage steps to +20 mV applied from -80 mV at 30-s intervals with or without depolarizing prepulses to -20 mV. Pipettes contained standard solution (see *Materials and Methods*). A to C, representative K<sup>+</sup> current traces recorded with (right traces) and without (left traces) depolarizing prepulses, and corresponding plot of bTREK-1 amplitudes with (○) and without (●) depolarizing pulses. Times indicated on traces correspond to those on the graph at right. Cells were either untreated (A) or were treated with 30 μM (B) or 200 μM (C) 6-Bnz-cAMP for 48 h before recording. D, summary of experiments as in A to C. Bars represent bTREK-1 current density expressed as mean ± S.E.M. of indicated number of determinations after 48 to 96 h of exposure to 6-Bnz-cAMP (30 or 300 μM), as indicated.

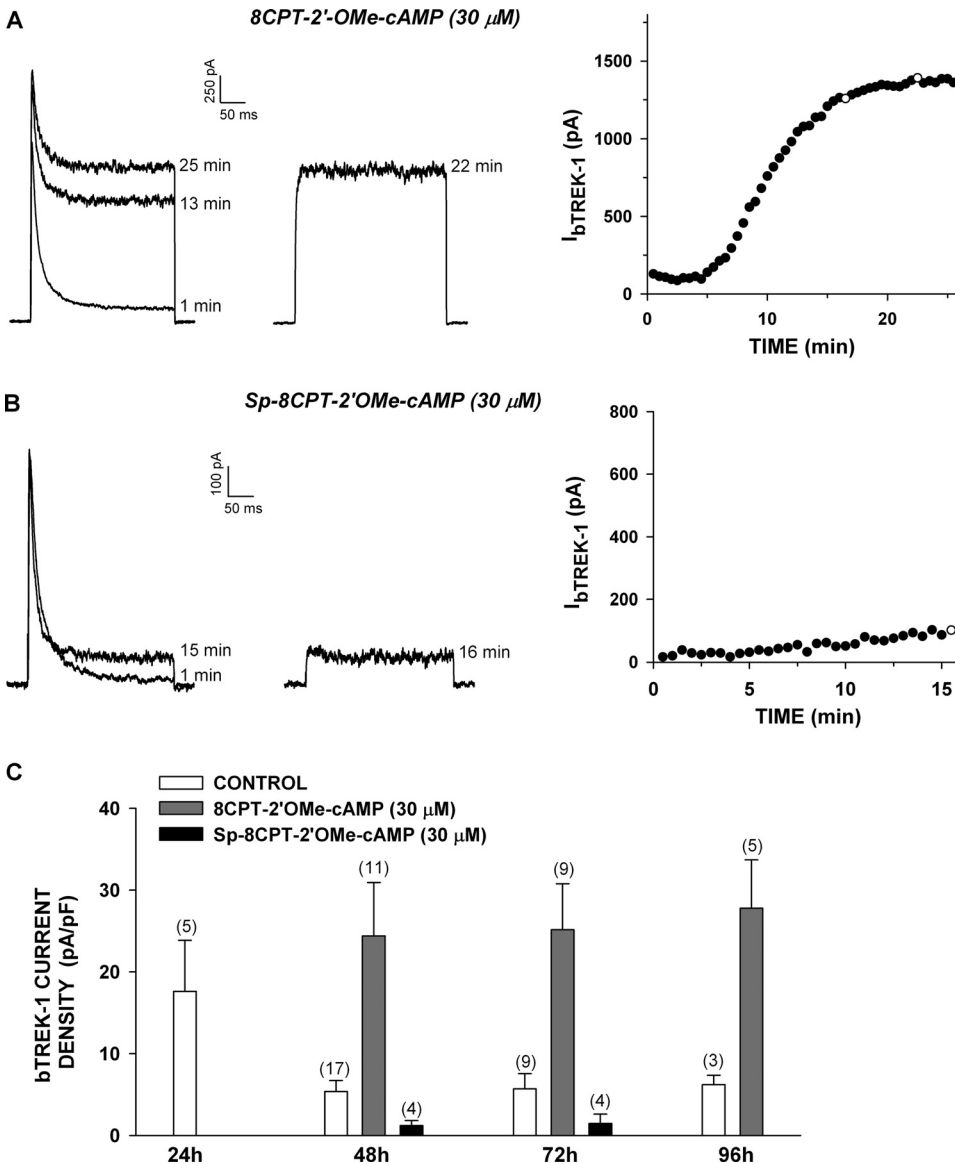
corded 1 to 3 h after plating (Fig. 1, A, B, and E). In contrast, when these cells were continuously exposed to adrenocorticotropin (2 nM) for 48 h after plating, bTREK-1 current was well maintained, compared with time-matched controls (Fig. 1, C and E). Overall, when AZF cells were cultured in serum-supplemented (control) media for 48 h, bTREK-1 current density decreased from its original density of  $47.1 \pm 6.5$  ( $n = 7$ ) to  $5.37 \pm 1.35$  pA/pF ( $n = 17$ ). In contrast, bTREK-1 current density in adrenocorticotropin-treated cells decreased to only  $32.5 \pm 12.3$  pA/pF ( $n = 7$ ) (Fig. 1D).

cAMP analogs substituted at the 8-position of the adenine ring activate both PKA and Epac2 (Christensen et al., 2003; Poppe et al., 2008). Although 8-substituted cAMP analogs bind to PKA and Epac proteins with  $K_d$  values in the low micromolar range, limited membrane permeability dictates that higher concentrations are required to produce effects in intact cells (Christensen et al., 2003; Liu et al., 2008). 8-Br-cAMP stimulates large increases in cortisol secretion by bovine AZF cells only when applied at concentrations greater than 100  $\mu$ M (Supplementary Fig. 1A).

We tested the effect of 8-Br-cAMP (300  $\mu$ M) on the

expression of bTREK-1 in AZF cells. As illustrated in Fig. 1, D and E, 8-Br-cAMP mimicked adrenocorticotropin in promoting the expression of bTREK-1 current. After a 48-h exposure to 8-Br-cAMP, bTREK-1 current density was 4.6 times greater than the time-matched control. As with adrenocorticotropin, exposing cells to 8-Br-cAMP for prolonged periods suppressed the expression of Kv1.4 (Fig. 1, C and D). This response was not further explored in the present study.

**6-Bnz-cAMP Enhances bTREK-1 Expression.** The increases in bTREK-1 current density induced by adrenocorticotropin and 8-Br-cAMP could have occurred through activation of Epac2, PKA, or perhaps an unidentified signaling pathway. cAMP derivatives with substitutions at the 6-position of the adenine ring selectively activate PKA over Epac proteins (Christensen et al., 2003). As with 8-Br-cAMP, limited membrane permeability is likely to diminish the potency of 6-Bnz-cAMP when it is applied to intact cells (Liu et al., 2009). 6-Bnz-cAMP stimulates large increases in cortisol secretion from AZF cells only at concentrations greater than 100  $\mu$ M (Supplemental Fig. 1B).



**Fig. 3.** Effect of Epac2-selective cAMP analogs on bTREK-1 current expression. Whole-cell K<sup>+</sup> currents were recorded from AZF cells in response to voltage steps to +20 mV applied from -80 mV at 30-s intervals with or without depolarizing prepulses to -20 mV. Pipettes contained standard solution (see *Materials and Methods*). A and B, representative K<sup>+</sup> current traces recorded with (right traces) and without (left traces) depolarizing prepulses, and corresponding plot of bTREK-1 amplitudes with (○) and without (●) depolarizing pulses. Time indicated on traces corresponds to those plotted on graph at right. AZF cells with treated with 30  $\mu$ M 8CPT-2'-OMe-cAMP (A) or 30  $\mu$ M Sp-8CPT-2'-OMe-cAMP (B) for 48 h before recording. C, summary of experiments as in A and B: bars specify bTREK-1 current density expressed as mean  $\pm$  S.E.M. of an indicated number of determinations after 24 to 96 h of exposure to 8CPT-2'-OMe-cAMP (30  $\mu$ M) or Sp-8CPT-2'-OMe-cAMP (30  $\mu$ M) as indicated.

