Reciprocal Regulation of TREK-1 Channels by Arachidonic Acid and CRH in Mouse Corticotropes

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Arachidonic acid (AA) is generated in the anterior pituitary gland upon stimulation by the ACTH secretagogue, CRH. Using the patch clamp technique, we examined the action of AA on the excitability of single pituitary corticotropes obtained from a transgenic mouse strain that expresses the enhanced green fluorescent protein driven by the proopiomelanocortin promoter. CRH evoked depolarization, but AA caused hyperpolarization. Under voltage clamp condition, AA caused a rapid inhibition of the delayed rectifier K^+ current and then increased a background K^+ current. Inhibition of AA metabolism did not prevent the activation of the K^+ current by AA, suggesting a direct action of AA. The sensitivity of the AA-activated K^+ current to fluoxetine, chlorpromazine, extracellular acidification, diphenylbutylpiperidine antipsychotics, and the membrane permeable cAMP analog [8-(4-chlorophenylthio)-cAMP] suggest that the current is mediated via TWIK-related K^+ channel (TREK)-1 channels. Activation of the CRH receptors that are coupled to the adenylate cyclase pathway suppressed the activation of TREK-1 current by AA and reversed the AA-mediated hyperpolarization. Intracellular acidification (pH 7.0) increased the basal amplitude of TREK-1 current and resulted in hyperpolarizaton. CRH suppressed the basal TREK-1 current in cells with intracellular acidification and caused depolarization. Our finding indicates that TREK-1 channels are important in setting the resting potential in corticotropes. The opposing actions of CRH and AA on the excitability of corticotropes raise the possibility that AA may act as a negative feedback regulator to reduce the stimulatory action of CRH and thus prevent excessive ACTH release during chronic stress. (***Endocrinology*** **152: 1901–1910, 2011)**

The endocrine response to stress is primarily controlled by the hypothalamic-pituitary-adrenal axis. During stress, neurons in the paraventricular nucleus of the hypothalamus release CRH into the portal circulation. CRH stimulates the release of the stress hormone, ACTH from pituitary corticotropes. The CRH-stimulated ACTH secretion is dependent on cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) elevation (1). We have shown previously in rat corticotropes that the stimulation of CRH receptors causes closure of a background K^+ current, which in turn leads to membrane depolarization and activation of voltage-gated Ca^{2+} channels (2). The depolarization-evoked rise in $[Ca^{2+}]_i$ in turn triggered exocytosis in rat corticotropes (3). Thus, the release of ACTH during CRH stimulation is regulated by the electrical excitability of the corticotropes. However, the identity of the CRH-sensitive background K^+ current in corticotropes remains elusive. CRH also evokes the release of arachidonic acid (AA) from rat pituitary cells (4, 5). The role of AA in ACTH secretion is controversial. At high concentrations (>100 μM), AA stimulated basal ACTH release (4, 6), and inhibition of AA metabolism was reported to either enhance or reduce the CRH-mediated ACTH release (4–6). The complexity of the effects of AA on ACTH secretion may be partially related to the diverse

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Abbreviations: AA, Arachidonic acid; AVP, arginine vasopressin; AZF, adrenal zona fasciculata; 8CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; DPBP, diphenylbutylpiperidine; eGFP, enhanced green fluorescent protein; ETYA, eicosatetraynoic acid; pH_{ext}, pH in the extracellular solution; pH_{int}, intracellular pH; POMC, proopiomelanocortin; TASK, TWIK-related acid sensing K^+ channel; TEA, tetraethylammonium; TRAAK, TWIK-related arachidonic acid activated K^+ channel; TREK, TWIK-related K^+ channel; TWIK, tandem of pore domains in a weak rectifying K^+ channel.

