Bovine adrenal zona fasciculata (AZF) cells express Cav3.2 T-type Ca\(^{2+}\) channels that function pivotally in adrenocorticotropic hormone (ACTH)-stimulated cortisol secretion. The regulation of Cav3.2 expression in AZF cells by ACTH, cAMP analogs, and their metabolites was studied using Northern blot and patch clamp recording. Exposing AZF cells to ACTH for 3–6 days markedly enhanced the expression of Cav3.2 current. The increase in Cav3.2 current was preceded by an increase in corresponding CACNA1H mRNA. O-Nitrophenylsulfenyl-adrenocorticotropin, which produces a minimal increase in cAMP, also enhanced Cav3.2 current. cAMP analogs, including 8-bromoadenosine cAMP (600 \(\mu\)M) and 6-benzoyladenosine cAMP (300 \(\mu\)M) induced CACNA1H mRNA, but not Cav3.2 current. In contrast, 8-(4-chlorophenylthio) (8CPT)-cAMP (10–50 \(\mu\)M) enhanced CACNA1H mRNA and Cav3.2 current, whereas nonhydrolyzable Sp-8CPT-cAMP failed to increase either Cav3.2 current or mRNA. Metabolites of 8CPT-cAMP, including 8CPT-adenosine and 8CPT-adenine, increased Cav3.2 current and mRNA with a potency and effectiveness similar to the parent compound. The Epac activator 8CPT-2'-O-methyl-cAMP and its metabolites 8CPT-2'-OMe-5'-AMP and 8CPT-2'-O-methyl-adenosine increased CACNA1H mRNA and Cav3.2 current; Sp-8CPT-2'-O-methyl-cAMP increased neither Cav3.2 current nor mRNA. These results reveal an interesting dichotomy between ACTH and cAMP with regard to regulation of CACNA1H mRNA and Ca\(^{2+}\) current. Specifically, ACTH induces expression of CACNA1H mRNA and Cav3.2 current in AZF cells by mechanisms that depend at most only partly on cAMP. In contrast, cAMP enhances expression of CACNA1H mRNA but not the corresponding Ca\(^{2+}\) current. Surprisingly, chlorophenylthio-cAMP analogs stimulate the expression of Cav3.2 current indirectly through metabolites. ACTH and the metabolites may induce Cav3.2 expression by the same, unidentified mechanism.

Bovine adrenal zona fasciculata (AZF)\(^{2}\) cells express low voltage-activated T-type Ca\(^{2+}\) channels and bTREK-1 K\(^{+}\) channels that function pivotally in ACTH-stimulated cortisol secretion (1–4). bTREK-1 K\(^{+}\) channels set the resting potential of these AZF cells. ACTH receptor activation is coupled to bTREK-1 inhibition, membrane depolarization, T-type Ca\(^{2+}\) channel activation, and cortisol secretion (2, 3, 5).

ACTH-stimulated cortisol production includes both rapid and delayed components (6, 7). The rapid effects of ACTH on cortisol secretion occur within minutes and do not require the synthesis of new proteins. In contrast, delayed increases occur only after a period of hours and involve transcriptional activation of genes coding for steroid hydroxylases and other steroidogenic proteins (6–8).

Parallel rapid and delayed responses to ACTH occur in the regulation of ion channel function and gene expression in AZF cells. The rapid actions of ACTH leading to depolarization-dependent Ca\(^{2+}\) entry occur in seconds or minutes and are mediated by the inhibition of pre-existing bTREK-1 channels (3, 9). Treating AZF cells with ACTH for prolonged periods also regulates bTREK-1 gene expression (10).

Three types of low voltage-activated T-type Ca\(^{2+}\) channels have been identified with molecular cloning and patch clamp techniques (11). One of these, Cav3.2, is coded for by the CACNA1H gene and is distinctive in its sensitivity to Ni\(^{2+}\) (12). The T-type Ca\(^{2+}\) channel in bovine AZF cells is blocked by Ni\(^{2+}\) with an IC\(_{50}\) of 20 \(\mu\)M, characteristic of Cav3.2 (1). In whole cell patch clamp recordings, ACTH has no rapid effect on the activity of pre-existing Cav3.2 channels (supplemental Fig. S1). It is not known whether ACTH exerts long term control over CACNA1H gene expression in these cells.

The signaling mechanisms by which ACTH produces rapid and delayed effects in the adrenal cortex are complex. Early studies identified cAMP as the principal intracellular messenger for ACTH in AZF cells (13–16). Accordingly, bovine AZF cells express a high affinity MC2R melanocortin receptor coupled to adenylate cyclase through G\(_{\alpha}\) (17, 18). Until recently, nearly all of the actions of cAMP in eukaryotic cells were explained by adenylate cyclase through G\(_{\alpha}\) (17, 18). Until recently, nearly all of the actions of cAMP in eukaryotic cells were believed to be mediated through the activation of PKA. However, two cAMP-activated guanine nucleotide exchange factors (Epac1 and Epac2 also known as cAMP-GEF1 and cAMP-GEF2) (19) have been identified as alternative mechanisms of cAMP action, especially in vertebrate systems.

