Phase-Dependent Resetting of the Adrenal Clock by ACTH in vitro

Abbreviated title: ACTH Resets the Adrenal Clock

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The adrenal cortex has a molecular clock that generates circadian rhythms in glucocorticoids, yet how the clock is synchronized to the external environment is unknown. Using mPER2::Luciferase (mPER2Luc) knockin mice, in which luciferase is rhythmically expressed under the control of the mouse Per2 clock gene, we hypothesized that ACTH transmits entrainment signals to the adrenal. Adrenal explants were administered ACTH at different phases of the mPER2Luc rhythm. Treatment with ACTH 1-39 produced a phase delay that was phase-dependent, with a maximum at circadian time (CT)18; ACTH did not alter the period or amplitude of the rhythm. Forskolin produced a parallel response, suggesting that the phase delay was cyclic AMP-mediated. The response to ACTH was concentration-dependent and peptide-specific. Pulse administration (60 min) of ACTH 1-39 also produced phase delays restricted to late CTs. In contrast to ACTH 1-39, other ACTH fragments including alpha melanocyte-stimulating hormone that do not activate the melanocortin 2 (MC2/ACTH) receptor had no effect. The finding that ACTH in vitro phase delays the adrenal mPER2Luc rhythm in a monophasic fashion argues for ACTH as a key resetter, but not the sole entrainer, of the adrenal clock.
Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is characterized by a robust circadian variation in glucocorticoids. This rhythm is paralleled by rhythms in plasma ACTH [6], and adrenal sensitivity to ACTH [10] mediated in part by sympathetic activity [49]. Rhythmic ACTH secretion is regulated by corticotropin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus (PVN) [6]. Circadian timing is dependent on a clock in the suprachiasmatic nucleus (SCN) [41] that is reset daily by photic information transmitted from the retina [28] with SCN neurons relaying these signals to the PVN [19]. This mechanism guarantees that glucocorticoid rhythms are entrained to the daily light-dark (LD) cycle.

A molecular clock exists in the SCN [39] and peripheral tissues [12] including the adrenal [4]. The clock consists of interlocking feedback loops of gene transcription and translation [39]. The "positive" limb involves CLOCK and BMAL1, which heterodimerize and activate transcription of period genes, Per1 and 2, and cryptochrome genes, Cry1 and 2. The Per and Cry transcripts increase with the protein products, PER and CRY, functioning as the "negative limb" by forming heterodimers that act on the CLOCK:BMAL1 complex to repress their own transcription. The feedback loops are self-regulating with an ~24h periodicity.

The adrenal clock controls steroidogenesis by gating sensitivity to ACTH. In Per2/Cry1 mutant mice that lack a functioning clock, rhythms in adrenal sensitivity are absent and the corticoid rhythm is blunted [35]. The steroidogenic acute regulatory protein (StAR) participates in the gating mechanism, as it is required for steroidogenesis [18], displays a circadian rhythm [16] and is activated by CLOCK:BMAL1 [47]. When the adrenal clock is knocked down by expressing antisense to BMAL1 using the ACTH (melanocortin 2) receptor (MC2R) promoter, the MC2R-AS-BMAL mice show no corticoid rhythm in constant dark indicating that the adrenal clock drives the free-running rhythm [47]. Since SCN lesions abolish corticoid rhythmicity, SCN-dependent outputs are required to synchronize the adrenal clock and the resultant glucocorticoid rhythms to LD cycles [29]. We hypothesize that ACTH and
sympathetic neural activity are SCN-dependent outputs that mediate corticoid rhythms via LD entrainment of an autonomous adrenal clock.

To assess whether ACTH entrains the adrenal clock, we used adrenals from mPER2::Luciferase (mPER2Luc) knockin mice [53]. This approach allows us to test direct responses of the adrenal clock to potential entraining signals in vitro, as shown for other peripheral clocks [31, 40]. If entrainment occurs, the phase of the PER2Luc rhythm should shift in response to ACTH in a phase-dependent fashion.