actions of AA on ion channels. For example, AA was found to modulate multiple types of voltage-gated K^+ channels, including the inhibition of the A-type and the delayed rectifier K^+ channels (7). In addition, AA was reported to inhibit some members of the tandem of pore domains in a weak rectifying K^+ channel (TWIK)-related acid-sensing K^+ channels (TASK) family but activated all three members [TWIK-related K^+ channel (TREK)-1, TREK-2, and TWIK-related arachidonic acid activated K^+ channel (TRAAK)] of the TREK (TWIK-related K^+ channels) family (8). Both the TWIK-related acid-sensing K^+ channels and TREK families belong to the tandem-pore class of K^+ (K_{2P}) channels, which are important in the setting of the resting potential in many cell types (8). In view of the importance of membrane excitability in the stimulatory action of CRH in rat corticotropes (2), it is possible that AA may affect corticotropes via its actions on ion channels and cellular excitability. In the current study, we employed mouse corticotropes obtained from the pro-opiomelanocortin (POMC)-enhanced green fluorescent protein (eGFP) transgenic mice (9–11). Because the POMC promoter sequence targets eGFP expression to all cell types that normally express POMC gene products (*e.g.* ACTH, β-lipotropin, α-MSH, and β-endorphin), the ACTH containing pituitary corticotropes and MSH containing intermediate lobe melanotropes in this transgenic mouse model also express GFP fluorescence (12). We found that AA caused hyperpolarization in GFP-labeled mouse corticotropes via the activation of TREK-1 channels. Most importantly, CRH acting via the cAMP-dependent pathway inhibited the same channel and caused depolarization. Inhibition of TREK-1 channels at basal condition by CRH or fluoxetine resulted in depolarization. In the presence of fluoxetine, the CRH-mediated depolarization was attenuated. Overall, our findings indicate that TREK-1 channels have major roles in the setting of the resting potential in corticotropes, and the activities of the TREK-1 channels are reciprocally regulated by CRH and AA. The generation of AA in the corticotropes after CRH stimulation can cause hyperpolarization, which in turn limits the depolarizing actions of CRH. This raises the possibility that AA may act as an intrinsic negative regulator of ACTH secretion during chronic stress.

Materials and Methods

Cell culture

The generation of the POMC-eGFP transgenic mice was as described previously (9). To identify mice that express POMC-eGFP from the mixed litter, the pituitary gland was removed from mice killed in accordance with standards of the Canadian Council on Animal Care. Because the intermediate lobe of the pituitary gland comprises mostly melanotropes that express α-MSH, a POMC gene product, the intermediate lobe of the pituitary gland in the POMC-eGFP mouse exhibits intense green fluorescence when viewed under a dissecting microscope. The anterior lobes of the pituitary glands were dissected from these mice and dissociated enzymatically as previously described (13). Dissociated pituitary cells were plated on poly-lysine (0.1 mg/ml; Sigma-Aldrich Canada, Oakville, Ontario, Canada) coated glass coverslips and maintained in standard culture condition in DMEM supplemented with 10% (vol/vol) horse serum, 50 U/ml penicillin G, and 50 mg/ml streptomycin (all from Sigma) for 1–3 d. Individual corticotropes were identified from the heterogeneous pituitary cell population by their GFP fluorescence (observed with an excitation filter at 400–440 nm and an emission filter at 520–560 nm).

Reverse hemolytic plaque assay

The procedure for the reverse hemolytic plaque assay was similar to that described previously (2, 14, 15). Briefly, dissociated mouse pituitary cells were suspended in DMEM supplemented with 0.1% (wt/vol) BSA. The pituitary cell suspension was then mixed with one-third the volume of 12% (vol/vol) sheep erythrocytes (Colorado Serum Co., Denver, CO) in 0.9% (wt/vol) NaCl. The erythrocytes were previously conjugated with *Staphylococcus aureus*-derived protein A (Sigma), using 0.2 mg/ml $CrCl_3$ as a catalyst. The combined cell suspension was incubated with a mixture of 10 nM CRH and 100 nM arginine vasopressin (AVP) and rabbit polyclonal antibodies to rat ACTH (1:20 dilution; gift from R. J. Kemppainen, Auburn University, Auburn, AL) for 3 h at 37 C. Plaques (zone of lysed erythrocytes) were formed by a 30-min exposure to guinea pig complement at 1:50 dilution.

Electrophysiological recording

Membrane potential or current was recorded with the whole-cell patch clamp technique with an EPC-9 patch clamp amplifier (HEKA Electronics, Mahone Bay, Nova Scotia, Canada). The standard bath solution contained (in mM): 150 NaCl, 10 HEPES, 8 glucose, 2.5 KCl, 2 $CaCl_2$, and 1 $MgCl_2$ (pH 7.4). For most experiments, the whole-cell pipette solution contained (in mM): 120 K-aspartate, 20 HEPES, 20 KCl, 1 $MgCl_2$, 10 EGTA, 2 Na_2ATP, and 0.1 Na_4GTP (pH 7.4). Because TREK-1 current can be increased by intracellular acidification (16), we employed a whole-pipette solution that was buffered to pH 7.0 in some experiments. All experiments were performed at room temperature (20–23 C). A −10 mV, junction potential was corrected throughout. For the generation of IV plots, individual cell was voltage clamped at −70 mV and then stepped to different potentials (−80 to 40 mV in 10-mV increment) for 200 msec. The current amplitude at different potentials was averaged from the last 40 msec of the voltage step.