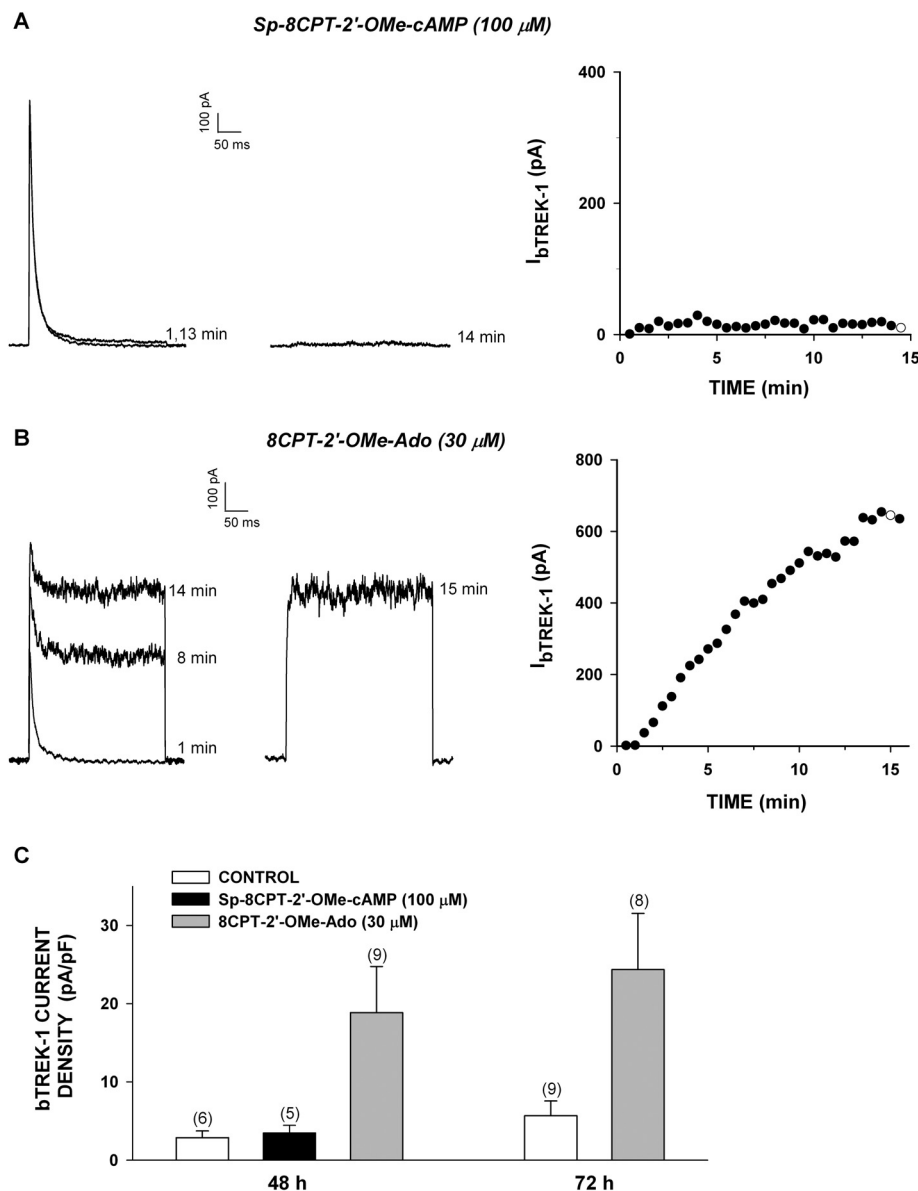
Accordingly, we found that prolonged incubation of AZF cells with 6-Bnz-cAMP (200  $\mu$ M), but not 30  $\mu$ M, markedly enhanced the expression of bTREK-1 current measured at times from 48 to 96 h compared with time-matched controls (Fig. 2, C and D). After 48 h in the presence of 6-Bnz-cAMP (200  $\mu$ M), bTREK-1 current density was 9-fold greater than that of untreated cells (Fig. 2D). These results suggest that cAMP can enhance the expression of bTREK-1 by activating PKA alone.

**Epac-Selective cAMP Analogs and Metabolites Promote bTREK-1 Expression.** Experiments with 6-Bnz-cAMP indicated that activation of PKA was sufficient to stimulate bTREK-1 current expression. Additional studies were done to determine the role of Epac2 in the expression of this current. The ESCA 8CPT-2'-OMe-cAMP has been shown to activate Epac proteins when applied to intact cells at concentrations from 10 to 100  $\mu$ M (Enserink et al., 2002; Holz et al., 2008). We found that 8CPT-2'-OMe-cAMP (30  $\mu$ M) effectively stimulated bTREK-1 current expression measured at times from 48 to 96 h (Fig. 3, A and

C). As illustrated in Fig. 3C, in control media, bTREK-1 current density decreased spontaneously with time, reaching an apparent minimum of  $5.27 \pm 1.35$  pA/pF ( $n = 17$ ) by 48 h, after which no further decay occurred. However, in the presence of 8CPT-2'-OMe-cAMP, bTREK-1 current density was significantly increased at each time point. By 48 h, bTREK-1 current density reached a nearly constant value that was approximately 4.5-fold greater than that of the time-matched controls at 48, 72, and 96 h (Fig. 3C).

The marked stimulation of bTREK-1 expression by 8CPT-2'-OMe-cAMP suggested that cAMP could induce the expression of bTREK-1 channels by activation of Epac2. However, at the same concentration, Sp-8CPT-2'-OMe-cAMP, a membrane-permeable ESCA that is resistant to hydrolysis by cyclic nucleotide phosphodiesterase, failed to stimulate the expression of bTREK-1 current at 48 and 72 h. In fact, in the presence of this ESCA, bTREK-1 current was significantly reduced compared with controls (Fig. 3, B and C).

The effective stimulation of bTREK-1 current expression by 8CPT-2'-OMe-cAMP combined with the failure of its nonhydro-



**Fig. 4.** Long-term effect of Sp-8CPT-2'-OMe-cAMP and 8CPT-2'-OMe-Ado on expression of bTREK-1 current. AZF cells were cultured for 48 h in media containing no further addition (control), Sp-8CPT-2'-OMe-cAMP (100  $\mu$ M) (A), or 8CPT-2'-OMe-Ado (30  $\mu$ M) (B). Whole-cell  $K^+$  currents were recorded from AZF cells in response to voltage steps to +20 mV applied from -80 mV at 30-s intervals with or without depolarizing prepulses to -20 mV. Pipettes contained standard solution (see *Materials and Methods*). A and B, representative  $K^+$  current traces recorded with (right traces) and without (left traces) depolarizing prepulses, and corresponding plot of bTREK-1 amplitudes with (○) and without (●) depolarizing prepulses. Times indicated on traces correspond to those plotted on the graph at right. C, summary of experiments as in A and B; bars represent bTREK-1 current density expressed as mean  $\pm$  S.E.M. of indicated number of determinations after 48 or 72 h of exposure to Sp-8CPT-2'-OMe-cAMP (100  $\mu$ M) or 8CPT-2'-OMe-Ado (30  $\mu$ M) as indicated.