Materials and Methods

Animals
Homozygous male mPER2::Luc mice (2-5 months old) bred in-house were housed on a 12:12 LD cycle (lights on at 0600). Animals were maintained and cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Experimental procedures were approved by the University of Minnesota Animal Care and Use Committee.

Bioluminescence
Animals were killed by CO2 asphyxiation 3.5-4.0 h before lights out (at Zeitgeber time (ZT) 8-8.5). Adrenals were rapidly excised and placed in cold Hank’s Balanced Salt Solution. Cleaned and hemisected adrenals were placed on Millicell organotypic inserts in a 35 mm Petri dish with 1.5 mL of warmed culture media (Dulbecco’s Modified Eagle Media, high glucose w/o Phenol Red) supplemented with luciferin and penicillin/streptomycin as described by others [53]. Dishes were sealed with circular glass coverslips and silicon grease. Cultures were maintained at 36° C, and bioluminescence was measured using photomultiplier tubes in an Actimetrics Lumicycle.

Assessing mPER2Luc rhythms
Tissue was treated at the circadian time (CT) indicated by replacement with fresh media containing rat ACTH 1-39 (Bachem), forskolin (BioMol International), human alpha melanocyte-stimulating hormone (αMSH; Bachem), human ACTH 7-38 (Phoenix Pharmaceuticals), ACTH 11-24 (Bachem), vasoactive intestinal polypeptide (VIP; Bachem), CRH (Bachem) or vehicle (fresh media alone). Pulse experiments consisted of treatment at the specified CT followed by replacement with untreated fresh media after 60 min.

Data Analysis

Lumicycle data were smoothed and detrended using a 2-hour and 24-hour running average, then baseline subtracted, and the pre-treatment rhythm was fit to a damped sine wave using Lumicycle Analysis software (Actimetrics). The pre-treatment period was calculated from at least 50 hours of data beginning ~35 hours after the onset of tissue incubation to reduce the transient effects of tissue preparation as described by others [11]. The post-treatment period was calculated using at least 50 hours of recording beginning the 15 hours after treatment, the point at which the error introduced by opening the PMT chamber no longer affected analysis. Period changes were calculated for each explant relative to its pre-treatment period and expressed as hours lengthened (positive values) or shortened (negative values). To assess phase shifts, the "predicted" post-treatment peak phase was extrapolated using the damped sine wave fit to the pre-treatment rhythm, and compared to the "observed" post-treatment peak phase, defined as that occurring 12 to 36 hours after treatment. Phase shifts are expressed as hours advanced (positive values) or delayed (negative values). Amplitude changes are expressed relative to pre-treatment peak height [calculated as 100x (post-treatment bioluminescence peak-trough/pre-treatment peak-trough)] as described by others [34]. Only explants showing rhythms with a goodness of fit >85% for the pre-treatment and post-treatment conditions were accepted.

Statistical Analysis
Data are presented as means ± SEM. Statistical differences were determined using one-way ANOVA (using Tukey’s or Dunnett's correction for post hoc analysis), or two-way ANOVA (using Tukey's correction for post hoc analysis) where appropriate using Prism software (GraphPad). Differences were considered significant if $p < 0.05$.

**Results**

**Unstimulated mPER2Luc Adrenals**

Rhythms from left and right hemi-adrenals were compared under baseline conditions. Since there were no differences in period or peak phase between hemi-adrenals prior to treatment (Table 1), hemi-adrenals were assigned randomly to treatment conditions. Following pre-treatment data collection, adrenal explant cultures were replaced with fresh media (vehicle) or media containing ACTH or other treatments. Absolute (Fig. 1A) and detrended (Fig. 1B) bioluminescence for multiple adrenals is shown for a representative experiment consisting of vehicle treatment. Although amplitude dampening occurred over days in culture, the period of the rhythm for the entire duration of the experiment (22.8±0.1) did not differ from the period calculated for pre-treatment (22.5±0.1) and post-treatment (22.8 ±0.2).