The caveolin family of proteins (Cav1, Cav2, Cav3) play a critical role in the regulation of T-type Ca\(^{2+}\) channels. The Cav3.2 subfamily of T-type Ca\(^{2+}\) channels is encoded by the CACNA1H gene and is distinctive in its sensitivity to Ni\(^{2+}\) (12). The T-type Ca\(^{2+}\) channel in bovine AZF cells is blocked by Ni\(^{2+}\) with an IC\(_{50}\) of 20 \(\mu\)M, characteristic of Cav3.2 (1). In whole cell patch clamp recordings, ACTH has no rapid effect on the activity of pre-existing Cav3.2 channels (supplemental Fig. S1). It is not known whether ACTH exerts long term control over CACNA1H gene expression in these cells.

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8-bromoadenosine 3',5'-cyclic monophosphosphate; 6-Bnz-cAMP, N\(^{6}\)-benzoyladenosine 3',5'-cyclic monophosphate; 8CPT-2'-OMe-cAMP, 8-(4-chlorophenylthio)-2'-O-methyl-cAMP; Sp-8CPT-2'-OMe-cAMP, 8-(4-chlorophenylthio)-2'-O-methyl-cAMP, Sp-isomer; 8CPT-2'-OMe-5'-AMP, 8-(4-chlorophenylthio)-2'-O-methyladenosine 5'-O-monomophosphate; 8CPT-2'-OMe-Ado, 8-(4-chlorophenylthio)-2'-O-methyladenosine; 8CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; Sp-8CPT-2'-OMe-cAMP, 8-(4-chlorophenylthio)-2'-O-methyladenosine; 8CPT-Ado, 8-(4-chlorophenylthio)adenine.

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**ACTH Induces Ca\(_{\nu}\)3.2 Current and mRNA by cAMP-dependent and cAMP-independent Mechanisms**

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2. The abbreviations used are: AZF, adrenal zona fasciculata; ACTH, adrenocorticotropic hormone; NPS-ACTH, O-nitrophenylsulfenyl-adrenocorticotropic; PKA, cAMP-dependent protein kinase; ESCA, Epac-specific cAMP analog; DMEM, Dulbecco’s modified Eagle’s medium; 8-Br-cAMP,
GEFII that activate Rap1 and Rap2 GTPases have been identified (19, 20). Epac proteins regulate a variety of cellular processes by mechanisms that, in some cases, include regulation of gene expression (21–23). Although Epac1 is widely expressed, Epac2 is robustly expressed mainly in selected areas of the brain and the adrenal glands of rats and humans (19, 20).

Elucidating the signaling pathways by which ACTH and cAMP function in the adrenal cortex has been facilitated by the discovery of cAMP derivatives that, at appropriate concentrations, selectively activate Epacs or PKA (24–26). These derivatives have been used to independently study the role of these cAMP-activated proteins in the regulation of cell function. In this regard, we previously found that cAMP inhibits the activity of bTREK-1 K⁺/H11001 current by a mechanism that is independent of PKA but mimicked by Epac-specific cAMP analogs (ESCAs) (27, 28).

Recently, we discovered that the most widely used ESCA 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8CPT-2'-OMe-cAMP), after a delay of many hours, stimulated large increases in the expression of steroid hydroxylase mRNAs and cortisol synthesis by a mechanism that is independent of PKA but mimicked by Epac proteins (32). In contrast, in rat AZF cells, it was reported that prolonged treatment with a different 8-substituted cAMP analog, the PKA and Epac activator 8-Br-cAMP, or forskolin failed to enhance the expression of the T-type Ca²⁺ current (33). Neither of these studies measured the effect of these specific cAMP analogs on CACNA1H mRNA.

In this study, we examined the regulation of CACNA1H mRNA and corresponding Ca₃.2 Ca²⁺ currents in AZF cells by ACTH, cAMP analogs, and their metabolites using Northern blot and patch clamp techniques. It was discovered

**FIGURE 1.** Long term effect of ACTH on expression of Ca₃.2 T-type Ca²⁺ current in bovine AZF cells. AZF cells were cultured up to 144 h in media containing 2 nM ACTH. Whole cell Ca²⁺ currents were activated by either long (300 ms) or short (10 ms) voltage steps applied at 30-s intervals from a holding potential of −80 mV. A, effect of time in culture on Ca₃.2 T-type Ca²⁺ current. Summary of experiments in which AZF cells were cultured and T-type Ca²⁺ current was measured after the indicated times. The values are the means ± S.E. of the indicated number of determinations. B, representative Ca²⁺ current traces recorded from AZF cells that were cultured for 96 h either without (control) or with ACTH (2 nM). Ca²⁺ current traces were activated by long (left panel) and short (right panel) depolarizing steps. C, current-voltage relationship. Peak current amplitudes from cells incubated for 48 h either without (control) or with ACTH (2 nM) are plotted against test potential. D, summary of experiments in which AZF cells were either left untreated (control) or treated with ACTH (2 nM) for times from 48 to 144 h. The bars represent Ca₃.2 T-type Ca²⁺ current density expressed as the means ± S.E. of the indicated number of determinations.
that ACTH and metabolites of chlorophenylthio-cAMP analogs induce the expression of CACNA1H mRNA and Ca\(^{2+}\) current. In contrast, other cAMP analogs enhanced the expression of CACNA1H mRNA but not the associated current.