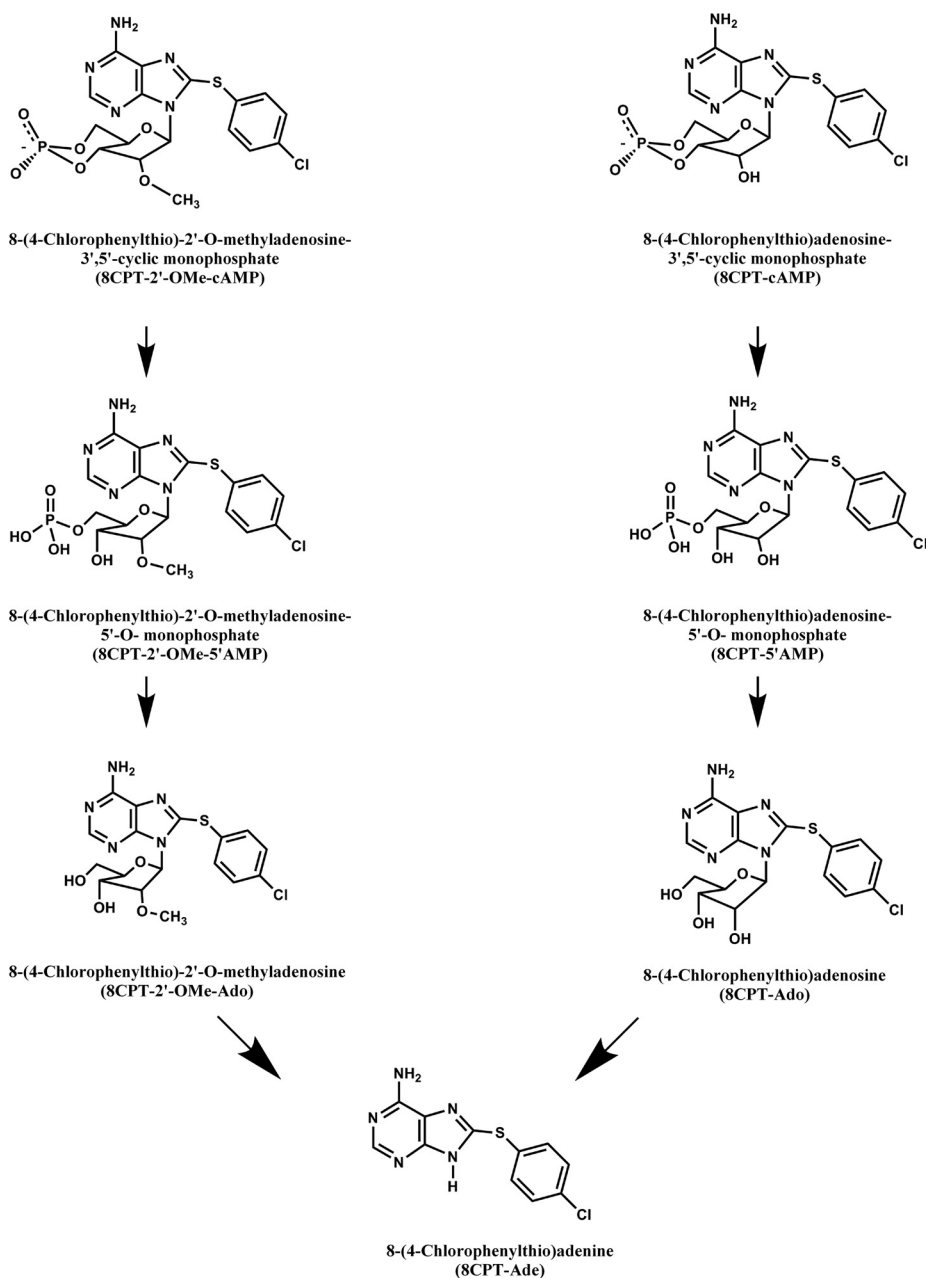
lyzable analog to mimic this action indicated that, at low concentrations, 8CPT-2'-OMe-cAMP acts independently of Epac2. In this regard, it has become apparent that, in some cells, significantly higher concentrations are required for effective activation of Epac2 (Holz et al., 2008; Chepurny et al., 2009). We have shown previously that in AZF cells, Sp-8CPT-2'-OMe-cAMP (100  $\mu$ M) activated Rap1, a downstream effector of Epac2 (Enyeart and Enyeart, 2009). However, we now report that treating AZF cells for 48 h with Sp-8CPT-2'-OMe-cAMP (100  $\mu$ M) did not enhance the expression of bTREK-1 currents (Fig. 4, A and C).

The results of experiments with the ESCAs indicated that activation of Epac2 does not induce the expression of bTREK-1 current. They further suggested that 8CPT-2'-OMe-cAMP increased bTREK-1 K<sup>+</sup> current expression indirectly by the generation of one or more active metabolites.

8CPT-2'-OMe-cAMP can be sequentially converted to

8CPT-2'-OMe-5'AMP and 8CPT-2'-OMe-Ado by cyclic nucleotide phosphodiesterase and 5' nucleotidases, respectively (Price and Stevens, 1999; Holz et al., 2008) (Fig. 5). We discovered that 8CPT-2'-OMe-Ado potently and effectively stimulated the expression of bTREK-1 current. After exposing cells for 48 h to this metabolite at a concentration of 30  $\mu$ M, bTREK-1 current density reached a value six times that of its time-matched control (Fig. 4C). This large increase in bTREK-1 current persisted after 72 h.

**Effect of cAMP Analogs and Metabolites on bTREK-1 mRNA.** We have shown previously that adrenocorticotropin-induced increases in bTREK-1 expression were associated with increases in bTREK-1 mRNA (Enyeart et al., 2003). Experiments were done to determine whether the increases in bTREK-1 current induced by cAMP analogs and their presumptive metabolites were mediated through the enhancement of bTREK-1 gene expression. In Northern



**Fig. 5.** Chemical structures of 8CPT-2'-OMe-cAMP, 8CPT-cAMP, and their metabolites.

blots using RNA isolated from AZF cells, a 1414-base pair cDNA probe that included the entire coding sequence of bTREK-1 hybridized to separate mRNA transcripts of ~4.9, 3.6, and 2.8 kb (Fig. 6A) (Enyeart et al., 2002). When bovine AZF cells were cultured in serum-supplemented media for 5 to 48 h, the quantity of bTREK-1 mRNA decreased over time (Fig. 6A).

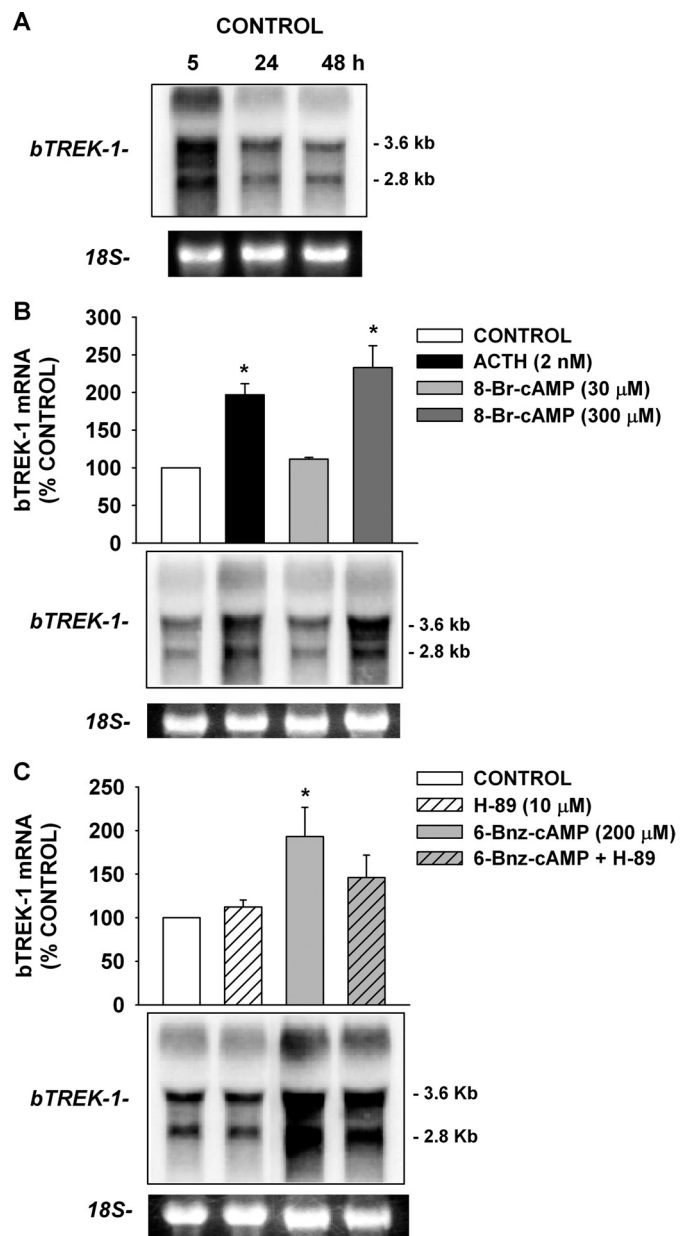
8-Br-cAMP (300  $\mu$ M), similar to adrenocorticotropin, markedly increased the expression of bTREK-1 mRNA in AZF cells. In the experiment illustrated in Fig. 6B, AZF cells were cultured overnight in serum-supplemented media before exposing them for 48 h to adrenocorticotropin or 8-Br-cAMP at the indicated concentrations. Adrenocorticotropin (2 nM) and 8-Br-cAMP (300  $\mu$ M) increased mRNA expression by  $2.0 \pm 0.1$ - and  $2.3 \pm 0.3$ -fold, respectively. Although each of the three bTREK-1 transcripts were induced by 8-Br-cAMP and adrenocorticotropin, the smaller 3.6- and 2.8-kb transcripts were preferentially increased. At a 10-fold lower concentration, 30  $\mu$ M 8-Br-cAMP failed to stimulate bTREK-1 mRNA expression (Fig. 6B).

