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# N6-substituted cAMP Analogs Inhibit bTREK-1 $K^+$ Channels and Stimulate Cortisol Secretion by a PKA-Independent Mechanism

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**Running Title**: 6-Bnz-cAMP Inhibits bTREK-1 by a PKA-independent mechanism

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**List of non-standard abbreviations**: AZF – adrenal zona fasciculata, 6-Bnz-cAMP - 6-benzoyl-cAMP, 6-MB-cAMP - 6-monobutyrl-cAMP, ACTH - adrenocorticotropic hormone, PKA-cAMP-dependent protein kinase, Epac -exchange proteins activated by cAMP, CaMK - calmodulin-dependent protein kinase, BAPTA - 1,2 *bis*-(2-aminophenoxy)ethane-*N,N,N',N''*-tetraacetic acid, DMEM - Dulbecco's modified eagle medium, DMEM/F12+ - DMEM/F12 (1:1) containing 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and the antioxidants 1 μM tocopherol, 20 nM selenite and 100 μM ascorbic acid, FBS, fetal bovine sera, $IC_{50}$ - the concentration that produces 50% inhibition of the response.

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## ABSTRACT

Bovine adrenal zona fasciculata (AZF) cells express bTREK-1 $K^+$ channels whose inhibition by cAMP is coupled to membrane depolarization and cortisol secretion through complex signaling mechanisms. cAMP analogs with substitutions in the 6 position of the adenine ring selectively activate cAMP-dependent protein kinase (PKA), but not exchange proteins activated by cAMP (Epacs). In whole cell patch clamp recordings from AZF cells, we found that 6-benzoyl-cAMP (6-Bnz-cAMP) and 6-monobutyryl-cAMP (6-MB-cAMP) potently inhibit bTREK-1 $K^+$ channels, even under conditions where PKA activity was abolished. Specifically, when applied through the patch electrode, 6-Bnz-cAMP inhibited bTREK-1 with an $IC_{50}$ of less than 0.2 μM. Inhibition of bTREK-1 by 6-Bnz-cAMP was not diminished by PKA antagonists including H-89, Rp-cAMPS, PKI(6-22) amide, and myristoylated PKI(14-22) applied alone or in combination, externally and intracellularly through the patch pipette. Under similar conditions, these same antagonists completely blocked PKA activation by 6-Bnz-cAMP. Inhibition of bTREK-1 by 6-Bnz-cAMP was voltage-independent and eliminated in the absence of ATP in the pipette solution. 6-Bnz-cAMP also produced delayed increases in cortisol synthesis and the expression of CYP11a1 mRNA that were only partially blocked by PKA antagonists. These results indicate that 6-Bnz-cAMP and other 6-substituted cAMP analogs can inhibit bTREK-1 $K^+$ channels and stimulate delayed increases in cortisol synthesis by AZF cells through a PKA- and Epac-independent mechanism. They also suggest that ACTH and cAMP function in these cells through a third cAMP-dependent protein. Finally, although 6-modified cAMP analogs exhibit high selectivity in activating PKA over Epac, they also may interact with other unidentified proteins expressed by eukaryotic cells.

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## INTRODUCTION

Bovine adrenal zona fasciculata (AZF) cells express bTREK-1 $K^+$ channels that set the resting membrane potential and couple ACTH receptor activation to membrane depolarization (Mlinar et al., 1993;Enyeart et al., 1996;Enyeart et al., 2002;Enyeart et al., 1993). ACTH inhibits bTREK-1 channels through both PKA-dependent and independent signaling pathways (Enyeart et al., 1996;Liu et al., 2008).

In addition to PKA, bovine AZF cells express the cyclic AMP-activated guanine nucleotide exchange factor Epac2 (also known as cAMP-GEFII) which is found in a limited number of tissues (Kawasaki et al., 1998;Liu et al., 2008). The binding site for cAMP on the Epac proteins differs from that of PKA (Christensen et al., 2003). Using this information and rational drug design, novel cAMP analogues have been developed that activate Epac proteins, but not PKA (Enserink et al., 2002;Holz et al., 2008). In patch clamp experiments, bTREK-1 is inhibited by the Epac activator 8CPT-2'-OMe-cAMP even under conditions where PKA has been completely inhibited (Liu et al., 2008).

These results demonstrate that cAMP inhibits bTREK-1 $K^+$ channels through at least two different signaling pathways. However, they do not exclude the possibility that bTREK-1 inhibition by cAMP could be mediated by pathways independent of both PKA and Epac2. In bovine adrenal glomerulosa cells, cAMP has been reported to activate calmodulin-dependent protein kinase (CaMK) and stimulate aldosterone secretion through a PKA- and Epac-independent mechanism(Gambaryan et al., 2006). In these same cells, aldosterone secretion is coupled to membrane depolarization by bTREK-1 inhibition (Enyeart et al., 2004).

It has been discovered that cAMP analogs modified at the 6 position of the adenine ring, including 6-Bnz-cAMP and 6-MB-cAMP, activate PKA but not Epac proteins, as measured in

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vitro and in several types of cultured cells (Christensen et al., 2003;Rehmann et al., 2003). cAMP derivatives, including 6-Bnz-cAMP and 6-monobutyryl-cAMP (6-MB-cAMP) can effectively discriminate between PKA- and Epac-dependent actions of cAMP. It isn't known if these cAMP derivatives interact with other cAMP-dependent proteins that may be present in cells. In whole cell patch clamp recordings from bovine AZF cells, we found that 6-Bnz-cAMP and 6-MB-cAMP potently inhibited bTREK-1 channels under conditions where PKA activity was blocked. Further, 6-Bnz-cAMP stimulated large, delayed increases in cortisol secretion and the expression of CYP11a1 steroid hydroxylase mRNA, both of which were only partially inhibited by PKA antagonists.

## MATERIALS AND METHODS

*Materials* - Tissue culture media, antibiotics, fibronectin, and fetal bovine sera (FBS) were obtained from Invitrogen (Carlsbad, CA). Coverslips were from Bellco (Vineland, NJ). Phosphate-buffered saline (PBS), enzymes, 1,2 *bis*-(2-aminophenoxy)ethane-*N,N,N',N''*-tetraacetic acid (BAPTA), MgATP, collagenase, DNase, H-89, and ACTH (1-24), UTP, and Rp-cAMPS were obtained from Sigma (St. Louis. MO). PKI (6-22) amide and PKI(14-22)myristoylated were purchased from EMD Biosciences, Inc. (San Diego, CA). 6-Bnz-cAMP and 6-MB-cAMP were purchased from Axxora,LLC (San Diego, CA). SignaTect cAMP-dependent protein kinase (PKA) assay system was from Promega (Madison,WI). [$^{32}$P]ATP and [$^{32}$P]dCTP were purchased from Perkin Elmer (Waltham, MA). Cortisol EIA (DSL-10-200) kit was from Diagnostic Systems Laboratories (Webster, TX). Ultrahyb was purchased from Ambion (Austin, TX) and Prime-It II kit for random priming was from Stratagene (La Jolla, CA).

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*Isolation and Culture of AZF Cells* - Bovine adrenal glands were obtained from steers (age 2-3 yr) at a local slaughterhouse. Isolated AZF cells were obtained and prepared as previously described (Enyeart et al., 1997). After isolation, cells were either resuspended in DMEM/F12+ (DMEM/F12 with 10 % FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and the antioxidants α-tocopherol (1 μM), selenite (20 nM) and ascorbic acid (100 μM)) and plated for immediate use, or resuspended in FBS/5% DMSO, divided into 1 ml aliquots, and stored in liquid nitrogen for future use.. For patch clamp experiments, cells were plated in DMEM/F12+ in 35 mm dishes containing 9 $mm^2$ glass coverslips. To ensure cell attachment, coverslips were treated with fibronectin (10 μg/ml) at 37$^oC$ for 30 minutes then rinsed with warm, sterile PBS immediately before adding cells. Cells were maintained at 37$^oC$ in a humidified atmosphere of 95% air-5% $CO_2$.

*Patch Clamp Experiments*- Patch clamp recordings of $K^+$ 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 $CaCl_2$, 2 mM $MgCl_2$, 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 $CaCl_2$, 2 mM $MgCl_2$, 11 mM BAPTA, 10 mM HEPES, 5 mM MgATP, and 200 μM GTP, with pH titrated to 6.8 using KOH.

*Recording Conditions and Electronics*- AZF cells were used for patch clamp experiments 2–12 h after plating. Typically, cells with diameters <15 μm and capacitances of 10–15 pF were selected. 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 3–5 ml/min. For whole cell recordings,

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patch electrodes with resistances of 1.0–2.0 MΩ were fabricated from Corning 0010 glass (World Precision Instruments, Sarasota, FL). These electrodes routinely yielded access resistances of 1.5–4.0 MΩ and voltage-clamp time constants of <100 µs. $K^+$ currents were recorded at room temperature (22–25 °C) according to the procedure of Hamill *et al.* (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 Digidata 1200 interface (Molecular Devices, Sunnyvale, CA). Currents were digitized at 2–10 KHz after filtering with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using summed scaled hyperpolarizing steps of ½ to ¼ pulse amplitude. Data were analyzed using CLAMPFIT 9.2 (Molecular Devices, Sunnyvale, CA) and SigmaPlot (version 10.0) software. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

*PKA Assay*- AZF cells were plated on 60-mm fibronectin-treated dishes in DMEM/F12+ at a density of ~ 4 x $10^6$ cells/dish. After 24 h, the cells were washed 4X with ice-cold PBS, and suspended in 500 µl of cold extraction buffer (25 mM Tris-HCl pH 7.4, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.5 mM Pefabloc-SC (Roche Applied Science, Indianapolis, IN), and protease inhibitors with EDTA (Complete Mini protease inhibitor cocktail tablet, 1 per 10 mls lysis solution, Roche Applied Science, Indianapolis, IN). Lysates were homogenized using a cold Dounce homogenizer then centrifuged for 5 min at 4 ºC at 14,000 X *g*. 5 µl samples of lysate supernatant were assayed using the SignaTECT cAMP-Dependent Protein Kinase Assay system (Promega, Madison, WI). This kit uses PKA-dependent phosphorylation of biotinylated

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peptides as a measure of PKA activity. Each experimental condition was assayed in quadruplicate.