**EXPERIMENTAL PROCEDURES**

DMEM/F12, antibiotics, and fetal bovine serum were obtained from Invitrogen. Phosphate-buffered saline, bovine plasma fibronectin, tocopherol, selenite, ascorbic acid, 8-CPT-cAMP, adenosine, and ACTH (1–24) were obtained from Sigma. O-Nitrophenylsulfenyl-adrenocorticotropin (NPS-ACTH) was custom synthesized by Celtek Peptides (Nashville, TN). 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; Biolog B007), N\(^6\)-benzoyladenosine 3',5'-cyclic monophosphate (6-Bnz-cAMP; Biolog B009), 8CPT-2'-OMe-cAMP (Biolog C041), hydrolysis-resistant 8-(4-chlorophenylthio)-2'-O-methyl-cAMP, Sp-isomer (Sp-8CPT-2'-OMe-cAMP; Biolog C052), 8-(4-chlorophenylthio)-2'-O-methyladenosine 5'-O-monophosphate (8CPT-2'-OMe-5'-AMP; Biolog C078), 8-(4-chlorophenylthio)-2'-O-methyladenosine (8CPT-2'-OMe-Ado; Biolog C070), 8-(4-chlorophenylthio)-cAMP (8CPT-cAMP; Biolog C010), hydrolysis-resistant 8-(4-chlorophenylthio)-cAMP (Sp-8CPT-cAMP; Biolog C012), 8-(4-chlorophenylthio)-adenosine (8CPT-Ado; Biolog C086), and 8-(4-chlorophenylthio)adenine (8CPT-Ade; Biolog C069) were purchased from Axxora, LLC (San Diego, CA).

**Isolation and Culture of AZF Cells**—Bovine adrenal glands were obtained from steers (age, 2–3 years) at a local slaughterhouse. Isolated AZF cells were either left untreated (control) or treated with ACTH (2 nM) or NPS-ACTH (50 or 100 nM) for 48 or 72 h. The bars represent Ca\(_{3.2}\) T-type Ca\(^{2+}\) current density expressed as the means ± S.E. of the indicated number of determinations.

**Induction of Cav3.2 Ca\(^{2+}\) Current and mRNA**

**FIGURE 2.** Long term effect of 8-Br-cAMP and NPS-ACTH on expression of Ca\(_{3.2}\) T-type Ca\(^{2+}\) current in bovine AZF cells. AZF cells were cultured up to 96 h in media containing no further addition (control), ACTH (2 nM), 8-Br-cAMP (300 or 600 µM), or NPS-ACTH (50 or 100 nM). Whole cell Ca\(^{2+}\) currents were recorded after activation by either long (300 ms) or short (10 ms) voltage steps applied at 30-s intervals from a holding potential of −80 mV. A, representative Ca\(^{2+}\) current traces recorded from AZF cells that were cultured for 96 h with ACTH (2 nM), 8-Br-cAMP (600 µM), or NPS-ACTH (50 nM). Ca\(^{2+}\) current traces were activated by long (left) and short (right) depolarizing steps. B, summary of experiments in which AZF cells were either left untreated (control) or treated with ACTH (2 nM) or 8-Br-cAMP (300 or 600 µM) for 48–96 h. The bars represent Ca\(_{3.2}\) T-type Ca\(^{2+}\) current density expressed as the means ± S.E. of the indicated number of determinations. C, summary of experiments in which AZF cells were either left untreated (control) or treated with ACTH (2 nM) or NPS-ACTH (50 or 100 nM) for 48 or 72 h. The bars represent Ca\(_{3.2}\) T-type Ca\(^{2+}\) current density expressed as the means ± S.E. of the indicated number of determinations.
FIGURE 3. 8CPT-cAMP but not Sp-8CPT-cAMP enhances expression of Ca_{3.2} T-type Ca\(^{2+}\) current in bovine AZF cells. AZF cells were cultured up to 144 h in media containing no further addition (control), 8CPT-cAMP (50 or 250 μM), or Sp-8CPT-cAMP (50 μM). Whole cell Ca\(^{2+}\) currents were recorded after activation by either long (300 ms) or short (10 ms) voltage steps applied at 30-s intervals from a holding potential of —80 mV. A, representative Ca\(^{2+}\) current traces recorded from AZF cells that were cultured for 48 h with 8CPT-cAMP (50 μM) or Sp-8CPT-cAMP (50 μM). Ca\(^{2+}\) current traces were activated by long (left) and short (right) depolarizing steps. B, summary of experiments in which AZF cells were either left untreated (control) or treated with 8CPT-cAMP (50 or 250 μM) or Sp-8CPT-cAMP (50 μM) for 48–144 h. The bars represent Ca_{3.2} T-type Ca\(^{2+}\) current density expressed as the means ± S.E. of the indicated number of determinations.

Induction of Ca_{3.2} Ca\(^{2+}\) Current and mRNA

and the antioxidants 1 μM tocopherol, 20 μM selenite and 100 μM ascorbic acid (DMEM/F12+) and plated for immediate use or were resuspended in fetal bovine serum, 5% Me\(_2\)SO, divided into 1-ml aliquots, and stored in liquid nitrogen for future use. To ensure cell attachment, the dishes were treated with fibronectin (10 μg/ml) for 30 min at 37 °C and then rinsed with warm, sterile phosphate-buffered saline immediately before adding cells. The cells were maintained at 37 °C in a humidified atmosphere of 95% air, 5% CO\(_2\).