**Responses to ACTH**

To establish whether there was a phase-dependent response, ACTH was administered at CT2, 6, 10, 14, 18 and 22. Absolute (Fig. 1C) and detrended (Fig. 1D) bioluminescence for multiple adrenals is shown for an experiment measuring the response to 10 nM ACTH administered at CT18. There were no differences in the period or amplitude of the rhythm between the pre-treatment and post-treatment conditions (see Table 2). Following ACTH treatment, the peak phase of the mPER2Luc rhythm occurred at ~118h (Fig. 1D); the phase was delayed compared to the peak phase following vehicle treatment (shown in Fig. 1B) of ~110h.

The pre-treatment peak phase and period of the mPER2Luc rhythm did not differ across treatment groups at any CT (Table 2). Treatment with ACTH 1-39 (10 and 100 nM) produced a phase delay in the...
mPer2::Luc rhythm in a phase-dependent fashion with differences from vehicle at CT10, 14, 18, and 22 (only 10.0 nM tested), but no response at CT2 or 6. The response to forskolin (20.0 uM) paralleled that of ACTH, producing a phase delay only at CT10, 14 and 18 (Figure 2). There was no effect of ACTH or forskolin on the period or amplitude of the rhythm except a change in period at CT2 (Table 2). The response to ACTH 1-39 at CT18 was concentration-dependent with a minimum effective concentration of 1.0 nM compared to vehicle (Figure 3A); there was no concentration-dependent change in period or amplitude following ACTH (data not shown). To assess peptide specificity, equimolar concentrations (10.0 nM) of ACTH 1-39 were compared to VIP 1-28 and CRH 1-41 at CT18. Results confirmed the phase delay produced by ACTH, but neither CRH nor VIP produced a similar response (Figure 3B). Changes in period were not observed in response to ACTH, CRH or VIP (data not shown).

These results show a phase-dependent delay in the mPER2Luc rhythm following treatment with ACTH 1-39; treatments were given at different CTs without wash out to avoid potential changes in bioluminescence produced by changing the media as noted by others [54]. To confirm the phase-dependent response to ACTH, pulses of ACTH 1-39 (1.0 nM) were administered at different CTs by replacement with fresh media after 1h of incubation. The pretreatment peak phase and period of the mPER2Luc rhythm did not differ between treatment groups at any CT (Table 3). In comparison to vehicle, ACTH pulses produced only a phase delay that was restricted to CT18 (Figure 4). To confirm that the response to ACTH was independent of time in culture, adrenal tissue was given an ACTH pulse at CT22 of the preceding cycle (CT(-2)). The response to ACTH at CT(-2) also was a phase delay, supporting the conclusion that ACTH produces a phase shift that is dependent on the phase of the rhythm not time in culture (Figure 4). There was no effect of ACTH 1-39 pulses at any CT on the period of the mPER2Luc rhythm (Table 3).

Finally, to examine whether the response is dependent on the ACTH receptor, pulses of ACTH 1-39 were given in the presence and absence of ACTH fragments that bind the MC2R but do not activate a steroidogenic response [21, 24]. Whereas pulses of ACTH 1-39 (1.0 nM) produced a phase delay at CT18, there was no response to high concentrations of either ACTH 7-38 (10.0 uM) or ACTH 11-24 (0.1
(Figure 5A and B). Co-administration did not affect the response to ACTH 1-39, showing that neither ACTH 11-24 nor ACTH 7-38 acts as an inhibitor of ACTH 1-39 under these culture conditions (Figure 5A and B). Since adrenal expression of the MC5R has been reported \[17\], it is possible that the ACTH response is mediated by the MC5R. However, neither 1 nM nor 10 nM alpha MSH produced a phase delay compared to 1 nM ACTH 1-39 (Figure 5C). Together these results support the selectivity of the ACTH 1-39 peptide, suggesting that the ACTH-induced phase delay requires activation of the MC2 receptor.