*Cortisol Assay*

Media from experiments was either assayed immediately after collection or frozen (-20 °C) until all samples were available. Cortisol secretion by AZF cells was measured in duplicate from duplicate experimental media samples using a Cortisol EIA (DSL-10-200) from Diagnostic Systems Laboratories (Webster, TX), according to the manufacturer's directions. If necessary, media samples were diluted using DMEM/F12+.

*Northern Blot and Measurement of mRNA*

Total RNA isolation and Northern blot procedures have been described previously (Enyeart et al., 2003). Briefly, 5-7 X $10^6$ AZF cells were plated on 60-mm fibronectin-treated dishes in DMEM/F12+. After 24 h, the serum-supplemented media were removed and replaced with DMEM/F12 containing PKA inhibitors, 6-Bnz-cAMP alone, or 6-Bnz-cAMP plus PKA inhibitors as required. Samples incubated with both 6-Bnz-cAMP plus PKA inhibitors were pre-incubated for 10 min with PKA inhibitors before addition of 6-Bnz-cAMP. At the end of the incubation period, total RNA was extracted using RNeasy columns (Qiagen, Valencia, CA), electrophoresed on a denaturing gel, and transferred to nylon transfer membrane (GeneScreen Plus, PerkinElmer Life Sciences, Waltham, MA). Probe was labeled with [$\alpha$-$^{32}$P]dCTP by random primer labeling (Prime-It II, Stratagene, La Jolla, CA). Probe was generated by RT-PCR using AMV reverse transcriptase (Promega, Madison WI), specific primers, and total RNA isolated from bovine AZF cells as described above. Specific probe for CYP11a1 was generated

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and included bases 679-1816 of NM_176644. Northern autoradiograms were imaged using a Typhoon 9200 variable mode imager and quantitated using ImageQuant TL v2003.3 software (GE Healthcare Life Sciences, Piscataway, NJ).

*Transient Transfection and Visual Identification of HEK293 Cells Expressing bTREK-1* - For patch clamp recording of bTREK-1 currents, HEK293 cells were co-transfected with a mixture of pCR®3.1-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 (Life Technologies). The p3-CD8 clone was kindly provided by Dr. Brian Seed, Department of Genetics, Massachusetts General Hospital, Boston,MA. Cells were visualized 1-2 days post transfection after a 15 min incubation with anti-CD8 antibody-coated beads (Dynal Biotech Inc., Lake Success, NY) as described (Jurman et al., 1994).

## RESULTS

*Effect of 6-Bnz-cAMP and ACTH on PKA Activity and bTREK-1 Current*

Bovine AZF cells express two types of $K^+$ channels: a voltage-gated, rapidly inactivating Kv1.4 channel and a two pore domain, four transmembrane spanning segment bTREK-1 background $K^+$ channel (Mlinar and Enyeart, 1993;Mlinar et al., 1993). In whole cell patch clamp recordings, bTREK-1 amplitude typically increases with time to a steady-state maximum. The absence of time- and voltage-dependent inactivation allows bTREK-1 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 $K^+$ current has fully

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inactivated. 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 (Figure 1A).

cAMP analogs modified in the 6-position of the adenine ring directly activate PKA. In vitro, 6-Bnz-cAMP activates PKA at submicromolar concentrations (Christensen et al., 2003;Poppe et al., 2008). However, in whole cell patch clamp recordings from bovine AZF cells, external application of 6-Bnz-cAMP (100 µM) for 10 min failed to reduce bTREK-1, while ACTH (200 pM) produced near complete inhibition within 3-5 min in the same experiments (Figure 1A,B). Overall, 6-Bnz-cAMP inhibited bTREK-1 by 5.2 ± 2.5 % (n=5) while ACTH inhibited bTREK-1 by 94.3 ± 1.7 % (n=6).

The failure of 6-Bnz-cAMP (100 µM) to inhibit bTREK-1 indicated that, in these experiments, this cAMP derivative did not reach the intracellular concentration necessary to activate PKA. In contrast to ACTH which generates cAMP intracellularly through the activation of adenylate cyclase, 6-Bnz-cAMP is transported across the cell membrane at a rate determined by its lipophilicity and diffusion constant (Pusch and Neher, 1988). The continuous dialysis of the cell with pipette solution in whole cell recordings constantly dilutes the cytoplasm, reducing the intracellular concentration of 6-Bnz-cAMP. Consequently, to further assess bTREK-1 inhibition by 6-Bnz-cAMP, this agent was applied intracellularly through the patch pipette. When applied through this route, 6-Bnz-cAMP potently and selectively suppressed the time-dependent expression of bTREK-1 with an $IC_{50}$ of less than 0.2 µM (Figure 2A-D). In contrast, the voltage-gated Kv1.4 current was not affected (Figure 2B).

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*PKA Inhibitors Do Not Block bTREK-1 Inhibition by 6-Bnz-cAMP*

When applied intracellularly through the patch pipette, 6-Bnz-cAMP potently inhibited bTREK-1. Experiments were done to determine whether bTREK-1 inhibition by the PKA-specific cAMP analog was mediated solely by PKA. 6-Bnz-cAMP (300 μM) produced a large increase in the PKA activity in AZF cells. H-89 and PKI (14-22) myristoylated are potent membrane permeable PKA antagonists (Hidaka et al., 1991;Glass et al., 1989). When AZF cells were preincubated for one hour with H-89 (10 μM) and PKI (14-22) myristoylated (4 μM), the large increase in PKA activity induced by 6-Bnz-cAMP (300 μM) was completely blocked (Figure 3A, left panel).

When added to cytoplasmic extracts of AZF cells, 6-Bnz-cAMP potently activated PKA at concentrations nearly identical to those that inhibited bTREK-1. At a concentration of 5 μM, 6-Bnz-cAMP activated nearly all of the available PKA in AZF cell lysates (Figure 3A, right panel). PKI(6-22) amide is a synthetic peptide patterned after a portion of the naturally occurring PKA inhibitory peptide. It inhibits PKA by binding to the substrate site with a reported $IC_{50}$ less than 2 nM (Glass et al., 1989). When added to cytoplasmic extracts from AZF cells, PKI(6-22) amide (4 μM), in combination with H-89 (10 μM), completely abolished PKA activation by 6-Bnz-cAMP (Figure 3A, right panel).

These same PKA inhibitors failed to suppress bTREK-1 inhibition by 6-Bnz-cAMP (1-30 μM) (Figure 3B). In these experiments, AZF cells were pre-exposed to PKI(14-22) myristoylated (4 μM) and H-89 (5 or 10 μM) for 15-60 min before recording with pipette solutions containing 6-Bnz-cAMP (1-30 μM), PKI(6-22)amide, and H-89. Thus, under conditions where PKA activation was abolished, 6-Bnz-cAMP continued to potently inhibit bTREK-1 activity. In particular, in the presence of the PKA inhibitors, 6-Bnz-cAMP at a

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concentration of only 1 μM reduced bTREK-1 current density from 87.4 ± 17.0 pA/pF (n=9) to 8.1 ± 2.3 pA/pF (n=12) (Figure 3B).

In whole cell recordings, intracellularly-applied 6-Bnz-cAMP suppressed bTREK-1 expression even when the AZF cells had been pre-incubated with a PKA inhibitor and the patch electrode also contained multiple inhibitors. To further demonstrate that 6-Bnz-cAMP inhibited bTREK-1 under conditions where PKA activity had been totally blocked in advance, AZF cells were sequentially patched with a pipette containing the PKA antagonists followed by one containing these antagonists as well as 6-Bnz-cAMP. As previously reported, AZF cells could often be consecutively patched with two pipettes with little or no decrease in the bTREK-1 current (Liu et al., 2008). However, when the second pipette contained 6-Bnz-cAMP (5 μM) bTREK-1 was rapidly inhibited (Figure 3C). Overall, in these double patch experiments, 6-Bnz-cAMP (5 μM) inhibited bTREK-1 current by 99.2 ± 0.4 % (n= 3).

In addition to 6-Bnz-cAMP, a second cyclic AMP derivative with a substitution at the 6-position of the adenine ring also potently inhibited bTREK-1 through a PKA-independent mechanism. 6-MB-cAMP (1 μM) nearly completely inhibited the expression of bTREK-1 current in the absence and presence of PKA inhibitors H-89 and PKI (6-22) amide in the pipette solution (Figure 4A).

Adenosine 3'-5'cyclic monophosphothiate, Rp-isomer (Rp-cAMPS) competitively inhibits cAMP activation of PKA, but not Epac, in living cells (Holz et al., 2008;Poppe et al., 2008). The presence of Rp-cAMPS (500 μM) in the pipette solution failed to blunt the near-complete inhibition of bTREK-1 activity by 1 or 5 μM 6-Bnz-cAMP, providing further evidence for PKA-independent inhibition of bTREK-1 by 6-Bnz-cAMP (Figure 4B).