Northern Blot Hybridization and Measurement of mRNA—Total RNA isolation and Northern blot procedures have been described previously (10). Briefly, 5–7 × 10\(^6\) AZF cells were plated on 60-mm fibronectin-treated dishes in DMEM/ F12+. After 24 h, the media were replaced with either control media (DMEM/F12+) or the same media containing ACTH (1–24), 6-Bnz-cAMP, 8-Br-cAMP, 8CPT-cAMP, Sp-8CPT-cAMP, or other agents as required. At the end of the incubation period, total RNA was extracted using RNasea columns (Qiagen), electrophoresed on a denaturing gel, and transferred to a nylon transfer membrane (GeneScreen Plus; PerkinElmer Life Sciences). The probes were generated by reverse transcription-PCR using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), specific primers for bovine CACNA1H, and total RNA isolated from bovine AZF cells as described above. CACNA1H primers (GGGGAGAGCCTGGCGGGAAGA and GGGTCTCTGGAGGGCCTGTGGA) were selected from GenBank\(^\text{TM}\) accession number XM_581124.4 (Predicted: Bos taurus calcium channel, voltage-dependent, T-type, α1H subunit (CACNA1H)) mRNA. This ~800-bp probe is specific for bovine CACNA1H mRNA and has low homology to either CACNA1G or CACNA1I. The CACNA1H probe was labeled with [α-\(^{32}\)P]dCTP by random primer labeling (Prime-It II; Stratagene, La Jolla, CA). Northern autoradiograms were imaged using a Typhoon 9200 variable mode imager and quantitated using ImageQuant TL v2003.3 software (GE Healthcare). The mRNA values are presented as the means ± S.E. of at least three independent experiments normalized to densitometric values for the corresponding 18S rRNA bands.

Ca\(^{2+}\) Current Recording—Patch clamp recordings of voltage-gated Ca\(^{2+}\) currents from bovine AZF cells were made in the whole cell configuration as previously described (1). The standard pipette solution was 120 mM CsCl, 1 mM CaCl\(_2\), 2 mM MgCl\(_2\), 11 mM 1,2-bis(2-aminophenoxy) ethane-N,N′,N″,N‴-tetraacetic acid, 10 mM HEPES, and 1 mM MgATP with pH titrated to 7.2 with CsOH. The external solution consisted of 117 mM tetraethylammonium chloride, 5 mM CsCl, 20 mM CaCl\(_2\), 2 mM MgCl\(_2\), 5 mM HEPES, and 5 mM glucose, with pH adjusted to 7.4 with tetraethylammonium hydroxide. All of the solutions were filtered through 0.22-μm cellulose acetate filters.

Recording Conditions and Electronics—AZF cells were used for patch clamp experiments 24–144 h after plating. Typically, cells with diameters of <15 μm and capacitances of 10–15 pF were selected. Coverslips upon which cells were plated (Belco, Vineland NJ) were transferred from 35-mm culture dishes to a recording chamber (volume, 1.5 ml) that was continuously perfused by gravity at a rate of 3–5 ml/min. For whole cell recordings, 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. Ca\(^{2+}\) currents were recorded at room temperature (22–25 °C) according to
Induction of Ca\textsubscript{v}3.2 Ca\textsuperscript{2+} Current and mRNA

**FIGURE 4.** Long term effect of metabolites of 8CPT-cAMP on the expression of Ca\textsubscript{v}3.2 T-type Ca\textsuperscript{2+} current in bovine AZF cells. AZF cells were cultured up to 96 h in media containing no further addition (control), 8CPT-Ado (50 \textmu M), or 8CPT-Ade (50 \textmu M). Whole cell Ca\textsuperscript{2+} currents were activated by either long (300 ms) or short (10 ms) voltage steps applied at 30-s intervals from a holding potential of −80 mV. A, representative Ca\textsuperscript{2+} current traces recorded from AZF cells that were cultured for 48 h with 8CPT-Ado (50 \textmu M) or 8CPT-Ade (50 \textmu M). Ca\textsuperscript{2+} current traces were activated by long (left panel) and short (right panel) depolarizing steps. B, summary of experiments in which AZF cells were either left untreated (control, white bars) or treated with 8CPT-Ado (50 \textmu M, black bars) or 8CPT-Ade (50 \textmu M, gray bars) for 48–96 h. The bars represent Ca\textsubscript{v}3.2 T-type Ca\textsuperscript{2+} current density expressed as the means ± S.E. of the indicated number of determinations.

by the procedure of Hamill et al. (35) 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 (Axon Instruments, Inc., Burlingame, CA). The 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 ¼ pulse amplitude. The data were analyzed using PCLAMP (CLAMPFIT 9.2) and SigmaPlot (version 11.0) software. For selected experiments (supplemental Fig. S1), ACTH (200 \textmu M) was applied by bath perfusion, controlled manually by a six-way rotary valve.