In addition to 8-Br-cAMP, we found that 6-Bnz-cAMP (200  $\mu$ M) also enhanced the expression of bTREK-1 mRNA (Fig. 6C). However, the PKA antagonist H-89 (10  $\mu$ M) only partially inhibited 6-Bnz-cAMP-stimulated increases in bTREK-1 expression. In three similar independent experiments, H-89 reduced 6-Bnz-cAMP-stimulated bTREK-1 expression by  $56 \pm 2\%$ . These results indicate that 6-Bnz-cAMP-stimulated increases in bTREK-1 expression are mediated only in part by PKA.

Patch-clamp experiments indicated that the ESCA 8CPT-2'-OMe-cAMP induced bTREK-1 current indirectly after conversion to one or more active metabolites (Figs. 3 and 4). Accordingly, we found that 8CPT-2'-OMe-cAMP potently and effectively induced the expression of bTREK-1 mRNA, but this effect was again mediated through hydrolysis products of this cAMP analog, rather than by activation of Epac2.

8CPT-2'-OMe-cAMP stimulated the expression of bTREK-1 mRNA at the same concentrations that enhanced the expression of bTREK-1 current. In the experiment illustrated in Fig. 7A, bTREK-1 mRNA was measured after exposing AZF cells to 8CPT-2'-OMe-cAMP at concentrations ranging from 10 to 50  $\mu$ M for 48 h. A significant increase was observed at 20  $\mu$ M, whereas 50  $\mu$ M 8CPT-2'-OMe-cAMP induced a maximum  $2.8 \pm 0.4$ -fold increase in this mRNA. The 8CPT-2'-OMe-cAMP-induced increases in bTREK-1 transcripts could be observed after a delay of 5 to 10 h, and bTREK-1 mRNA continued to increase for at least 48 h (Fig. 7B). The temporal pattern of these increases in mRNA was therefore well correlated with the corresponding enhancement in bTREK-1 current.

In contrast to 8CPT-2'-OMe-cAMP, Sp-8CPT-2'-OMe-cAMP failed to promote the expression of bTREK-1 mRNA when applied to cells at 30  $\mu$ M or at higher concentrations at which this nonhydrolyzable ESCA produces significant activation of Rap1 (Fig. 7C, left and right) (Enyeart and Enyeart, 2009). Accordingly, we found that the two putative metabolites 8CPT-2'-OMe-5'AMP and 8CPT-2'-OMe-Ado increased the expression of bTREK-1 mRNA with potency similar to that of the parent compound (50  $\mu$ M 8CPT-2'-OMe-5' AMP,  $2.4 \pm 0.1$ -fold; 50  $\mu$ M 8CPT-2'-OMe-Ado,  $2.1 \pm 0.6$ -fold; Fig. 7D). In contrast, adenosine



**Fig. 6.** Effects of adrenocorticotropin, 8-Br-cAMP, and 6-Bnz-cAMP on bTREK-1 gene expression. AZF cells were incubated either without (control) or with adrenocorticotropin, 8-Br-cAMP, 6-Bnz-cAMP, or 6-Bnz-cAMP + H-89, as indicated. Total RNA was isolated as described under *Materials and Methods*. Membranes were hybridized with specific probe for bTREK-1. 18S rRNA bands from representative gels are shown as evidence of even loading. bTREK-1 mRNA levels are expressed as percent of the 3.6-kb control band value. A, effect of time in culture on bTREK-1 mRNA. AZF cells were plated and total RNA isolated after 5, 24, or 48 h in culture, as indicated. B, effect of adrenocorticotropin (ACTH) and 8-Br-cAMP on bTREK-1 mRNA expression. AZF cells were cultured overnight before either no addition (control, □) or addition of adrenocorticotropin (2 nM, ■) or 8-Br-cAMP (30  $\mu$ M, light gray bar; 300  $\mu$ M, dark gray bar) for 48 h before isolating total RNA. \*, statistically significant difference between control and treated cells (\*,  $P < 0.02$ ). C, effect of 6-Bnz-cAMP and H-89 on bTREK-1 mRNA. AZF cells were plated and cultured overnight before either no addition (control, open bar) or addition of H-89 (10  $\mu$ M, striped bar), 6-Bnz-cAMP (200  $\mu$ M, gray bar), or 6-Bnz-cAMP + H-89 (gray striped bar) for 24 h before isolating total RNA. Cells were preincubated with H-89 (10  $\mu$ M) for 1 h before the 6-Bnz-cAMP (200  $\mu$ M) addition (\*,  $P < 0.03$ ).



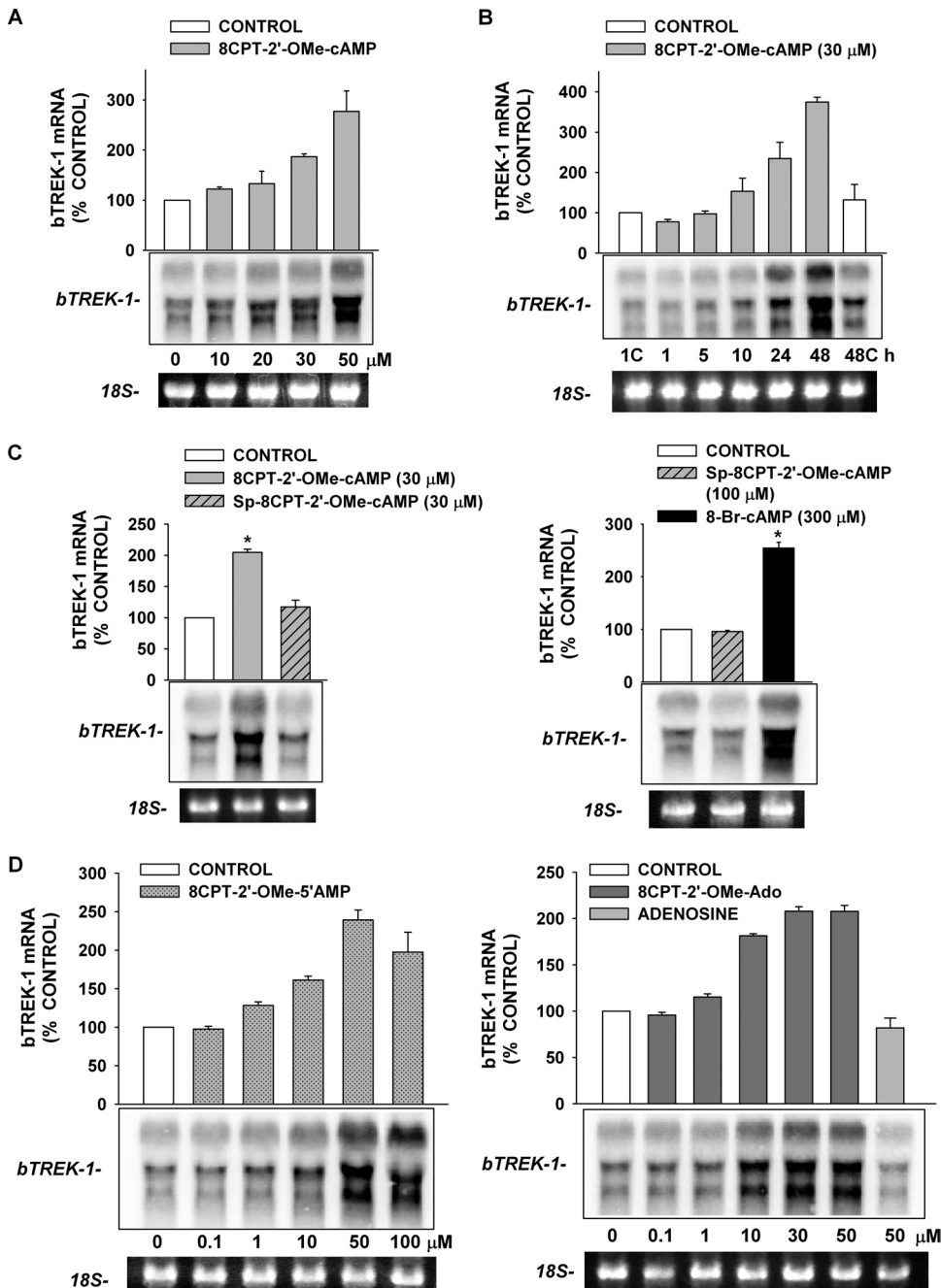
(50  $\mu$ M) failed to stimulate any increase in bTREK-1 expression ( $0.9 \pm 0.1$ -fold, Fig. 7D, right).