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Rp-cAMPS (500 μM) also failed to alter inhibition of bTREK-1 by 6-Bnz-cAMP in twice-patched cells wherein PKA is pre-inhibited by a pipette containing Rp-cAMPS (500 μM) before recording currents with a separate pipette containing both Rp-cAMPS and 6-Bnz-cAMP (Figure 4C). In 5 similar experiments, 6-Bnz-cAMP inhibited bTREK-1 by 84.5 ± 7.1 % when PKA had been pre-inhibited with Rp-cAMPS.

PKI(6-22) amide, H-89, and Rp-cAMPS each inhibit PKA by separate mechanisms (Glass et al., 1989;Hidaka et al., 1991;Dostmann, 1995). When all three of these inhibitors were added to the pipette solution at concentrations many times higher than their reported $IC_{50}$s, they failed to suppress the inhibition of bTREK-1 by 6-Bnz-cAMP, even when this cAMP derivative was applied at concentrations as low as 1 μM (Figure 5A).

Results of experiments with multiple PKA inhibitors provided compelling evidence that 6-Bnz-cAMP and 6-MB-cAMP can inhibit bTREK-1 by a PKA-independent mechanism. cAMP has bee reported to activate other kinases in adrenocortical cells and cell lines. cAMP activates calmodulin-dependent protein kinase (CaMK) in bovine adrenal zona glomerulosa cells by a PKA-independent mechanism (Gambaryan et al., 2006). The addition of the specific calmodulin antagonist KN-93 (5 μM) to the pipette solution in addition to the PKA inhibitors H-89 and PKI(6-22) amide did not blunt bTREK-1 inhibition by 6-Bnz-cAMP (Figure 5B). cAMP has also been reported to activate the MAP kinase pathway in adrenocortical cells (Gyles et al., 2001). However, the potent MAP kinase inhibitor U0126 (Favata et al., 1998) also failed to diminish bTREK-1 inhibition by 6-Bnz-cAMP (Figure 5B).

The PKA- CaMK-, and MAPK-independent inhibition of bTREK-1 by 6-Bnz-cAMP could occur through activation of another unidentified kinase. If so, then this inhibition should be prevented by substituting UTP for ATP in the patch pipette. UTP promotes bTREK-1

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channel activity even though it does not function as a substrate for protein kinases (Enyeart et al., 1997). UTP (2 mM) markedly suppressed the inhibition of bTREK-1 expression by 6-Bnz-cAMP in whole-cell recordings (Figure 5C). With 2 mM UTP in the patch pipette, maximum bTREK-1 current density was reduced by 5 μM 6-Bnz-cAMP from 97.1 ± 16.6 pA (n=5) to 74.7 ± 59.2pA (n=7) (5C). Interestingly, in the presence of UTP, 6-Bnz-cAMP slowed the time-dependent increase in bTREK-1 expression. These results indicate a requirement for hydrolyzable ATP in the inhibition of bTREK-1 activity by 6-Bnz-cAMP.

*bTREK-1 Inhibition by 6-Bnz-cAMP is Voltage-independent*

Our results indicated that 6-Bnz-cAMP can inhibit bTREK-1 by a PKA-independent mechanism. The PKA-dependent inhibition of hippocampal TREK-1 by cAMP was reported to occur through a mechanism that converted TREK-1 from a voltage-insensitive open leak channel into a voltage-gated outward rectifier (Bockenhauer et al., 2001). In this study, upon phosphorylation by PKA, TREK-1 channel open probability was markedly reduced at negative, but not positive, test potentials.

To determine whether 6-Bnz-cAMP converted native bTREK-1 channels into voltage-gated channels, we recorded bTREK-1 over a wide range of test potentials in twice-patched cells, first in the absence and then in the presence of 6-Bnz-cAMP. In these experiments, bTREK-1 currents were permitted to grow to a stable value before recording currents with voltage steps between -60 and +40 mV, and then voltage ramps between -100 mV and +100 mV (Figure 6). $K^+$ currents were then recorded from the same cell with a second pipette containing 6-Bnz-cAMP (10 μM) using the same voltage protocols. 6-Bnz-cAMP selectively inhibited bTREK-1 almost completely at every test potential, whether this current was activated by voltage steps or ramps

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(Figure 6B,C). These results demonstrate that 6-Bnz-cAMP does not inhibit bTREK-1 solely by converting it to a voltage-gated channel through PKA-dependent phosphorylation.

*Inhibition of bTREK-1 by 6-Bnz-cAMP in Transfected Cells*

The potent inhibition of native bTREK-1 channels in bovine AZF cells by 6-Bnz-cAMP in the presence of multiple PKA inhibitors indicates that these cells express a yet to be identified cAMP effector coupled to bTREK-1 inhibition. To determine whether this 6-Bnz-cAMP-activated pathway was specific to AZF cells, HEK293 cells were transfected with bTREK-1.

As in AZF cells, 6-Bnz-cAMP potently inhibited bTREK-1 channels expressed in HEK293 cells (Figure 7A). In contrast to AZF cells, the bTREK-1 current in HEK293 was typically quite large upon initiating whole cell recording and this current continued to increase in size over many minutes. However, when the recording pipette contained 6-Bnz-cAMP (1 μM), the $K^+$ current amplitude rapidly decreased to a new steady-state value (Figure 7A). The relatively small remaining $K^+$ current included an unknown fraction of endogenous current (Figure 7A). The inhibitory effect of 6-Bnz-cAMP on the activity of bTREK-1 channels expressed in HEK293 cells was insensitive to Rp-cAMPS (500 μM) or PKI (6-22) (4 μM) and H-89 (10 μM) in combination. At a concentration of 1 μM, 6-Bnz-cAMP was equally effective at inhibiting bTREK-1 in the absence or presence of these antagonists (Figure 7B).

*6-Bnz-cAMP Stimulates Cortisol Secretion and CYP11a1 Hydroxylase Gene Expression by PKA-dependent and independent Mechanisms*

Patch clamp experiments indicated that 6-Bnz-cAMP can inhibit bTREK-1 $K^+$ channels by a PKA-independent mechanism. Since bTREK-1 inhibition by cAMP has been linked to

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cortisol secretion, experiments were done to determine whether 6-Bnz-cAMP could also stimulate cortisol secretion from AZF cells through a mechanism that did not require PKA activation. As illustrated in Figure 8A, 6-Bnz-cAMP (200 μM) stimulated almost no increase in cortisol synthesis, measured at 1 and 3 h, but induced a large 33 fold increase by 24 h. Under conditions where protein kinase inhibitors produced near complete inhibition of PKA activity, the delayed increases in cortisol secretion were only partially blocked. In the experiment illustrated in Figure 8B, 6-Bnz-cAMP-stimulated cortisol secretion was reduced by only 40 ± 7%.

The delayed increases in cortisol synthesis induced by ACTH and cAMP are mediated through increases in the expression of genes coding for steroid hydroxylases (Simpson and Waterman, 1988;Waterman, 1994). CYP11a1 is a mitochondrial steroid hydroxylase that catalyzes the conversion of cholesterol to pregnenolone, the rate-limiting step in cortisol synthesis (Waterman, 1994;Payne and Hales, 2004). 6-Bnz-cAMP also stimulated pronounced increases in CYP11a1 mRNA by a mechanism that was largely insensitive to PKA antagonists. In the same experiment as that illustrated in Figure 8A, 6-Bnz-cAMP increased CYP11a1 mRNA to 439 % of its time-matched control after 24 h. The PKA antagonists reduced this response by only 8.5% (Figure 8C).

## DISCUSSION

In whole cell patch clamp recordings from bovine AZF cells, we found that cAMP analogs with substitutions at the 6 position of the adenine ring, when applied through the patch electrode, potently inhibited bTREK-1 $K^+$ channels at sub-micromolar concentrations. Further, although at these low concentrations 6-Bnz-cAMP and 6-MB-cAMP specifically activate PKA,

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but not Epac proteins, both inhibited bTREK-1, under conditions where PKA was shown to be completely inhibited. Further, in the presence of PKA inhibitors, 6-Bnz-cAMP induced a marked increase in cortisol synthesis and the expression of CYP11a1 mRNA. These results indicate that, in addition to Epac2 and PKA, bovine AZF cells express an additional cAMP-activated protein that is instrumental in regulating their electrical and secretory properties.

Although when applied through the patch pipette 6-Bnz-cAMP inhibited bTREK-1 almost completely, it was ineffective when applied externally at 100 fold higher concentrations. This disparity in potency that depends on the site of application almost certainly reflects the failure of the extracellularly applied drug to achieve concentrations comparable to those obtained when it is applied directly to the cytoplasm through pipette solution. When applied directly through the pipette solution, the intracellular concentration would approach that in the pipette within seconds (Pusch and Neher, 1988). Assuming that 6-Bnz-cAMP does not easily exit the cell through the plasma membrane, this concentration would remain constant since the pipette solution approximates an infinite reservoir. In contrast, when applied externally, the steady-state intracellular concentration would depend on the net inward transport, determined by the diffusion constant and lipid solubility, as well as the dilution produced by the constant dialysis of the cytoplasm by the pipette solution (Pusch and Neher, 1988). Accordingly, when applied externally, high concentrations of the lipid-soluble cAMP analog 8CPT-cAMP are required to inhibit bTREK-1 (Enyeart et al., 1996). By activating adenylate cyclase, ACTH generates a constant supply of intracellular cAMP that does not need to cross the cell membrane to reach its target. Further cAMP activates both PKA and Epac2. It is therefore much more effective at inhibiting bTREK-1.