**RESULTS**

**ACTH Enhances Expression of Ca\textsubscript{v}3.2 Ca\textsuperscript{2+} Current in Bovine AZF Cells**—When bovine AZF cells were cultured in serum-supplemented media, the Ca\textsubscript{v}3.2 current disappeared over a period of days, as determined from whole cell patch clamp recordings (Fig. 1A). This rapidly inactivating and slowly deactivating Ca\textsuperscript{2+} current was recorded in response to long (300 ms) or short (10 ms) voltage steps (Fig. 1B). After 96 h, Ca\textsubscript{v}3.2 current density diminished from a control value of 5.83 ± 1.14 pA/pF (n = 10) to 1.12 ± 0.08 pA/pF (n = 24) (Fig. 1A). Prolonged exposure of AZF cells to ACTH (2 nM) reversed the time-dependent decrease in the Ca\textsuperscript{2+} current that occurred in the absence of the peptide. By 48 h, the Ca\textsubscript{v}3.2 current began to increase, reaching a maximum by 96 h (Fig. 1, B–D). After 96 h, Ca\textsubscript{v}3.2 current density was 6.74 ± 1.06 pA/pF (n = 10) in ACTH-treated cells, compared with 0.99 ± 0.06 pA/pF (n = 18) in the time-matched controls (Fig. 1D). The T-type Ca\textsuperscript{2+} current induced by ACTH was blocked by Ni\textsuperscript{2+} with a potency that identified it as Ca\textsubscript{v}3.2 (supplemental Fig. S2).

NPS-ACTH but Not 8-Br-cAMP Induces Expression of Ca\textsubscript{v}3.2 Current in AZF Cells—cAMP has long been regarded as the principal intracellular messenger for ACTH. Experiments were done to determine whether ACTH-stimulated expression of Ca\textsubscript{v}3.2 current was mediated by cAMP. 8-Br-cAMP activates PKA and Epac proteins at concentrations above 100 \textmu M and stimulates rapid and delayed increases in cortisol secretion by bovine AZF cells (25, 26, 36). However, as illustrated in Fig. 2 (A and B), exposing cells to 8-Br-cAMP (300 or 600 \textmu M) for times from 48 to 96 h failed to increase the expression of T-type Ca\textsuperscript{2+} current, compared with time-matched controls. In contrast, ACTH (2 nM) increased the expression of T-type Ca\textsuperscript{2+} current 2–3-fold (Fig. 2, A and B).

NPS-ACTH stimulates large increases in [Ca\textsuperscript{2+}], and cortisol secretion in both rat and bovine AZF cells at concentrations that produce little or no increase in cAMP synthesis (30, 31). Although less potent than ACTH, at maximally effective concentrations NPS-ACTH produces increases in cortisol secretion comparable with those produced by ACTH (30). We compared ACTH to NPS-ACTH with respect to their effectiveness at enhancing the expression of Ca\textsubscript{v}3.2 current. At concentrations of 50 and 100 nM, NPS-ACTH increased Ca\textsubscript{v}3.2 current density by an amount similar to that produced by ACTH (10 nM) at 48 and 72 h (Fig. 2, A and C).
**Induction of Ca\textsubscript{3.2} Ca\textsuperscript{2+} Current and mRNA**

Experiments with the cAMP analog 8-Br-cAMP indicated that the activation of both PKA and Epac2 in AZF cells was not sufficient to induce the expression of functional Ca\textsubscript{3.2} channels. CAMP derivatives with substitutions at the 6-position of the adenine ring potently activate PKA but not Epac proteins (38, 39). At concentrations above 100 \( \mu \text{M} \), 6-Bnz-cAMP markedly enhances cortisol secretion (36). Treating bovine AZF cells with 6-Bnz-cAMP for prolonged periods failed to induce the expression of Ca\textsubscript{3.2} current. In the experiments illustrated in Fig. 6, the cells were treated with 6-Bnz-cAMP (50 and 300 \( \mu \text{M} \)) for periods of 48–144 h. At no time did 6-Bnz-cAMP significantly increase Ca\textsubscript{3.2} current density over the control value.

8CPT-2\textsuperscript{-}′-OMe-cAMP binds to Epac with an affinity greater than 100-fold that of its affinity for the cyclic nucleotide binding domain of the PKA regulatory subunit \( I_{\alpha} \) (39). In contrast to 6-Bnz-cAMP, 8CPT-2\textsuperscript{-}′-OMe-cAMP (50 \( \mu \text{M} \)) prevented the time-dependent down-regulation of Ca\textsubscript{3.2} expression and instead markedly enhanced the expression of this current at times from 72 to 144 h (Fig. 6). After exposing AZF cells to this Epac activator for 96 h, Ca\textsubscript{3.2} expression was increased from the control value of 0.99 ± 0.06 pA/pF (\( n = 18 \)) to 7.30 ± 1.86 pA/pF (\( n = 9 \)) (Fig. 6).

These results suggested that cAMP could induce the expression of Ca\textsubscript{3.2} by selectively activating Epac2 but not PKA. However, like 8CPT-cAMP, 8CPT-2\textsuperscript{-}′-OMe-cAMP can be hydrolyzed by cellular enzymes to produce similar metabolites (29, 40). The nonhydrolyzable ESCA Sp-8CPT-2\textsuperscript{-}′-OMe-cAMP (50 \( \mu \text{M} \)) failed to enhance the expression of Ca\textsubscript{3.2} current in AZF cells (Fig. 7).