Overall, these results indicate that cAMP induces bTREK-1 mRNA and K<sup>+</sup> current through activation of PKA but not Epac2. Remarkably, increases in bTREK-1 mRNA and K<sup>+</sup> current induced by the ESCA 8CPT-2'-OMe-cAMP seem to be produced indirectly by one or more metabolites of this compound.

**Effect of 8CPT-cAMP and Metabolites on bTREK-1 Expression.** In a previous study, we showed that 8CPT-cAMP (250  $\mu$ M) increased the expression of bTREK-1 mRNA (Enyeart et al., 2003). Although it probably activates both PKA and Epac2 at this concentration, this 8-(4-chlorophenylthio)-derivative of cAMP can be metabolized by the same enzymes that hydrolyze 8CPT-2'-OMe-cAMP (Price and

Stevens, 1999; Holz et al., 2008). This raised the possibility that metabolites of 8CPT-cAMP contributed to the observed increases in bTREK-1 mRNA.

To answer this question, we compared 8CPT-cAMP and its membrane-permeable nonhydrolyzable analog Sp-8CPT-cAMP with respect to their potency as enhancers of bTREK-1 current and mRNA (Dostmann et al., 1990). Exposing cells for 48 h to 30  $\mu$ M 8CPT-cAMP markedly increased bTREK-1 current density from  $5.85 \pm 1.22$  pA/pF ( $n = 20$ ) to  $35.2 \pm 7.6$  ( $n = 7$ ) (Fig. 8A). In contrast, treating cells with Sp-8CPT-cAMP (30  $\mu$ M) failed to increase bTREK-1 expression (Fig. 8, B and D). These results indicated that, at this low concentration, 8CPT-cAMP increased bTREK-1 expression by a mechanism independent of cAMP. At a 10-fold higher concentration, the poorly



**Fig. 7.** Effects of 8CPT-2'-OMe-cAMP and its metabolites on bTREK-1 gene expression. AZF cells were cultured overnight and then incubated either without (control) or with 8CPT-2'-OMe-cAMP, Sp-8CPT-2'-OMe-cAMP, 8CPT-2'-OMe-5'AMP, or 8CPT-2'-OMe-Ado as indicated. Total RNA was isolated as described under *Materials and Methods*. Membranes were hybridized with a specific probe for bTREK-1; bTREK-1 mRNA levels are expressed as a percentage of the 3.6-kb control band value. 18S rRNA bands from representative gels are shown as evidence of even loading. **A**, concentration-dependent effect of 8CPT-2'-OMe-cAMP on bTREK-1 mRNA expression. AZF cells were untreated (control,  $\square$ ) or treated with 8CPT-2'-OMe-cAMP (1–50  $\mu$ M,  $\blacksquare$ ) for 48 h before isolating total RNA. **B**, time-dependent effect of 8CPT-2'-OMe-cAMP on bTREK-1 mRNA expression. AZF cells were either untreated (control,  $\square$ ) or treated with 30  $\mu$ M 8CPT-2'-OMe-cAMP ( $\blacksquare$ ) for 1 to 48 h before isolating total RNA. **C**, effect of 8CPT-2'-OMe-cAMP, Sp-8CPT-2'-OMe-cAMP, and 8-Br-cAMP on bTREK-1 mRNA expression. AZF cells were either untreated (control, open bar) or treated with 30  $\mu$ M 8CPT-2'-OMe-cAMP (gray bar, left), 30  $\mu$ M Sp-8CPT-2'-OMe-cAMP (gray striped bar, left), 100  $\mu$ M Sp-8CPT-2'-OMe-cAMP (gray dotted bar, right), or 300  $\mu$ M 8-Br-cAMP (black bar, right) for 48 h before isolating total RNA (\*,  $P < 0.005$ ). **D**, effect of metabolites of 8CPT-2'-OMe-cAMP on induction of bTREK-1 mRNA. AZF cells were treated with either 8CPT-2'-OMe-5'AMP (0.1–100  $\mu$ M, gray dotted bars), 8CPT-2'-OMe-Ado (0.1–50  $\mu$ M, dark gray bars), or adenosine (50  $\mu$ M, light gray bar) for 48 h before isolating total RNA.

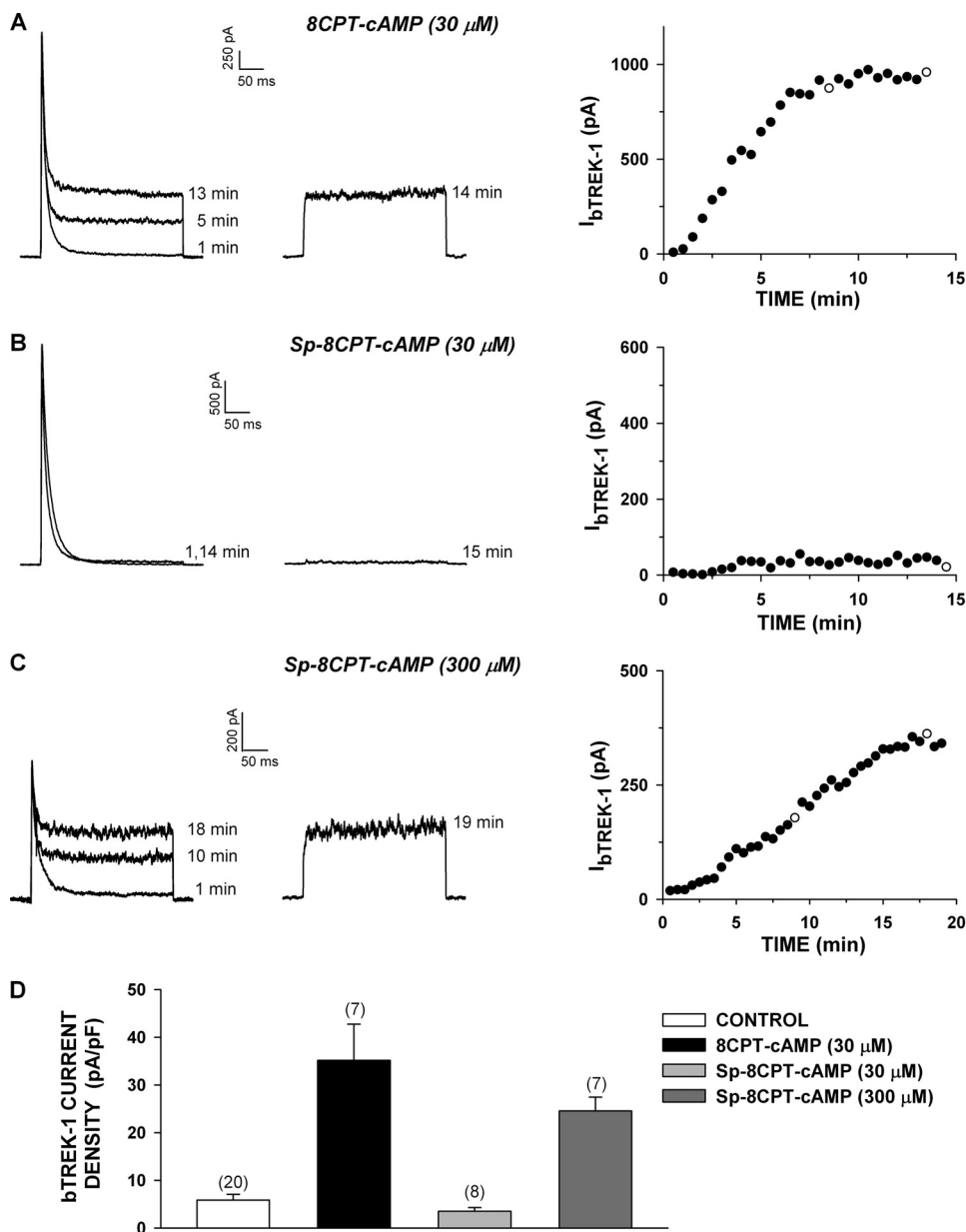
hydrolyzable Sp-8CPT-cAMP (300  $\mu\text{M}$ ) increased bTREK-1 current density 4.2-fold over the time-matched control to  $24.5 \pm 2.9$  pA/pF ( $n = 7$ ) (Fig. 8, C and D).