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*PKA-independent Inhibition of bTREK-1 by 6-Bnz-cAMP*

When applied intracellularly through the patch electrode, 6-Bnz-cAMP potently inhibited bTREK-1 with an $IC_{50}$ of approximately 0.1 μM, while raising the concentration to 1 μM was sufficient to produce near maximal inhibition. 6-Bnz-cAMP was slightly less potent at activating PKA when applied directly to AZF cell lysates.

These results show that 6-Bnz-cAMP is a potent PKA activator in AZF cells and that at a concentration as low as 1 μM, it could produce significant inhibition of bTREK-1 through activation of this enzyme. However, the failure of multiple specific PKA antagonists to alter the inhibition of bTREK-1 by even low concentrations of 6-Bnz-cAMP indicate that, in bovine AZF cells, cAMP can inhibit bTREK-1 through a PKA- and Epac2-independent mechanism. In this regard, there appears to be little doubt that PKA was totally inhibited by the PKA antagonists in the patch clamp experiments. H-89, Rp-cAMPS, and PKI (6-22) amide each inhibit PKA by different mechanisms (Hidaka et al., 1991;Botelho et al., 1988;Cheng et al., 1986). Each was used at concentrations 10-1000 fold greater than their reported $IC_{50}$s. In AZF cell lysates, these antagonists completely inhibited 6-Bnz-cAMP-induced increases in PKA activity. Finally, preincubating cells with the PKA antagonists and the use of the "double-patch" technique insured that PKA was inhibited before 6-Bnz-cAMP was applied intracellularly.

Overall, the combined results reported in this and previous studies indicate that ACTH can inhibit bTREK-1 in bovine AZF cells by three cAMP-dependent pathways. These include a PKA-induced phosphorylation at serine 333 in the carboxyterminal domain, an Epac2-dependent mechanism, and an unknown ATP hydrolysis-dependent mechanism (Patel et al., 1998;Enyeart and Enyeart, 2009;Honoré, 2007). Apparently, the novel pathway can be activated by cAMP derivatives with additions at the 6-position of the adenine ring, but not by Epac-selective cAMP

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analogs. When Epac2 is down-regulated, the Epac activator 8CPT-2'-OMe-cAMP fails to inhibit bTREK-1 in AZF cells (Enyeart and Enyeart, 2009).

*TREK-1 Inhibition by 6-Bnz-cAMP is Voltage-independent*

The voltage-independent inhibition of bTREK-1 by 6-Bnz-cAMP provided additional evidence that this response was not mediated through PKA. Previous studies showed that inhibition of neuronal TREK-1 by PKA-dependent phosphorylation occurred through a rightward shift in the voltage-dependent activation of these channels (Bockenhauer et al., 2001). Consequently, open probability was reduced only at relatively negative test potentials. In contrast, we found that 6-Bnz-cAMP inhibited bTREK-1 equally, even at test potentials up to +100 mV. Thus, it appears that bTREK-1 inhibition by 6-Bnz-cAMP can occur through a voltage- and therefore PKA-independent mechanism..

*bTREK-1 Inhibition by 6-Bnz-cAMP in Transfected Cells*

The potent inhibition of bTREK-1 channels by 6-Bnz-cAMP expressed in HEK293 cells in the presence of PKA antagonists indicated that these cells also express an alternative mechanism for bTREK-1 inhibition by cAMP. Interestingly, the Epac-selective cAMP agonist 8CPT-2'-OMe-cAMP doesn't inhibit bTREK-1 expressed in HEK293 cells, a result that is consistent with the observed absence of Epac2 expression in these cells (Liu et al., 2008). These findings also indicate that 6-Bnz-cAMP, but not 8CPT-2'-OMe-cAMP can activate this third, unidentified mechanism for bTREK-1 inhibition by cAMP. This conclusion is supported by our previous observation that 8CPT-2'-OMe-cAMP does not inhibit bTREK-1 in AZF cells where Epac2 expression has been down-regulated (Liu et al., 2008).

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*PKA-independent Cortisol Secretion by 6-Bnz-cAMP*

The stimulation of delayed increases in cortisol secretion and CYP11a1 mRNA expression by 6-Bnz-cAMP under conditions where PKA was inhibited is consistent with the findings of our patch clamp experiments and the hypothesis that this cAMP-derivative activates a protein in addition to PKA in AZF cells. They further suggest that ACTH and cAMP can induce the delayed component of cortisol synthesis through this unknown protein.

Further, although it has been marketed and used for years as a selective PKA antagonist, H-89 inhibits many protein kinases, some far more potently than PKA (Murray, 2008). Therefore, even the partial inhibition of 6-Bnz-cAMP-stimulated increases in cortisol synthesis by H-89 (10 μM) may have been due to effects on enzymes in addition to PKA.

*Molecular Mechanism for PKA-independent Actions of 6-Bnz-cAMP*

The signaling mechanism for PKA-independent inhibition of bTREK-1 and stimulation of cortisol secretion by 6-Bnz-cAMP is currently unknown. However, the requirement for ATP in bTREK-1 inhibition suggests the involvement of a protein kinase, ATPase or other energy-requiring enzyme. The failure of KN-93 and U0126 to block the inhibition of bTREK-1 by 6-Bnz-cAMP in the presence of PKA antagonists indicates that neither calmodulin kinase nor MAP kinase mediates this inhibition. However, the possibility that either of these kinases may be involved in 6-Bnz-cAMP-stimulated cortisol secretion is not ruled out. cAMP has been reported to stimulate steroid synthesis in mouse Y1 adrenal cortical cells by the activation of MAP kinase (Gyles et al., 2001).

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 (Stork and

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Schmitt, 2002;Busca et al., 2000;Iocovelli et al., 2001;Fujita et al., 2002;Ivins et al., 2004). These results indicate that other cAMP-binding proteins exist in eukaryotic cells in addition to PKA, Epac, and cAMP-modulated ion channels.

Finally, although cAMP analogs with substitutions in the 6 position of the adenine ring exhibit high selectivity in activating PKA over Epac, they also appear to potently interact with other unidentified proteins expressed by eukaryotic cells. Therefore, caution should be used when linking their effects exclusively to PKA.

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**FOOTNOTE:**

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## FIGURE LEGENDS

*Figure 1.* **Inhibition of bTREK-1 $K^+$ channels by ACTH and 6-Bnz-cAMP.** Whole cell $K^+$ currents were recorded from bovine AZF cells in response to voltage steps applied from -80 mV to +20 mV at 30 s intervals with or without depolarizing prepulses to -20 mV. After bTREK-1 reached a stable maximum amplitude, cells were superfused with 6-Bnz-cAMP (100 μM) or ACTH (200 pM), as indicated. A) $K^+$ current traces recorded with (right traces) or without (left traces) depolarizing prepulses and corresponding plot of bTREK-1 amplitudes with (open circles) or without (closed circles) depolarizing pulses. Numbers on traces correspond to those on plots. B) Summary of experiments as in (A). Values are mean ± SEM of bTREK-1 inhibition.

*Figure 2.* **Concentration-dependent inhibition of bTREK-1 by intracellular 6-Bnz-cAMP**. Whole cell $K^+$ currents were recorded from bovine AZF cells in response to voltage steps applied from -80 mV to +20 mV at 30 s intervals with or without depolarizing prepulses to -20 mV. Patch pipettes contained standard solution or the same solution supplemented with 6-Bnz-cAMP at concentrations from 0.2 to 30 μM. A,B) Time-dependent increase in bTREK-1 and inhibition by 6-Bnz-cAMP. Current traces recorded with (right) and without (left) depolarizing prepulses at indicated times. bTREK-1 amplitudes are plotted at right. Open circles on plots indicate traces recorded with depolarizing prepulse. C) Summary of experiments as in A,B. Bars indicate bTREK-1 current density in pA/pF expressed as the mean ± SEM of the indicated number of determinations.

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*Figure 3.* **Effect of PKA inhibitors on PKA activity and bTREK-1 inhibition by 6-Bnz-cAMP.** The effect of 6-Bnz-cAMP on PKA activity and bTREK-1 current expression was measured in the absence and presence of PKA inhibitors. A) Effect of 6-Bnz-cAMP and PKA inhibitors applied extracellularly (left) or to cell lysates (right) on PKA activity. Left: PKA activity was determined as described in the Methods after incubation either without (control, white bar), or with H-89 (10 μM) + myristoylated PKI(14-22) amide (4 μM) (striped white bar), 6-Bnz-cAMP (300 μM, grey bar) or 6-Bnz-cAMP after preincubation with H-89 and myristoylated PKI(14-22) amide for 60 min (grey, striped bar). Right: PKA activity was determined from AZF cell lysates with no addition (white bar), 6-Bnz-cAMP (0.2 to 5 μM, grey bars), or 6-Bnz-cAMP (1 and 5 μM) with H-89 (10 μM) and PKI (6-22) amide (4 μM) (grey, cross-hatched bars). B) Effect of PKA antagonists on bTREK-1 inhibition by 6-Bnz-cAMP. $K^+$ currents were recorded from AZF cells in response to voltage steps applied from -80 mV to +20 mV at 30 S intervals with or without depolarizing prepulses to -20 mV. AZF cells were preincubated for 15-60 min with H-89 (10 μM) + myristoylated PKI(14-22) (4 μM) before recording. Pipettes contained standard solution or the same solution supplemented with PKA(6-22)amide (4 μM) and H-89 ( 5 or 10 μM) and 6-Bnz-cAMP (1,5, or 30 μM). Current amplitudes are plotted against time at left. Bar graphs at right indicate bTREK-1 current density in pA/pF expressed as mean ± SEM. C) Effect of PKA inhibitors on bTREK-1 inhibition by 6-Bnz-cAMP in twice patched cells. $K^+$ currents were recorded as above. Cells were sequentially patched with two pipettes- the first contained PKI (6-22) amide and H-89. When bTREK-1 reached a stable amplitude, the first pipette was withdrawn and the cell patched with a pipette containing the antagonists and 6-Bnz-cAMP. Current traces and plots of bTREK-1 amplitude against time for cells patch clamped with pipettes containing the indicated additions. Pipette #1 (antagonists

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only, circles), Pipette #2 (antagonists plus 6-Bnz-cAMP, inverted triangles). Numbers on traces correspond to those on plots.