The failure of Sp-8CPT-2\textsuperscript{-}′-OMe-cAMP to increase the expression of Ca\textsubscript{3.2} suggested that activation of Epac2 alone did not enhance the expression of this current. This result further suggested that 8CPT-2\textsuperscript{-}′-OMe-cAMP induced the expression of Ca\textsubscript{3.2} indirectly through a metabolite. Accordingly, 8CPT-2\textsuperscript{-}′-OMe-adenosine (8CPT-2\textsuperscript{-}′-OMe-Ado), a metabolite of the hydrolyzable Epac activator, markedly increased the expression of Ca\textsubscript{3.2} measured at 48, 72, and 96 h. At each of these times, current density was 3.5–4-fold greater than that of the time-matched control (Fig. 7).

**ACTH and cAMP Derivatives and Metabolites Induce Ca\textsubscript{3.2} mRNA Expression**—In bovine AZF cells, ACTH enhanced the expression of a Ni\textsuperscript{2+}-sensitive T-type Ca\textsuperscript{2+} current presumed to be Ca\textsubscript{3.2}. If these increases in current occurred through activation of gene transcription or enhanced
stability of the specific mRNA, then they would be accompanied by a corresponding increase in CACNA1H mRNA. We found that, at concentrations from 10 to 1000 pM, ACTH induced a concentration-dependent increase of a ~8-kb CACNA1H transcript (Fig. 8A, B and C). ACTH-induced increases in CACNA1H mRNA were present by 5 h and reached a maximum by 24–48 h (Fig. 8F).

Patch clamp experiments showed that the cAMP analogs 8-Br-cAMP and 6-Bnz-cAMP at concentrations up to 600 pM failed to stimulate an increase in functional Ca$_{3.2}$ current. However, both of these analogs did induce concentration-dependent increases in CACNA1H mRNA. Significant increases were observed only at concentrations above 100 pM (Fig. 8B and C).

At low concentrations, 8CPT-cAMP and its nonhydrolyzable analog Sp-8CPT-cAMP had effects on CACNA1H mRNA similar to their effects on the associated Ca$_{3.2}$ current. At a concentration of 50 pM, 8CPT-cAMP increased expression of the CACNA1H transcript, whereas 50 pM Sp-8CPT-cAMP did not (Fig. 8D).

These results suggested that 8CPT-cAMP (50 µM) enhanced the expression of CACNA1H mRNA indirectly, after conversion to an active metabolite. Accordingly, at concentrations from 1 to 50 µM, its putative metabolite 8CPT-Ade stimulated a concentration-dependent increase in CACNA1H mRNA (Fig. 8E).

With respect to temporal pattern, the 8CPT-Ade-induced increases in CACNA1H mRNA lagged behind that induced by ACTH. Although no 8CPT-Ade-mediated increase in the CACNA1H transcript could be observed after 5 h, by 48 h the increase was similar in magnitude to those stimulated by ACTH (Fig. 8F).

Enhancement of CACNA1H mRNA Expression by ESCAs and Metabolites—The hydrolyzable and nonhydrolyzable ESCAs 8CPT-2’-OMe-cAMP and Sp-8CPT-2’-OMe-cAMP produced effects on CACNA1H mRNA similar to their effects on the associated membrane current. Measured over a 48-h period, 8CPT-2’-OMe-cAMP (10–50 µM) induced a delayed, concentration-dependent increase in the CACNA1H mRNA (Fig. 9). In contrast, nonhydrolyzable Sp-8CPT-2’-OMe-cAMP (100 µM) failed to enhance CACNA1H mRNA expression, just as it failed to increase Ca$_{3.2}$ current (Fig. 9C).

These results again indicated that one or more metabolites of 8CPT-2’-OMe-cAMP enhanced CACNA1H mRNA expression by an Epac2-independent mechanism. Accordingly, we discovered that both 8CPT-2’-OMe-5’-AMP and 8CPT-2’-OMe-Ado stimulated concentration-dependent increases in the expression of CACNA1H mRNA with potencies similar to the parent compound (Fig. 9). In contrast, adenosine (50 µM) did not increase the expression of the CACNA1H transcript (Fig. 9E).

**DISCUSSION**

In this study, we have reported several novel findings regarding the physiological mechanisms that regulate Ca$_{3.2}$ expression in bovine AZF cells. First, ACTH induces the expression of CACNA1H mRNA and Ca$_{3.2}$ current through mechanisms that are, at most, only partially dependent on cAMP. cAMP analogs that activate PKA and Epac, as well as those that activate only PKA, increase the expression of CACNA1H mRNA, but not the associated Ca$_{3.2}$ current. Epac activation alone is not sufficient to induce the expression of either mRNA or current. Chlorophenylthio-cAMP analogs potentely induce the
expression of CACNA1H mRNA and ionic current after conversion to one or more active metabolites. It is possible that ACTH and the cAMP metabolites stimulate the expression of CACNA1H mRNA and current through a common cAMP-independent mechanism.