These results suggest that, at low concentrations at which 8CPT-cAMP does not effectively activate PKA, it stimulates bTREK-1 current expression only after it is converted to one or more active metabolites. In contrast, at higher concentrations, the nonhydrolyzable Sp-8CPT-cAMP increases bTREK-1 current expression by activation of PKA.

8CPT-cAMP can be metabolized to 8CPT-5'AMP and 8CPT-Ado by cyclic nucleotide phosphodiesterase and 5'-nucleotidase, respectively (Price and Stevens, 1999). 8CPT-Ado can then be converted to 8CPT-Ado by purine nucleoside phosphorylase (Price and Stevens, 1999). It is interesting that 8CPT-Ado may be synthesized from both 8CPT-2'-OMe-cAMP and 8CPT-cAMP in AZF cells (Fig. 5). Therefore, we examined the effects of 8CPT-Ado and 8CPT-Ado on bTREK-1 expression.

bTREK-1 current was markedly enhanced in response to a 48-h incubation with both 8CPT-Ado (30  $\mu\text{M}$ ) and 8CPT-Ado (30  $\mu\text{M}$ ) (Fig. 9, A–C). The 4.6- and 7.2-fold increases in current density induced by these metabolites compare with the 6.0-fold increase produced by 8CPT-cAMP at the same concentration. Thus, patch-clamp experiments indicated that, at low concentrations, 8CPT-cAMP enhanced the expression of bTREK-1  $\text{K}^+$  current indirectly after conversion to one or more active metabolites.

**Effect of 8CPT-cAMP, Sp-8CPT-cAMP, and 8CPT-Adenine on bTREK-1 mRNA Expression.** In Northern blot analysis, we found that the effects of 8CPT-cAMP and Sp-8CPT-cAMP on bTREK-1 mRNA expression were consistent with their effects on bTREK-1  $\text{K}^+$  current. As in results seen in experiments with 8CPT-2'-OMe-cAMP and Sp-8CPT-2'-OMe-cAMP (Fig. 7C), exposing AZF cells to 30  $\mu\text{M}$  8CPT-cAMP for 48 h significantly increased bTREK-1 mRNA ( $1.8 \pm 0.2$ -fold), whereas nonhydrolyzable Sp-8CPT-cAMP was ineffective in this regard (Fig. 10A).



**Fig. 8.** Long-term effect of 8CPT-cAMP and Sp-8CPT-cAMP on the expression of bTREK-1 current. AZF cells were cultured overnight and then incubated either without (control) or with 8CPT-cAMP (30  $\mu\text{M}$ ) or Sp-8CPT-cAMP (30 or 300  $\mu\text{M}$ ), as indicated. Whole-cell  $\text{K}^+$  currents were recorded from AZF cells in response to voltage steps to +20 mV applied from  $-80$  mV at 30-s intervals with or without depolarizing prepulses to  $-20$  mV. Pipettes contained standard solution as described under *Materials and Methods*. A to C, representative  $\text{K}^+$  current traces recorded with (right traces) and without (left traces) depolarizing prepulses and corresponding plot of bTREK-1 amplitudes with ( $\circ$ ) and without ( $\bullet$ ) depolarizing pulses. Time indicated on traces corresponds to those plotted on the graph at right. Cells were treated with 30  $\mu\text{M}$  8CPT-cAMP (A), 30  $\mu\text{M}$  Sp-8CPT-2'-OMe-cAMP (B), or 300  $\mu\text{M}$  Sp-8CPT-cAMP (C) for 48 h before recording. D, summary of experiments as in A to C. Bars represent bTREK-1 current density expressed as mean  $\pm$  S.E.M. of indicated number of determinations after 48-h exposure to 8CPT-cAMP (30  $\mu\text{M}$ ) or Sp-8CPT-2'-OMe-cAMP (30 or 300  $\mu\text{M}$ ) as indicated.

8CPT-Ado stimulated a concentration-dependent increase in bTREK-1 mRNA transcripts with a potency, temporal pattern, and effectiveness similar to that of other cAMP metabolites used in this study (Fig. 10B). In the experiment illustrated in Fig. 10B, 8CPT-Ado increased bTREK-1 mRNA at concentrations from 10 to 50  $\mu\text{M}$ . With respect to temporal pattern, adrenocorticotropin induced the expression of bTREK-1 mRNA more rapidly than 8CPT-Ado, but by 48 h, these two agents were similarly effective. In the experiment illustrated in Fig. 10C, at 48 h, 8CPT-Ado and adrenocorticotropin induced  $2.9 \pm 0.1$ - and  $2.4 \pm 0.1$ -fold increases in bTREK-1 transcript relative to the time-matched control.

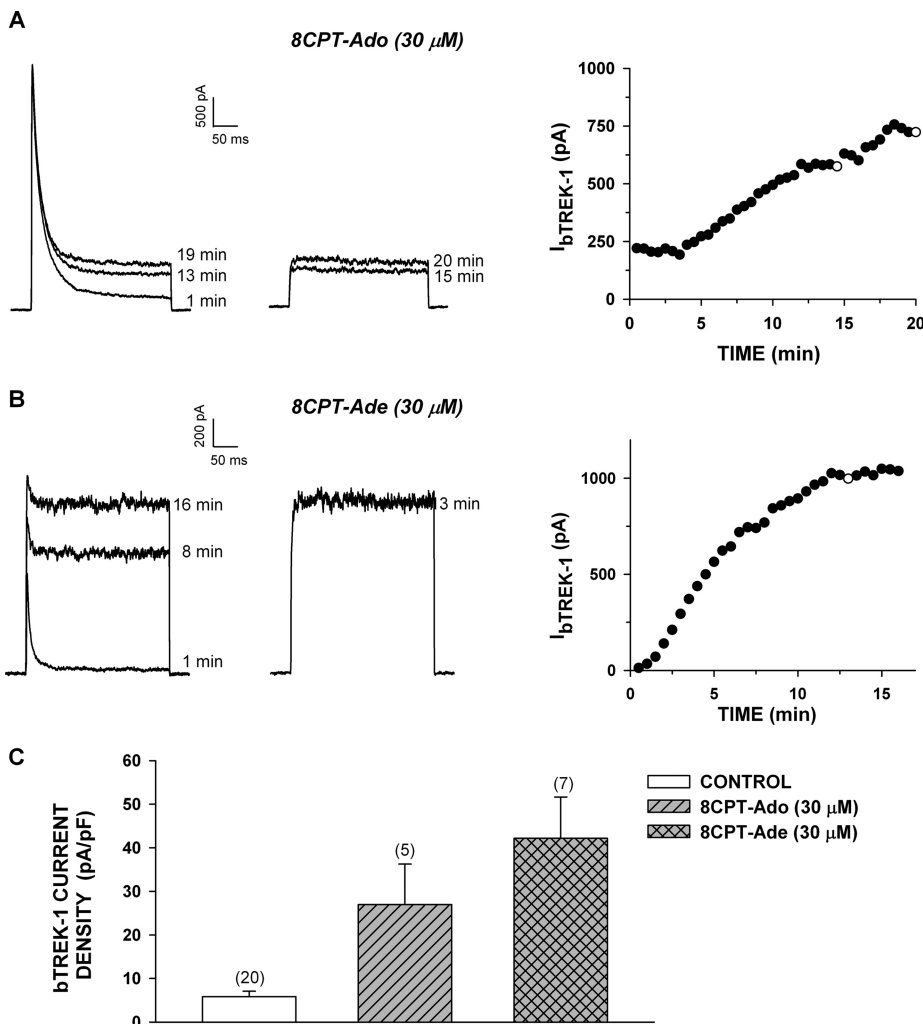
## Discussion

In this study, we presented evidence indicating that cAMP enhanced the expression of bTREK-1 mRNA and K<sup>+</sup> current and prevented their disappearance by activating PKA but not Epac2. Furthermore, hydrolysis products of 8-(4-chlorophenylthio)-cAMP analogs enhanced bTREK-1 mRNA transcripts and corresponding membrane current. The hydrolyzable 8-(4-chlorophenylthio)-cAMP analogs and their putative metabolites were significantly more potent than other cAMP analogs at promoting bTREK-1 expression. These metabolites all induced bTREK-1 with a potency, temporal pattern,

and effectiveness similar to that of the parent compound, but by an unknown cAMP-independent pathway.