**Discussion**

Using adrenal explants from mPER2Luc mice, we found that ACTH produces a phase delay in the mPER2Luc rhythm that is phase-dependent, concentration-dependent, peptide-specific and ACTH 1-39-selective. The monophasic nature of the ACTH response eliminates the possibility that it is the sole mechanism for entraining the adrenal clock to a LD cycle. However, these data support the possibility that ACTH induced by stress could reset the adrenal clock in a phase-dependent fashion.

The adrenal cortex expresses a clock that generates a circadian rhythm in glucocorticoids by inducing StAR, a clock-controlled gene, in a cyclical fashion \[47\]. Although this clock is sufficient to elicit rhythmic steroidogenesis independently of environmental cues, it requires extrinsic input for entrainment to environmental stimuli. The circadian glucocorticoid rhythm is dependent on the SCN \[38\] that is entrained by LD transitions. By constructing a phase response curve (PRC), we tested the hypothesis that ACTH mediates photic entrainment of the adrenal clock, defined by a phase delay followed by a phase advance \[15\]. Although the adrenal cannot respond directly to light, the term "photic entrainment" is used to refer to the process by which extrinsic signals to the adrenal convey light-dark information from the SCN to entrain adrenal rhythms. Our results showed that ACTH produced a phase delay only, suggesting that ACTH alone does not mediate photic entrainment. The circadian rhythm in ACTH shows a low amplitude (2-fold) relative to that of corticosterone (10-fold), requiring a rhythm in adrenal sensitivity to amplify the ACTH signal \[10\]. Sympathetic denervation reduces adrenal sensitivity,
evidence for adrenal innervation as an underlying mechanism [49]. Since rhythms in sympathetic activity
are driven by SCN-dependent pathways [5], and neural input stimulates adrenal Per expression [20],
sympathetic input in concert with rhythmic ACTH could mediate photic entrainment of the adrenal clock.
Adrenocortical innervation includes VIPergic fibers [23]. Since corticosterone rhythms are suppressed in
constitutive VIP knockout [26, 27] and VIP receptor knockout mice [13, 45], ACTH could complement
VIPergic innervation to mediate photic entrainment of the adrenal clock.

The PRC using adrenal explants showed that treatment with ACTH at 4h intervals produced a
phase delay with a peak response at CT18. Neither the period nor the amplitude of the rhythm was
affected by ACTH. The magnitude of the phase shift at CT18 was concentration-dependent and peptide-
specific; responses were not observed following CRH 1-41 or VIP 1-28, peptides that bind receptors in
the adrenal cortex to elicit steroid responses [32, 50]. Since ACTH is degraded within 2 hours in adrenal
tissue culture [3], treatment at CTs separated by 4h should provide "pulse-like" exposure to ACTH;
however, the resolution of the PRC could be masked by lingering bioactivity. To more precisely define
the phase shift, the PRC was reexamined using 60 min pulses of ACTH, yielding a similar curve with a
significant delay limited to CT18. Pulses of ACTH at CT(-2), corresponding to CT22 of the previous
cycle, also produced a phase delay. These data show that ACTH produces a phase delay in late CTs that is
dependent on the phase of the rhythm and not the duration of explant culture.

Multiple approaches were used to determine whether ACTH was acting via the MC2R to phase
shift the adrenal clock. Since the MC2R is a Gi protein-coupled receptor linked to activation of adenylyl
cyclase and cAMP generation [9, 43], clock entrainment by ACTH may be cAMP-dependent. It is well
established that cAMP is involved in resetting the SCN and peripheral clocks [33, 34, 37, 48]. Forskolin,
an activator of adenylyl cyclase, produced a PRC similar to that of ACTH. In contrast, neither VIP nor
CRH elicited a phase shift when administered at CT18, although both affect adrenal steroidogenesis via
Gs-coupled receptors [32, 50]. A more thorough examination of these peptides is warranted, yet these
findings further support the specificity of MC2R activation by ACTH in resetting the adrenal clock.