ACTH and Ca,_{3.2} Expression—ACTH reversed the spontaneous time-dependent decrease in Ca,_{3.2} current typically observed in cultured bovine AZF cells and induced a delayed increase in expression of functional channels that lasted for at least 6 days. With respect to temporal pattern, maximum increases in Ca,_{3.2} current were similar to those observed for ACTH-induced increases in steroid hydroxylases and bTREK-1 current in these same cells (6, 7, 10, 41). ACTH also induced CACNA1H mRNA with a temporal pattern and potency similar to that previously observed for steroid hydroxylase messages (6, 7, 41). ACTH increases the steroid hydroxylases by increasing the rate of transcription of each of these genes (6–8, 42). It is likely that ACTH-induced increases in Ca,_{3.2} expression involve increases in transcription of the CACNA1H gene. However, we have not eliminated the possibility that ACTH increases CACNA1H mRNA expression by stabilizing this transcript. The signaling pathway(s) by which ACTH regulates CACNA1H mRNA and Ca,_{3.2} ionic current expression appear to be complex and may involve transcriptional and post-transcriptional mechanisms activated through cAMP-dependent and -independent pathways.

In this regard, the contrasting effects of ACTH compared with the cyclic AMP analogs 8-Br-cAMP and 6-Bnz-cAMP on CACNA1H mRNA and ionic currents are particularly interesting. Specifically, at concentrations above 100 μM, both of these cAMP analogs stimulate the expression of CACNA1H mRNA, but not that of functional Ca,_{2+} channels. Therefore, ACTH-stimulated increases in cAMP alone are not sufficient to enhance Ca,_{3.2} expression, even though they may contribute to this response. ACTH-induced effects may be mediated through additional or entirely separate signaling mechanisms that function at the transcriptional or post-transcriptional level. ACTH and NPS-ACTH increase intracellular Ca,_{2+} and stimulate cortisol secretion from bovine AZF cells at concentrations that trigger little or no increase in cAMP synthesis (30, 31, 43). Perhaps in bovine AZF cells, Ca,_{2+} and cAMP function cooperatively to regulate CACNA1H transcription and Ca,_{3.2} translation and trafficking.

These results show that the mechanisms by which ACTH regulates the expression of Ca,_{3.2} differ fundamentally from those that control the expression of steroid hydroxylases and bTREK-1 K⁺ channels. Specifically, ACTH-stimulated increases in cAMP alone appear to be sufficient to induce the expression of steroid hydroxylase and bTREK-1 mRNAs and their corresponding functional proteins (6, 7, 36).

cAMP, PKA, Epac2, and CACNA1H mRNA Expression—Experiments with 8-Br-cAMP showed that increasing cAMP in AZF cells was sufficient to increase CACNA1H mRNA, but not the corresponding Ca,_{2+} current. Further, the robust concentration-dependent increase in CACNA1H mRNA induced by 6-Bnz-cAMP suggests that the increase is mediated through activation of PKA. However, it is possible that 6-Bnz-cAMP functions through a third, unidentified cAMP receptor. In patch clamp experiments, we found that, when included in the pipette solution at very low concentrations (i.e. <10 μM), 6-Bnz-cAMP completely blocks the activity of bTREK-1 channels, even in the presence of several PKA antagonists (36).

Experiments with the nonhydrolyzable ESCA Sp-8CPT-2′-OMe-cAMP clearly demonstrated that cAMP does not enhance the expression of CACNA1H mRNA by activation of Epac2. Specifically, at concentrations that activate Rap1 in
these cells, Sp-8CPT-2'-OMe-cAMP failed to increase the quantity of CACNA1H mRNA (29). Thus, cAMP-stimulated expression of CACNA1H mRNA may be mediated entirely or in part by PKA but is independent of Epac2. Furthermore, a third cAMP-activated protein may be involved. In a number of cells, cAMP synthesized in response to activation of G-protein-coupled receptors produces effects that are independent of Epac and PKA proteins (44–48).

**8-Chlorophenylthio-cAMP Analogs and CACNA1H Expression**—In contrast to 8-Br-cAMP and 6-Bnz-cAMP, 8-chlorophenylthio-cAMP analogs, including 8CPT-cAMP and the ESCA 8CPT-2'-OMe-cAMP (each at 50 μM), induced CACNA1H mRNA and Cav3.2 current expression. It is not likely that these effects were mediated through cAMP, because at identical concentrations, the nonhydrolyzable monophosphothioate derivatives of these two compounds failed to increase either CACNA1H mRNA or Cav3.2 current. Accordingly, we found that four potential metabolites of these 8-chlorophenylthio-cAMP analogs induced the expression of CACNA1H mRNA transcripts and Cav3.2 ion current with effectiveness and potency similar to the parent compounds. Enzymes that catalyze the conversion of these two cAMP derivatives to each of these putative metabolites are expressed in mammalian cells (37). The active metabolite(s), receptors, and signaling pathways that mediate the increases in CACNA1H mRNA and the corresponding current have not been identified. In this regard, it is interesting that both 8CPT-cAMP and the ESCA 8CPT-2'-OMe-cAMP can be converted to 8CPT-adenine, which may be the common active metabolite. Regardless of their identity, it is clear that the active metabolite(s) do not function in the AZF cell through cAMP. First, we have previously shown that neither 8CPT-2'-OMe-5'-AMP, 8CPT-2'-OMe-adenosine,
nor 8CPT-adenine activates PKA when applied to bovine AZF cells (29). PKA activation is an extremely sensitive measure of cAMP synthesis in AZF cells (49). In addition, 8-Br-cAMP failed to increase Ca$_{3.2}$ current in AZF cells, even at concentrations that have been shown to activate PKA and Epac2, stimulate large increases in cortisol synthesis and steroid hydroxylase mRNAs, and increase both bTREK-1 mRNA and K$^+$ current (36).