*Figure 4.* **PKA-independent inhibition of bTREK-1 channels by 6-MB-cAMP and Effect of Rp-cAMPS, PKI(6-22) amide and H-89 on bTREK-1 inhibition by 6-Bnz-cAMP.** A) Whole cell $^{K+}$ currents were recorded from AZF cells in response to voltage steps applied from -80 to +30 at 30 s intervals. AZF cells were pre-incubated for 15-60 min with H-89 (10 μM) and myristoylated PKI (14-22) amide (4 μM) before recording currents. Pipettes contained standard saline (control) or the same saline supplemented with 6-MB-cAMP (1 μM), or 6-MB-cAMP, H-89 (10 μM) and PKI (6-22) amide. Bars indicate bTREK-1 current density in pA/pF expressed as mean ± SEM of indicated number of determinations. B) Effect of Rp-cAMPS on bTREK-1 inhibition by 6-Bnz-cAMP. $K^+$ currents were recorded with pipettes containing standard solution (control) or the same solution supplemented with 6-Bnz-cAMP (1 or 5 μM) and Rp-cAMPS (500 μM). Current amplitudes are plotted against time. Bars at right indicate bTREK-1 current density in pA/pF expressed as the mean ± SEM of the indicated number of determinations. C) Effect of Rp-cAMPS on bTREK-1 inhibition by 6-Bnz-cAMP in twice patched cells. Cells were sequentially patched with two pipettes. When bTREK-1 reached a stable amplitude, the first pipette (Rp-cAMPS alone, circles) was withdrawn and the cell was patched with the second pipette (Rp-cAMPS plus 5 μM 6-Bnz-cAMP, inverted triangles). Numbers on traces correspond to those on plots.

*Figure 5.* **Effect of multiple kinase inhibitors or UTP on bTREK-1 inhibition by 6-Bnz-cAMP**. $K^+$ currents were recorded from AZF cells in response to voltage steps applied from -80

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mV to +20 mV at 30 s intervals with or without depolarizing prepulses to -20 mV. A) Effect of multiple PKA inhibitors on bTREK-1 inhibition by 6-Bnz-cAMP. $K^+$ currents were recorded with pipettes containing standard solution (control, circles) or the same solution supplemented with 6-Bnz-cAMP (1 μM), PKI(6-22)amide (4 μM), H-89 (10 μM) and Rp-cAMPS (500 μM) (triangles). Current amplitudes are plotted against time. Bars at right indicate bTREK-1 maximum current density in pA/pF expressed as mean ± SEM of indicated number of determinations. B) Effect of PKA, MAPK and CaMK antagonists on bTREK-1 inhibition by 6-Bnz-cAMP. $K^+$ currents were recorded with pipettes containing standard solution (control, circles), or the same solution supplemented with 6-Bnz-cAMP (1 μM), PKI (6-22) amide (4 μM), H-89 (10 μM), and KN-93 (5 μM) (open triangles), or 6-Bnz-cAMP (1 μM), PKI (6-22) amide (4 μM), H-89 (10 μM), and U0126 (10 μM) (closed triangles). Current amplitudes are plotted against time. Bars at right indicate bTREK-1 maximum current density in pA/pF expressed as mean ± SEM of indicated number of determinations. C) bTREK-1 inhibition by 6-Bnz-cAMP is ATP-dependent. $K^+$ currents were recorded with pipettes containing UTP (2 mM) in place of ATP, or this same solution plus 6-Bnz-cAMP (5 μM). bTREK-1 amplitudes are plotted against time at left in the absence (circles) and presence (triangles) of 6-Bnz-cAMP. Bars at right indicate maximum bTREK-1 current density in pA/pF expressed as mean ± SEM of indicated number of determinations.

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*Figure 6.* **bTREK-1 inhibition by 6-Bnz-cAMP is voltage independent.** Whole cell $K^+$ currents were recorded from AZF cells in response to voltage steps from -80 mV to +20 mV with a pipette containing standard solution. When bTREK-1 reached a stable maximum, $K^+$ currents were activated in response to voltage steps applied at 30 s intervals in 10 mV increments from a

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holding potential of -80 mV to test potentials from -60 to +40 mV or voltage ramps applied at 100 mV/s to potentials between +100 mV and -100 mV from a holding potential of 0 mV. $K^+$ currents were then recorded from the same cell using the same three voltage protocols with a second patch electrode that contained 6-Bnz-cAMP (10 μM). A) Current traces and plot of bTREK-1 amplitudes in response to voltage steps from -80 to +20 mV. Cell was sequentially patched with pipettes containing standard saline (circles) and then saline containing 6-Bnz-cAMP (10 μM, triangles). Numbers on traces correspond to those on plots. B) Left: Current traces recorded in response to voltage steps to test potentials from -60 to +40 mV in 10 mV increments in control saline and in the presence of 6-Bnz-cAMP (10 μM). Right: plot of current amplitudes against test potential for cell at left. C) Non-leak subtracted current traces recorded in response to ramp voltage steps between -100 and 100 mV at 0.5 mV/s before (black trace) and after superfusing cell with 6-Bnz-cAMP (10 μM, grey trace).

*Figure 7*. **6-Bnz-cAMP inhibits bTREK-1 channels expressed in HEK293 cells**. Whole cell $K^+$ currents were recorded from HEK293 cells that had been transiently transfected with pCR®3.1-Uni-bTrek-1 as described in the Methods. $K^+$ currents were activated by voltage steps to +20 mV applied at 30 s intervals from a holding potential of -80 mV. Patch pipettes contained standard solution, or the same solution supplemented with 6-Bnz-cAMP alone or in combination with Rp-cAMPS (500 μM) or PKI(6-22) amide (4 μM) and H-89 (10 μM). A) Effect of 6-Bnz-cAMP on bTREK-1 current in HEK293 cells. bTREK-1 current traces and associated plots of current amplitudes. Numbers on traces correspond to those on plot at right. B) 6-Bnz-cAMP and PKA inhibitors. Plots of bTREK-1 current amplitudes recorded with pipettes containing standard pipette solution (circles) or the same solution supplemented with 6-Bnz-cAMP and Rp-

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cAMPS (squares), or 6-Bnz-cAMP, PKI(6-22) amide and H-89 (triangles). C) Summary of experiments as in (A) and (B). Bars indicate maximum bTREK-1 current density expressed as pA/pF. Values are mean ± SEM for indicated number of determinations.

*Figure 8.* **6-Bnz-cAMP stimulates a delayed increase in cortisol secretion by a PKA-independent mechanism.** Bovine AZF cells were plated as described in the Methods. After 24 h, media was replaced with the same media with no addition (control), or with 6-Bnz-cAMP only, H-89 and myristoylated PKI(16-22), or 6-Bnz-cAMP plus H-89 and PKI(16-22) myristoylated, as indicated. A) Time-dependent effect of 6-Bnz-cAMP on cortisol secretion. Media was sampled and cortisol determined at 1, 3 and 24 h after treating AZF cells with 6-Bnz-cAMP (200 µM). B) Effect of PKA inhibitors on 6-Bnz-cAMP-induced cortisol secretion and CYP11a1 mRNA. Left: AZF cells were cultured in media containing no further additions (control, white bar), or with H-89 and PKI(16-22)myristoylated (white, cross-hatched bar), 6-Bnz-cAMP (200 µM, grey bar), or 6-Bnz-cAMP plus H-89 and PKI(16-22) myristoylated (grey, cross-hatched bars) for 24 hr before sampling media for cortisol determination as described in the Methods. Right: After media sampling, cells from experiment shown at left were lysed and total RNA analyzed by Northern blot. Blots were hybridized with specific probe for bovine CYP11a1. Summary of mRNA values from densitometric analysis are presented as mean ± SEM of 3 independent determinations. A representative Northern blot is shown for the set of experiments with 18S rRNA bands from gel shown as evidence of even loading.