Putative MC2R antagonists also were tested. Both ACTH 7-38 [24] and ACTH 11-24 [25] bind to the
MC2R, but show low potency relative to ACTH 1-39 in activating a steroidogenic response. Neither pulses of ACTH 7-38 nor ACTH 11-24 produced phase shifts, supporting the specificity of ACTH 1-39 in eliciting a response. However, neither peptide altered the response when co-administered with ACTH 1-39. As shown for other G protein-coupled receptors [1], multiple binding sites on the MC2R have been postulated that differentially affect agonist and antagonist activity [21]. Under the required experimental conditions of 3-4 days for explant culture, it is possible that antagonist affinity for the MC2R is reduced for these ACTH fragments rendering them ineffective. In addition to the MC2R, the rodent adrenal cortex expresses the MC5R [51], a receptor that can be activated by ACTH 1-39; in contrast, the MC1, MC3 and MC4 receptors have not been identified in the adrenal cortex [7]. Since alpha MSH is a more potent ligand for the MC5R compared to ACTH [14] and alpha MSH does not activate the MC2R [30], alpha MSH was tested. We found that alpha MSH produced no phase shift, suggesting that the response to ACTH is not MC5R-mediated. Although the absence of MC2R blockade is a limitation, the selectivity of ACTH 1-39 argues strongly for MC2R-dependency in the phase shift of the adrenal clock rhythm.

The mechanism for a phase-dependent response of the adrenal clock rhythm to ACTH is unclear. Since high concentrations of ACTH administered at early CTs were ineffective in phase shifting the mPER2Luc rhythm, it is likely that adrenal clock sensitivity to ACTH varies in a cyclical fashion, as shown for the steroidogenic responses to ACTH [10, 42]. Similar to light-induced phase shifts of the SCN clock [52], phase resetting of the adrenal clock may result from ACTH-induced increases in adrenal PER that vary with the time of stimulation to differentially change PER negative feedback on the molecular clock. Additional studies are warranted to assess this possibility.

**Perspectives and Significance**

The novel finding that ACTH shifted the phase of the mPER2Luc rhythm in a monophasic fashion at a concentration in the high physiological range (0.1 nM) suggests that stress-induced ACTH might be capable of resetting the adrenal clock *in vivo*. The adrenal clock drives the rhythm in circulating glucocorticoids [35, 47]. Thus, stress-induced increases in ACTH and subsequent resetting of the adrenal clock phase likely would shift the phase of the glucocorticoid rhythm. Since rhythmic glucocorticoids are
critical for maintaining homeostasis by controlling glucose metabolism [46], entrainment of other peripheral clocks [2, 22, 36] and regulating clock gene rhythms in brain nuclei implicated in control of motivation and emotional states [44], acute stress could have persistent deleterious effects on organ system function by altering the timing of glucocorticoid-dependent rhythms. Since the disruption of circadian rhythms in glucocorticoids is associated with multiple somatic and psychiatric disorders [8], understanding the mechanisms for ACTH-induced resetting of the adrenal clock rhythm could facilitate the development of optimal treatment strategies.
Acknowledgments

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Disclosure: The authors have nothing to disclose.
Figure Legends

Figure 1
Representative experiment showing the mPER2luc rhythm in multiple adrenal explants before (panels A and C) and after detrending (panels B and D) under pre-treatment conditions and after post-treatment with vehicle (panels A and B) or ACTH (10.0 nM) (panels C and D) at CT18. Note the peak phase of the rhythm following vehicle (panel B, black arrowhead in inset) of ~110h precedes the peak phase following ACTH of ~118h (panel D, black arrowhead in inset), reflecting an ACTH-induced phase delay in the mPER2Luc rhythm.

Figure 2
Phase response curve showing phase shifts in the adrenal mPER2luc rhythm following treatment with vehicle, ACTH or forskolin administered at multiple circadian times (CTs). p<0.05 vs Vehicle.