With respect to temporal pattern and potency, 8CPT-adenine and other metabolites in this study induced the expression of CACNA1H mRNA and Ca$^{2+}$ current with characteristics similar to those previously observed for bTREK-1 mRNA and K$^+$ current, as well as for steroid hydroxylase mRNAs and cortisol secretion (29, 36). These increases in gene expression were selective; total RNA synthesis was not significantly increased in metabolite-treated cells. Expression of Kv1.4 K$^+$ channel mRNA and current were also not altered by these metabolites.3

The mechanism by which a small molecule such as 8CPT-adenine could activate specific genes in AZF cells is unclear. Regardless, our findings are consistent with the hypothesis that 8CPT-adenine and perhaps other similar metabolites induce the expression of CACNA1H mRNA and Ca$^{2+}$ channels by a cAMP-independent pathway that is also activated by ACTH and NPS-ACTH.

**Regulation of Ca$_{3.2}$ Expression in Other Adrenal Cells**—The finding that in bovine AZF cells ACTH can induce the expression of CACNA1H mRNA and Ca$_{3.2}$ current, whereas cAMP analogs like 8-Br-cAMP increase only the mRNA was unexpected. However, it is in agreement with a previous patch clamp study in which a 3-day exposure of rat AZF cells to ACTH was found to increase T-type Ca$^{2+}$ current in these cells, whereas 8-Br-cAMP failed (33). Apparently, in both bovine and rat AZF cells, ACTH stimulates the expression of functional Ca$_{3.2}$ channels by a signaling pathway that includes a cAMP-independent component. Because 8-Br-cAMP increases CACNA1H mRNA but not Ca$^{2+}$ current, the cAMP-independent action of ACTH likely occurs at the level of translation or protein trafficking. Regardless, the results of the present study reveal that the control of CACNA1H expression is complex and involves multiple signaling pathways operating at transcriptional and post-transcriptional levels.

Our results also may clarify some potentially confusing findings regarding the control of T-type Ca$^{2+}$ channel expression in adrenal cells. In a patch clamp study on rat adrenal chromaffin cells, it was shown that prolonged treatment with 8CPT-cAMP or 8CPT-2'-OMe-cAMP induced the expression of a low voltage-activated Ni$^{2+}$-sensitive Ca$^{2+}$ current. Because the effects of these cAMP analogs on Ca$^{2+}$ channel expression were not blocked by the PKA inhibitor H-89, it was concluded that the Ca$^{2+}$ channel "recruitment" was mediated through cAMP by activation of an Epac protein (32). In view of our findings and those of Barbara and Takeda (33), it is quite possible that the effects of the chlorophenylthio-cAMP analogs on the Ni$^{2+}$-sensitive T-type current in rat adrenal chromaffin cells were mediated indirectly through the metabolites of these compounds.

**Physiological Significance**—The results of this and previous studies strongly suggest that ACTH exerts long term control over the electrical properties of AZF cells by regulating the

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3 H. Liu, J. A. Enyeart, and J. J. Enyeart, unpublished observations.
expression of genes that code for ion channels (10, 36, 50). Ca\textsubscript{3.2}^2+ channels function as the primary pathway for depolarization-dependent Ca\textsuperscript{2+} entry, leading to cortisol secretion by these cells (2). Prolonged exposure of AZF cells to ACTH, as occurs in chronic stress, may enhance cortisol production by increasing the number of Ca\textsubscript{3.2}^2+ channels.

Ca\textsubscript{3.2}^2+ channels are expressed in other tissues, including the brain, where they regulate the initiation of action potentials in neurons. Overexpression of this specific T-type Ca\textsuperscript{2+} channel in neurons has been shown to play a significant role in various forms of epilepsy, as well as neuropathic pain (51–53). To understand the molecular basis of the pathophysiology of diseases such as these, it will be essential to identify the signaling mechanisms that regulate Ca\textsubscript{3.2}^2+ expression. Our study indicates that ACTH likely induces the expression of CACNA1H mRNA by activating PKA. However, other post-transcriptional mechanisms must be activated by cAMP-independent pathways to produce functional Ca\textsubscript{3.2}^2+ channels. It will be important to identify this other ACTH activated pathway and to determine whether it is also activated by metabolites of 8-chlorophenylthio-cAMP analogs that increase CACNA1H mRNA and current.

The extent to which metabolites of the chlorophenylthio-derivatives of cAMP regulate gene expression in other cells is unknown. Hydrolysis products of selected cAMP analogs transform the morphology of the protozoa Trypanosoma brucei (40). It is not known whether these effects observed in distantly related eukaryotes are mediated by a common signaling pathway (36, 40).

Finally, 8CPT-cAMP and 8CPT-2’-OMe-cAMP have been used in many hundreds of studies to identify and characterize the roles of cAMP, PKA, and Epac proteins in cell signaling. The findings of this and several other recent studies indicate that the results of many earlier studies may require re-evaluation (29, 36, 40).

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