Overall, we have identified 10 separate cAMP analogs and metabolites that enhanced the expression of bTREK-1 mRNA and associated K<sup>+</sup> current (Supplemental Table 1). For each of these, the effects on TREK-1 mRNA and current were well correlated. These results are consistent with the hypothesis that each of these agents increases the rate of transcription of the bTREK-1 gene. However, our results do not exclude the possibility that post-transcriptional mechanisms are involved, including bTREK-1 mRNA stabilization and translational or post-translational control by these compounds. It is unlikely that the cAMP-dependent and -independent actions of these on bTREK-1 are produced by a single common mechanism.

**cAMP, PKA, Epac2, and bTREK-1 Expression.** Experiments with 8-Br-cAMP and Sp-8CPT-cAMP clearly established that cAMP stimulates the expression of bTREK-1 mRNA and associated K<sup>+</sup> current. These experiments were necessary because our previous study demonstrating that 8CPT-cAMP induced bTREK-1 mRNA was completed without the knowledge that hydrolyzable 8-(4-chlorophenylthio) derivatives of cAMP induce bTREK-1 mRNA indirectly through metabolites (Enyeart et al., 2003). As a consequence, it was important to determine whether cAMP



**Fig. 9.** Metabolites of 8CPT-cAMP induce bTREK-1 current. AZF cells were cultured overnight then incubated either without (control) or with 8CPT-Ado (30  $\mu\text{M}$ ) or 8CPT-Ado (30  $\mu\text{M}$ ) as indicated. Whole-cell K<sup>+</sup> currents were recorded from AZF cells in response to voltage steps to +20 mV applied from -80 mV at 30-s intervals with or without depolarizing prepulses to -20 mV. Pipettes contained standard solution as described under *Materials and Methods*. A and B, representative K<sup>+</sup> current traces recorded with (right traces) and without (left traces) depolarizing prepulses and corresponding plot of bTREK-1 amplitudes with (○) and without (●) depolarizing pulses. Time indicated on traces corresponds to those plotted on graph at right. Cells were treated with 30  $\mu\text{M}$  8CPT-Ado (A) or 30  $\mu\text{M}$  8CPT-Ado (B) for 48 h before recording. C, summary of experiments as in A and B. Bars represent bTREK-1 current density expressed as mean  $\pm$  S.E.M. of indicated number of determinations after 48-h exposure to 8CPT-Ado or 8CPT-Ado as indicated.

could induce bTREK-1 expression, because adrenocorticotropin seems to produce effects in bovine AZF cells by cAMP-dependent and -independent mechanisms (Moyle et al., 1973; Yamazaki et al., 1998, 2006).

The robust stimulation of bTREK-1 mRNA and  $K^+$  current expression by 6-Bnz-cAMP suggests that the expression of this  $K^+$  channel is controlled at the pretranslational level by cAMP through the activation of PKA. However, several questions remain regarding the molecular mechanisms and signaling pathways by which 6-Bnz-cAMP functions.

First, with regard to molecular mechanism, adrenocorticotropin and cAMP induce increases in steroid hydroxylase mRNAs by accelerating the rate of gene transcription (John et al., 1986; Waterman, 1994). Our results are consistent with a similar effect of 6-Bnz-cAMP on bTREK-1 gene transcription. However, they do not rule out an effect of this cAMP analog on bTREK-1 mRNA stability.

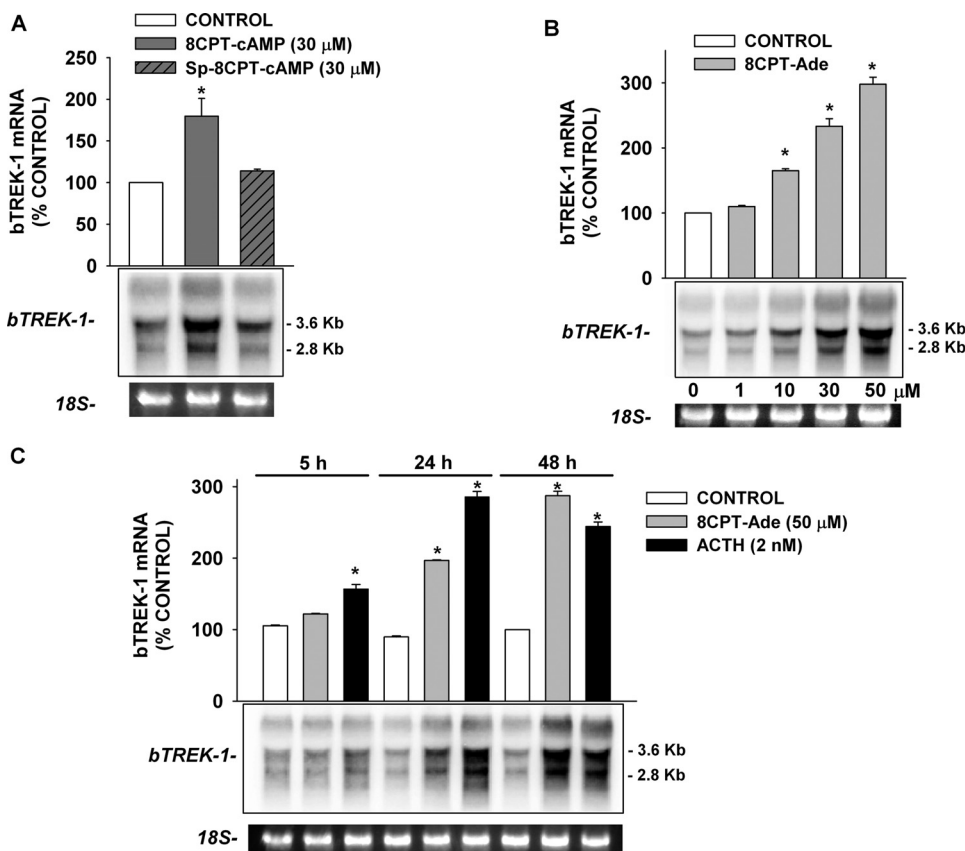
With respect to signaling mechanism, cAMP analogs substituted in the 6-position of the adenine ring selectively activate PKA over Epac proteins (Christensen et al., 2003). Therefore, our observation that 6-Bnz-cAMP robustly induced the expression of bTREK-1 mRNA and  $K^+$  current argue that cAMP-stimulated increases in the expression of this  $K^+$  channel were mediated through PKA rather than Epac2. However, several lines of evidence suggest that 6-Bnz-cAMP could function through a third unidentified cAMP receptor. First, when applied at concentrations that suppress PKA activity, H-89 only partially suppressed increases in bTREK-1 mRNA expression (Davies et al., 2000). Furthermore, in patch-clamp experiments, we found that, when included in the patch electrode at very low concentrations ( $<10 \mu\text{M}$ ), 6-Bnz-cAMP completely blocks

this activity of bTREK-1 channels, even in the presence of several PKA antagonists (Liu et al., 2009).

The steroidogenic actions of cAMP in AZF cells also suggest the presence of additional cAMP binding proteins. PKA-stimulated transcription typically occurs within minutes and does not require de novo protein synthesis (Parker and Schimmer, 1995). In contrast, cAMP-induced increases in steroid hydroxylase-specific mRNAs are cycloheximide-sensitive and require periods of up to several hours before they can be observed (Simpson and Waterman, 1988; Waterman and Simpson, 1989; Waterman, 1994). Furthermore, although the steroid hydroxylase genes are all induced by cAMP, they lack consensus cAMP response elements in their 5'-flanking regions and therefore do not bind PKA phosphorylated transcription factors (Simpson and Waterman, 1988; Ahlgren et al., 1990; Kagawa and Waterman, 1990; Lund et al., 1990; Parker and Schimmer, 1995; Payne and Hales, 2004).