Figure 3
A. Concentration-response curve showing phase shifts in the adrenal mPER2Luc rhythm following treatment with ACTH at circadian time (CT)18. *p < 0.05 vs Vehicle; ^p < 0.05 vs 0.1 nM ACTH; +p < 0.05 vs 1.0 nM ACTH.

B. Comparison of the phase response of the adrenal mPER2Luc rhythms to ACTH 1-39 (10.0 nM), VIP 1-28 (10.0 nM) and CRH 1-41 (10.0 nM) administered at CT18. *p < 0.05 vs Vehicle

Figure 4 Phase response curve showing phase shifts in the adrenal mPER2Luc rhythm following pulses (60 min) of ACTH (1.0 nM) compared to vehicle. *p < 0.05 vs Vehicle
A. Effect of ACTH 7-38 on phase shifts in the adrenal mPER2Luc rhythm induced by pulse (60 min) treatment with ACTH 1-39 at circadian time (CT)18. *p<0.05 vs Vehicle; ^p<0.05 vs ACTH 7-38.

B. Effect of ACTH 11-24 on phase shifts in the adrenal mPER2Luc rhythm induced by pulse treatment with ACTH 1-39 at CT18. *p<0.05 vs Vehicle; ^p<0.05 vs ACTH 11-24.

C. Effect of pulse treatment with alpha melanocyte-stimulating hormone (αMSH) compared to ACTH 1-39 (1.0 nM) on phase shifts in the adrenal mPER2Luc rhythm at CT18. *p<0.05 vs Vehicle; ^p<0.05 vs αMSH.
References


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A

Phaseshift (h)

Vehicle 0.1 1.0 3.0 10.0

ACTH (nM)

(15) (6) (15) (8) (15)

* ^ +

B

Vehicle ACTH CRH VIP

Phase shift (h)

n= 4-7

*
Peak Phase (h)

Vehicle (n=4-12)

ACTH (1.0 nM) (n=4-12)

Phase Shift (h)

CT (h)
Table 1. Rhythmic parameters across adrenal hemisections under pretreatment conditions.

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<th>Period (h)</th>
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<td>Right 2</td>
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Data are presented as means ± SEM.
Table 2. Rhythmic parameters under pretreatment conditions and treatment-induced changes in period and amplitude following phase-dependent treatment with ACTH or forskolin.

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Data are presented as mean ± SEM. *p<0.05 vs Vehicle.
Table 3. Rhythmic parameters under pretreatment conditions and ACTH-induced changes in period following phase-dependent pulse administration.

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<th>Period change (h)</th>
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<td>ACTH</td>
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<td>14.2 ± 0.3</td>
<td>22.5 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>Vehicle</td>
<td>4</td>
<td>14.0 ± 0.3</td>
<td>22.4 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>4</td>
<td>14.1 ± 0.2</td>
<td>22.8 ± 0.1</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>Vehicle</td>
<td>6</td>
<td>15.5 ± 0.4</td>
<td>24.1 ± 0.3</td>
<td>-1.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>7</td>
<td>15.7 ± 0.3</td>
<td>23.6 ± 0.3</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>14</td>
<td>Vehicle</td>
<td>6</td>
<td>15.9 ± 0.4</td>
<td>24.1 ± 0.1</td>
<td>-0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>7</td>
<td>16.3 ± 0.5</td>
<td>24.1 ± 0.3</td>
<td>-0.7 ± 0.2</td>
</tr>
<tr>
<td>18</td>
<td>Vehicle</td>
<td>9</td>
<td>15.1 ± 0.5</td>
<td>23.8 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>7</td>
<td>15.7 ± 0.5</td>
<td>24.1 ± 0.2</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>22</td>
<td>Vehicle</td>
<td>11</td>
<td>15.5 ± 0.3</td>
<td>23.6 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>12</td>
<td>15.7 ± 0.4</td>
<td>23.7 ± 0.2</td>
<td>0.3 ± 0.4</td>
</tr>
</tbody>
</table>

ACTH (1.0 nM) was administered as a 60 min pulse. Data are presented as mean ± SEM.