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FIGURE 1

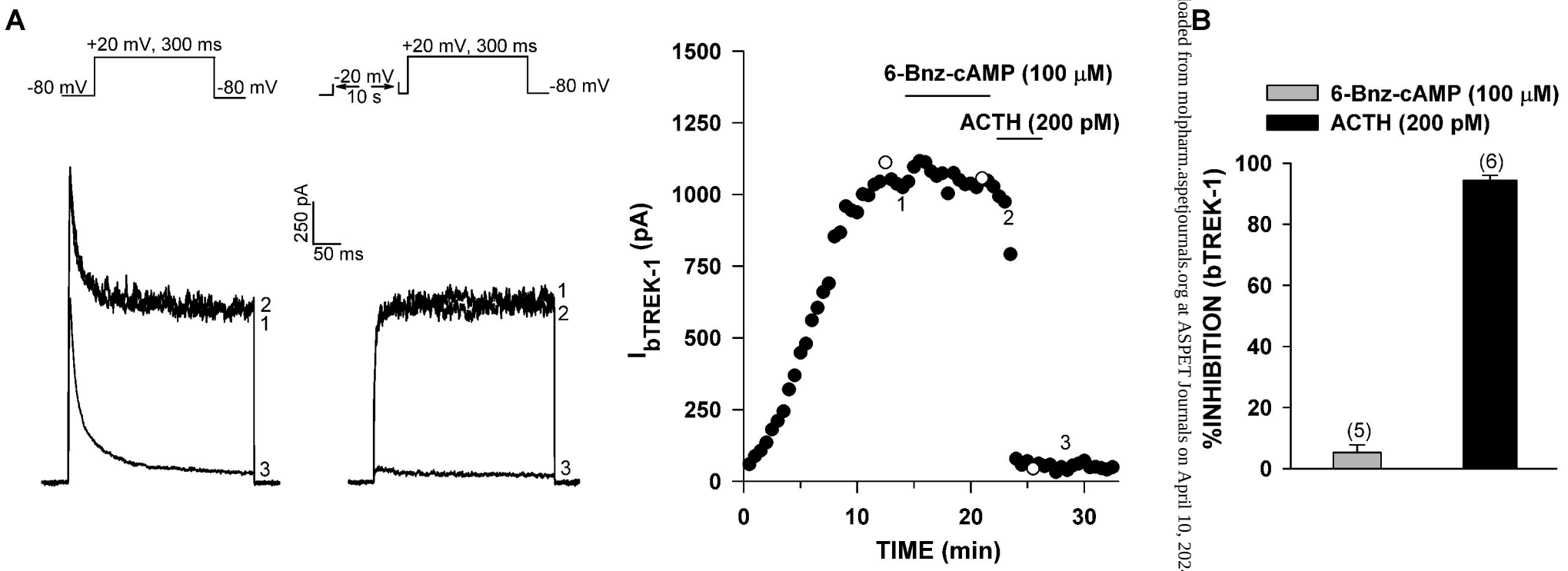


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FIGURE 2

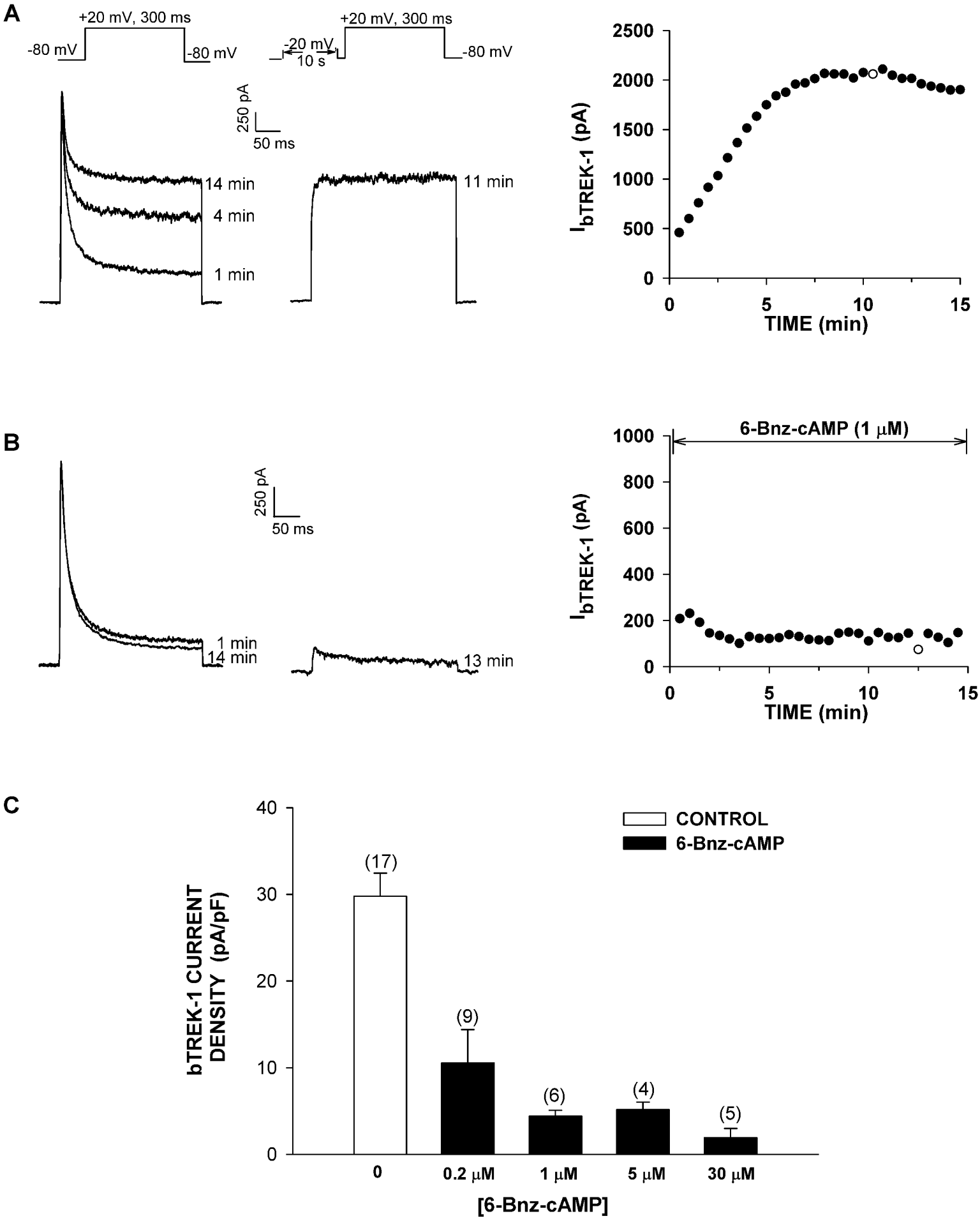


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FIGURE 3

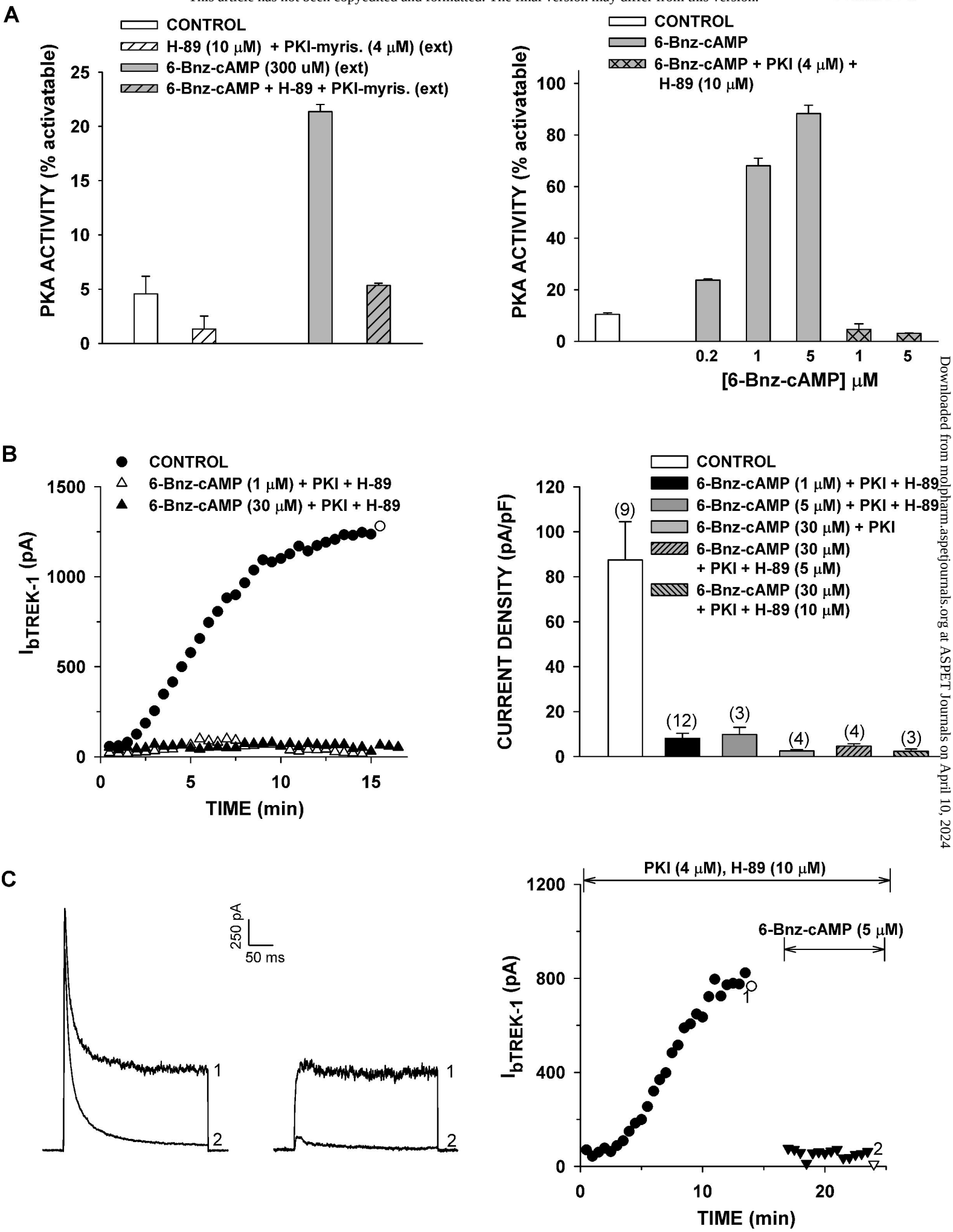