In a number of other cells, cAMP synthesized in response to the activation of G protein-coupled receptors produces effects that are independent of PKA or Epac proteins (Buscà et al., 2000; Iacovelli et al., 2001; Fujita et al., 2002; Stork and Schmitt, 2002; Ivins et al., 2004). Overall, these results suggest that 6-Bnz-cAMP may induce the expression of bTREK-1 through a cAMP-dependent pathway in addition to PKA.

Experiments with the ESCAs 8CPT-2'-OMe-cAMP and its nonhydrolyzable analog Sp-8CPT-2'-OMe-cAMP clearly demonstrated that cAMP does not enhance the expression of bTREK-1  $K^+$  channels by activation of Epac2. Specifically, 8CPT-2'-OMe-cAMP induced bTREK-1 mRNA and  $K^+$  current at concentrations that produce little or no



**Fig. 10.** Effects of 8CPT-cAMP, Sp-cAMP, and 8CPT-Ade on expression of bTREK-1 mRNA. AZF cells were cultured overnight then incubated either without (control) or with 8CPT-cAMP, Sp-cAMP, 8CPT-Ade, or adrenocorticotropin as indicated. Total RNA was isolated as described under *Materials and Methods*. Membranes were hybridized with a specific probe for bTREK-1; bTREK-1 mRNA levels are expressed as a percentage of the 3.6-kb control band value. 18S rRNA bands from representative gels are shown as evidence of even loading. A, 8CPT-cAMP but not Sp-8CPT-cAMP induces bTREK-1 mRNA. AZF cells were either untreated (control, open bar) or treated with 30  $\mu\text{M}$  8CPT-cAMP (dark gray bar) or 30  $\mu\text{M}$  Sp-8CPT-cAMP (dark gray striped bar) for 48 h before isolating total RNA (\*,  $P < 0.02$ ). B, concentration-dependent effect of 8CPT-Ade on bTREK-1 mRNA. AZF cells were either untreated (control,  $\square$ ) or treated with 8CPT-Ade (1–50  $\mu\text{M}$ ,  $\blacksquare$ ) for 48 h before isolating total RNA (\*,  $P < 0.001$ ). C, time-dependent effect of 8CPT-Ade and adrenocorticotropin (ACTH) on bTREK-1 mRNA expression. AZF cells were either untreated (control,  $\square$ ) or treated with 30  $\mu\text{M}$  8CPT-cAMP ( $\blacksquare$ ) or 2 nM adrenocorticotropin ( $\blacksquare$ ) for 5, 24, and 48 h before isolating total RNA (\*,  $P < 0.001$ ).

activation of the Epac2 effector Rap1, whereas Sp-8CPT-2'-OMe-cAMP failed to increase bTREK-1 expression at higher concentrations shown previously to activate Rap1 in these cells (Enyeart and Enyeart, 2009). Thus, adrenocorticotropin- and cAMP-stimulated expression of bTREK-1 seems to be mediated by in part by PKA but independently of Epac2.

**8-(4-Chlorophenylthio)-cAMP Metabolites and bTREK-1 Expression.** Results of experiments with hydrolyzable and nonhydrolyzable 8-(4-chlorophenylthio)-cAMP analogs strongly suggest that the hydrolyzable cAMP analogs stimulated bTREK-1 expression indirectly through metabolites. Accordingly, we found that a total of five potential metabolites of 8CPT-cAMP and 8CPT-2'-OMe-cAMP each induced the expression of bTREK-1 mRNA transcripts and ion current with potency and effectiveness similar to those of the parent compounds. Enzymes that could catalyze the conversion of these two cAMP derivatives to each of these putative metabolites are expressed in mammalian cells. These include cyclic nucleotide phosphodiesterases, 5'-nucleotidases, hypoxanthine phosphoribosyltransferase, and nucleotide phosphorylases (Price and Stevens, 1999).

The active metabolites, associated receptor, and signaling pathways that mediate the increases in bTREK-1 gene expression have not been identified. It is possible that each of the five metabolites stimulates bTREK-1 expression. In this regard, it is interesting to note that both 8CPT-cAMP and the ESCA 8CPT-2'-OMe-cAMP can be converted to the same 8-(4-chlorophenylthio)-adenine derivative (Fig. 5). It will be important to determine whether 8CPT-Ade is the final common active metabolite.

Regardless of the identity of the active metabolite(s), it is clear that it does not function by activating a cAMP-dependent mechanism. First, although the hydrolyzable 8-(4-chlorophenylthio)-cAMP analogs and their metabolites at low concentrations all induced bTREK-1 expression, none of these down-regulated the Kv1.4 current whose expression is inhibited by a PKA-dependent mechanism (Enyeart et al., 2000). In contrast, 8-Br-cAMP and 6-Bnz-cAMP, as well as adrenocorticotropin, at concentrations that induced bTREK-1 all inhibited the expression of Kv1.4 current (Figs. 1, 2, 3, 4, and 9). Furthermore, we showed previously that neither 8CPT-2'-OMe-cAMP, 8CPT-2'-OMe-Ado, nor 8CPT-Ade activates PKA when applied to bovine AZF cells (Enyeart and Enyeart, 2009). Finally, in the current study, we demonstrated that a nonhydrolyzable ESCA failed to induce bTREK-1 expression, even at concentrations that have been shown to activate Epac2.

The findings of this study are consistent with the possibility that cAMP metabolites induce bTREK-1 by activation of a cAMP-independent pathway that is also activated by adrenocorticotropin. An *O*-nitrophenyl-derivative of adrenocorticotropin, *O*-nitrophenylsulfenyl-adrenocorticotropin, blocks the activity of bTREK-1 K<sup>+</sup> channels, induces increases in [Ca<sup>2+</sup>]<sub>i</sub>, and stimulates large increases in cortisol synthesis at concentrations that produce little or no increase in cAMP synthesis (Moyle et al., 1973; Yamazaki et al., 1998; Liu et al., 2008). Although these effects are mediated through the MC2R receptor, the cAMP-independent signaling pathway has not been identified.

We have shown previously that metabolites of 8CPT-2'-OMe-cAMP induced large, delayed increases in the expres-

sion of genes coding for steroidogenic proteins, resulting in corresponding increases in cortisol synthesis (Enyeart and Enyeart, 2009). With respect to kinetics and concentration-dependence, the effects of the metabolites on the expression of steroidogenic proteins resemble those on bTREK-1, suggesting a common mechanism.

The range of genes whose expression might be modulated by this novel signaling pathway is yet to be determined. It is clear that the presumptive metabolites did not produce a nonspecific global increase in RNA synthesis. Total RNA synthesis was not increased in metabolite-treated cells. Expression of specific genes, including the transcriptional repressor Dax-1 and Kv1.4, were not increased by the metabolites (J. A. Enyeart and J. J. Enyeart, unpublished observations).

This and other studies demonstrate that adrenocorticotropin and cAMP exert long-term control over the electrical properties of AZF cells by regulating the expression of genes that code for ion channels (Enyeart et al., 2000, 2003). Adrenocorticotropin may function through cAMP-dependent and independent mechanisms. In this regard, it is possible that adrenocorticotropin and metabolites of 8-(4-chlorophenylthio)-cAMP analogs may induce the expression of genes coding for bTREK-1 K<sup>+</sup> channels and steroidogenic proteins by activating a common, but yet to be identified, cAMP-independent pathway.

The extent to which metabolites of 8-(4-chlorophenylthio)-cAMP derivatives regulate gene expression and cell function in other tissues and organisms is unknown. Hydrolysis products of selected cAMP analogs transform the protozoa *Trypanosoma brucei* from slender to stumpy-like forms (Laxman et al., 2006). It is possible that the effect of these metabolites in distantly related eukaryotes might be mediated through a common ancient signaling pathway.

Finally, 8CPT-cAMP analogs, including 8CPT-cAMP and 8CPT-2'-OMe-cAMP, have been used in hundreds of studies to determine the roles of cAMP, PKA, and Epac proteins in cell signaling. The findings of our study indicate that the results of some of these previous studies may require re-evaluation.

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