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FIGURE 4

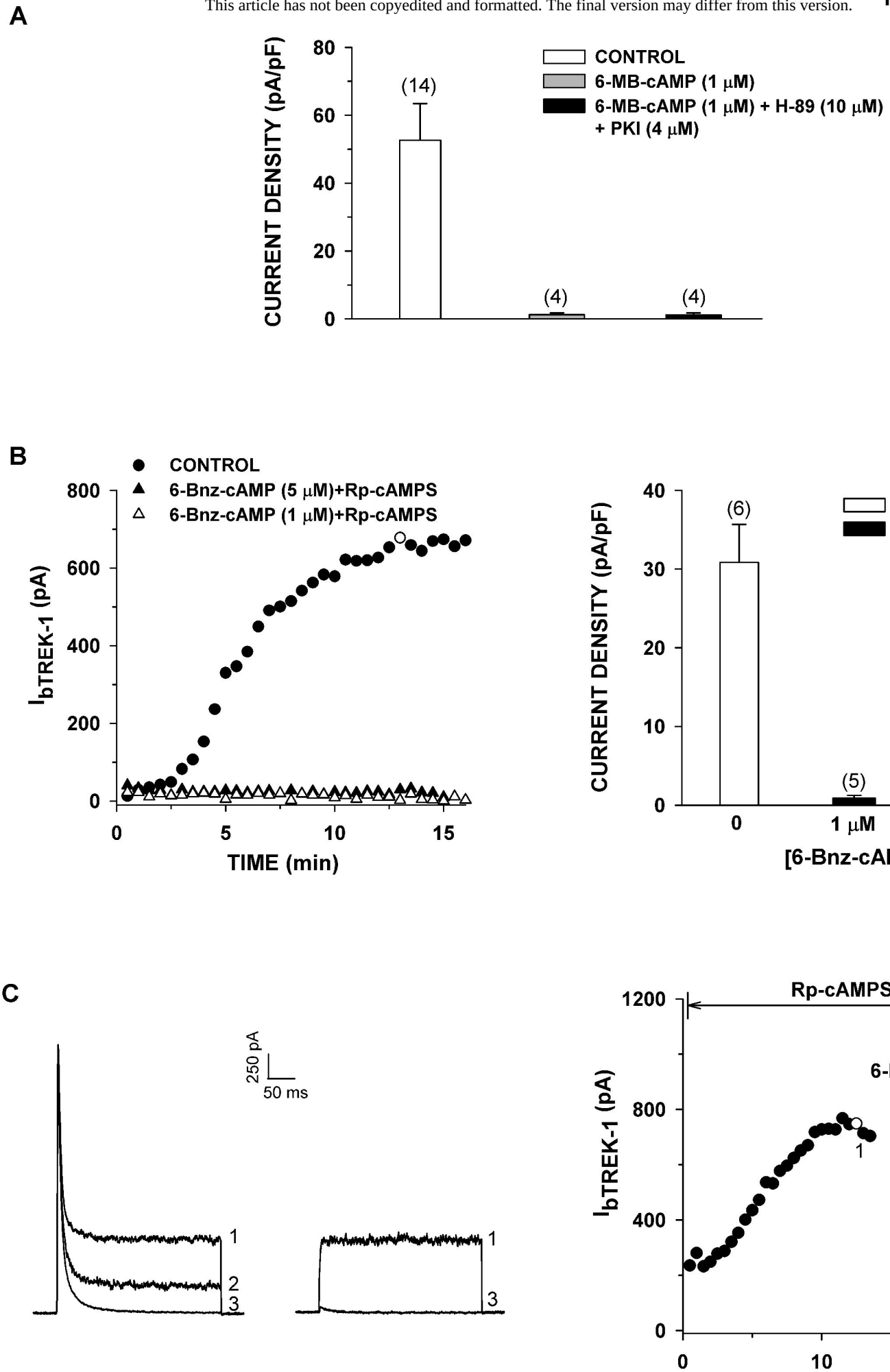


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FIGURE 5

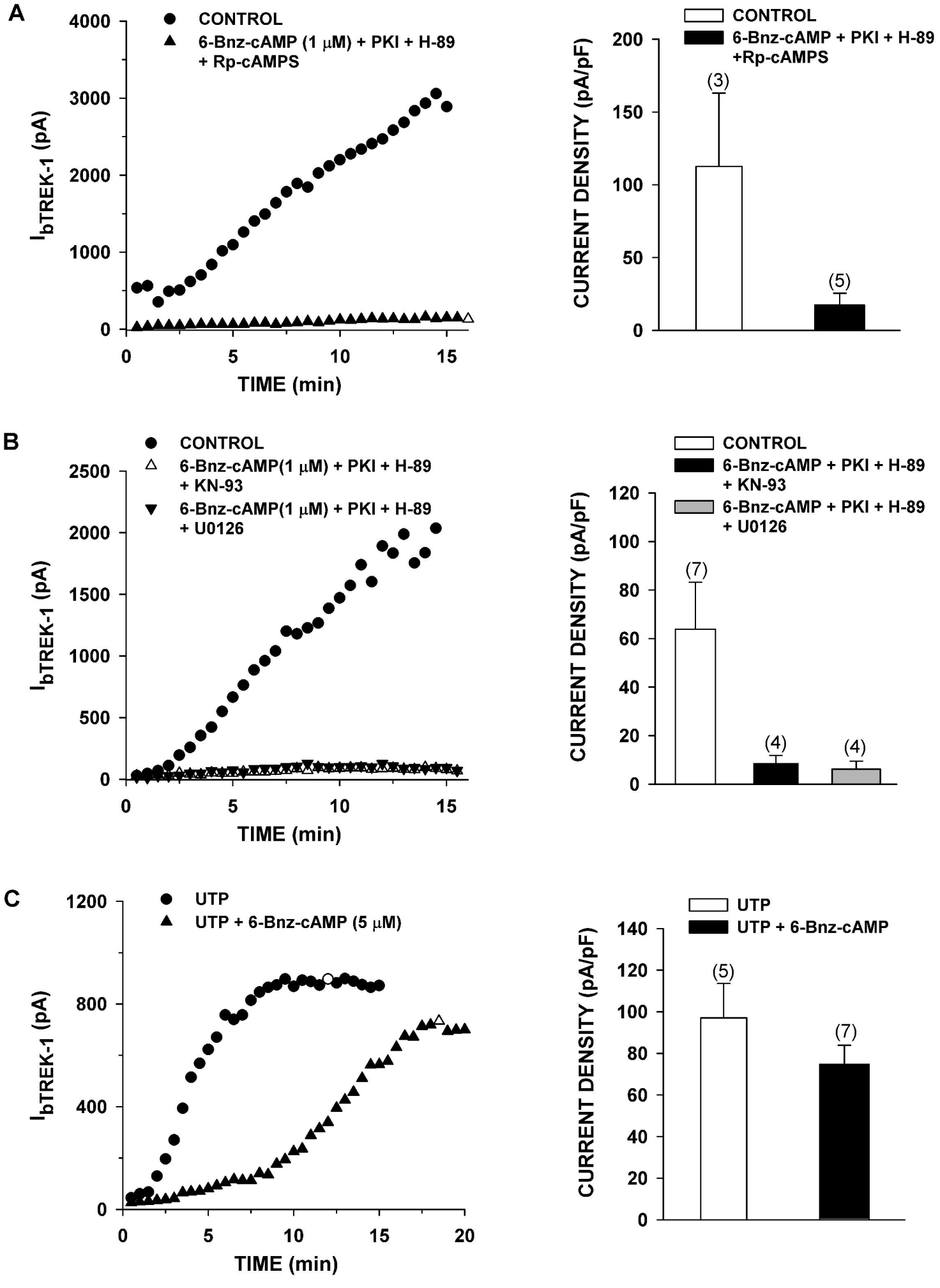


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FIGURE 6

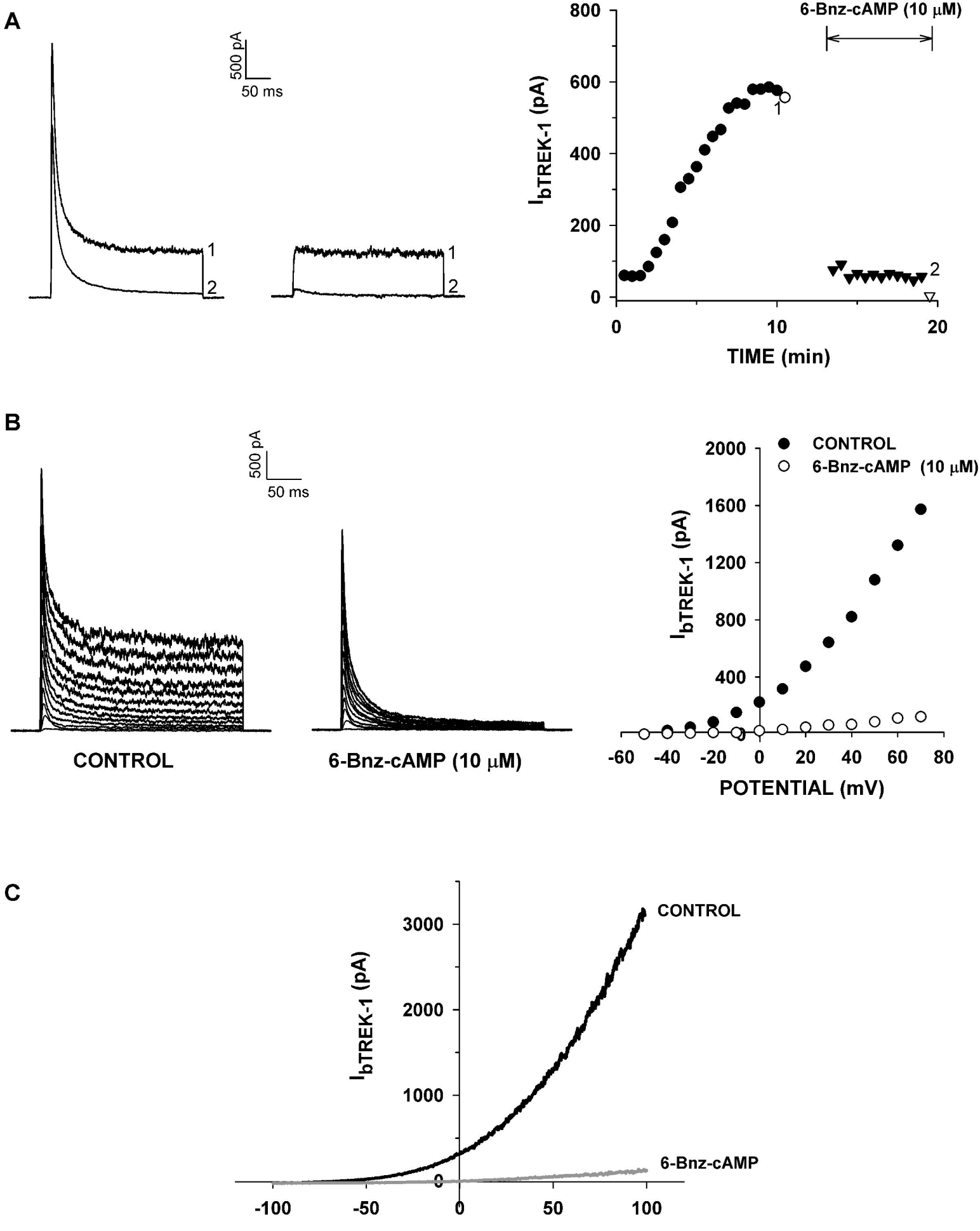


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FIGURE 7

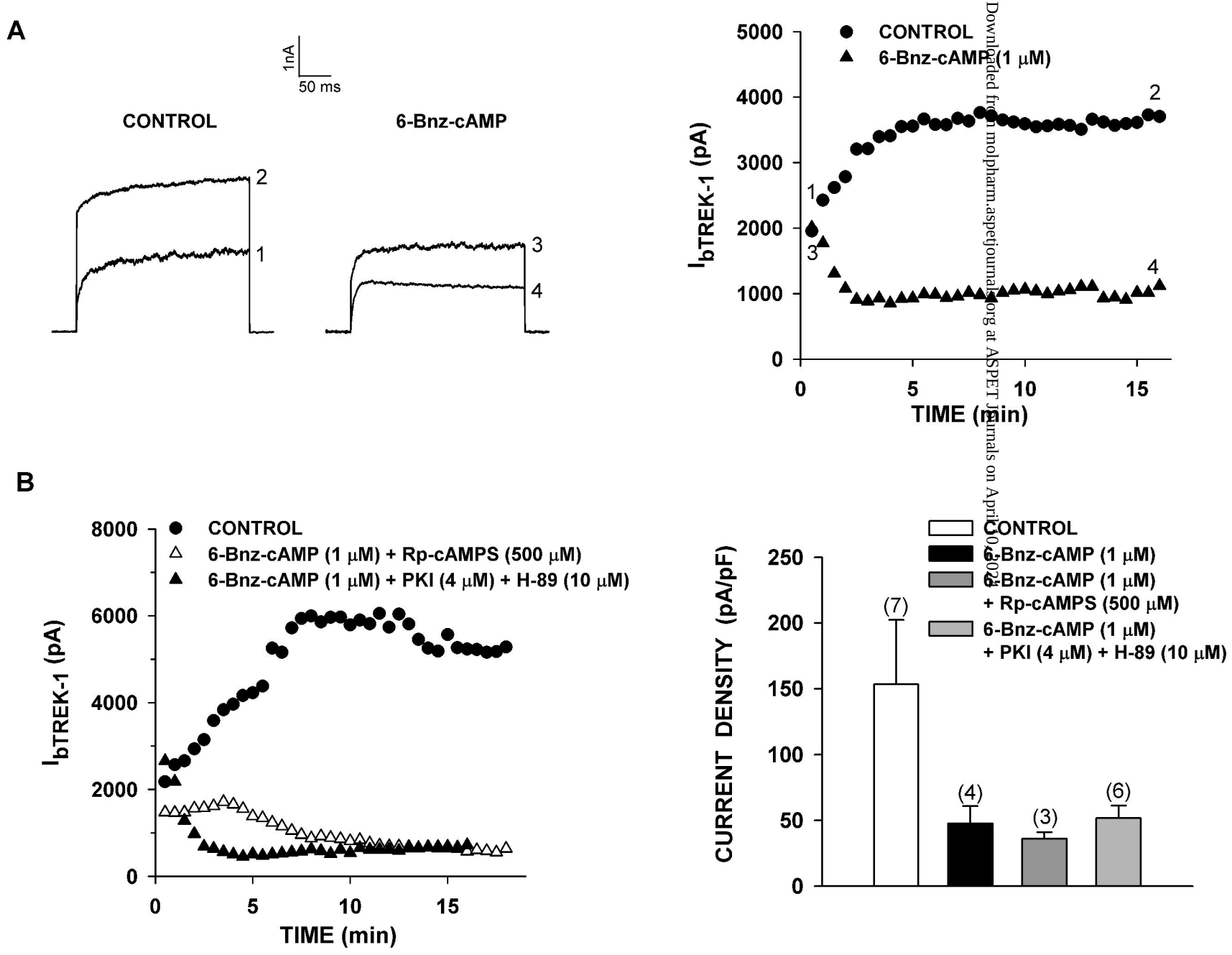


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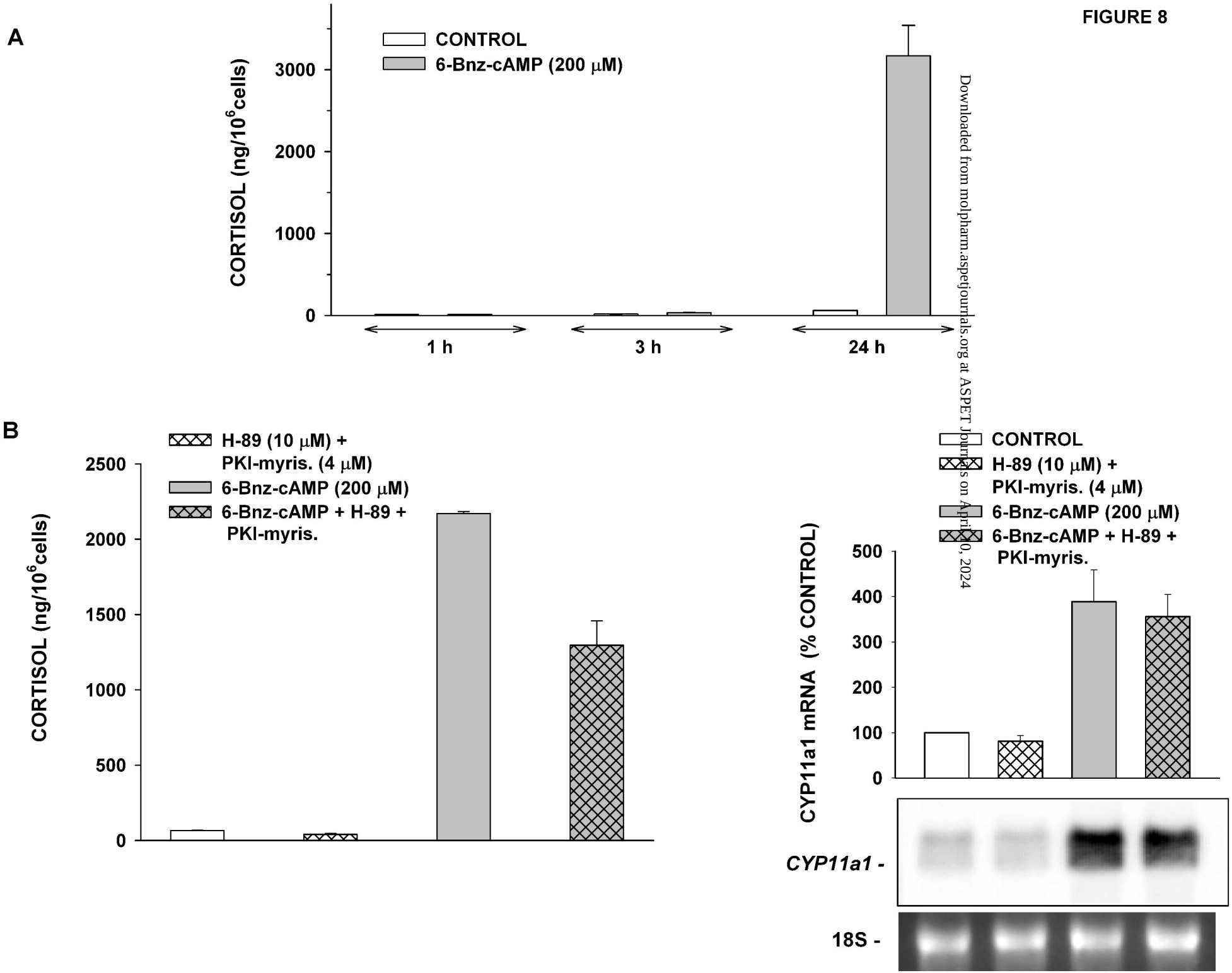
FIGURE 8

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