Statistical analysis

Origin program 8 (OriginLab Corp., Northampton, MA) was employed for plotting and statistical procedures. The two-sample Student's *t* test was used in comparisons of values between two populations of cells. Any difference with $P < 0.05$ was considered statistically significant and was marked with an asterisk in the figures. All values shown were means ± SEM.

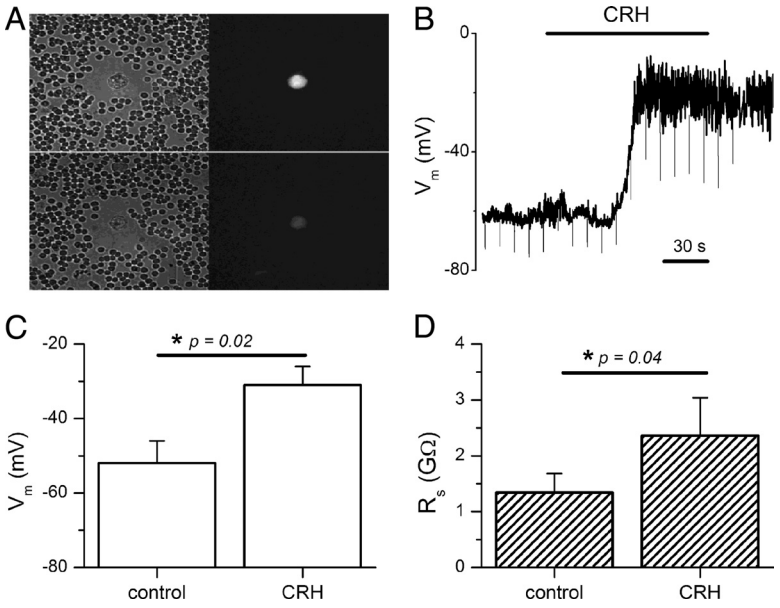

FIG. 1. GFP-fluorescent mouse corticotropes secreted ACTH and depolarized in response to CRH stimulation. A, The plaque-forming mouse pituitary cells in the reverse hemolytic plaque assay were GFP fluorescent. Brightfield (*left panels*) and fluorescent images (*right panels*) of two mouse pituitary cells that formed plaques. B, Application of CRH (20 nM) evoked depolarization in a GFP-fluorescent cell. The downward deflections in membrane potential, V_m, were induced by the injection of current pulses (−5 pA) to monitor changes in cell input resistance. C, CRH caused depolarization. The resting V_m (control) and V_m in the presence of CRH (20 nM) averaged from five corticotropes. D, The CRH-mediated depolarization was accompanied by an increase in cell input resistance. The average cell input resistance (R_s) before (control) and in CRH (same cells as in C).

Results

CRH caused membrane depolarization in GFP-fluorescent mouse corticotropes

To confirm that the GFP-fluorescent cells isolated from the anterior pituitary gland are corticotropes, we examined whether individual GFP-fluorescent cells secrete ACTH when challenged with CRH and AVP. To measure ACTH secretion from single cells, we employed the reverse hemolytic plaque assay using a polyclonal antibody to rat ACTH. Figure 1A shows that individual GFP-fluorescent mouse pituitary cells formed plaques (a zone of lysed erythrocytes), indicating that ACTH was released from the GFP-fluorescent cells. In 49 GFP-fluorescent cells examined, approximately 73% of the cells formed plaques, suggesting that upon stimulation, most of the GFP-fluorescent cells can secrete sufficient amount of ACTH that is detectable with the reverse hemolytic plaque assay. For the rest of this study, we identified individual corticotropes from POMC-eGFP transgenic mice based on their GFP fluorescence. Using the whole-cell patch technique, we examined whether CRH affected the membrane potential. For the majority of the experiments described here (except for figure 8, C and D), the whole-cell pipette solution was buffered to pH 7.4. Figure 1B shows that CRH evoked depolarization in a GFP-fluorescent mouse corticotrope. To examine whether the depolarization was accompanied by changes in cell input resistance, we injected small current pulses (5 pA) into the cell. In five cells examined, CRH depolarized the cell membrane potential from -52 ± 6 to -31 ± 5 mV (ΔV_m of ~21 mV) (Fig. 1C) and increased the cell input resistance from 1.3 ± 0.3 to 2.4 ± 0.8 GΩ (Fig. 1D). This result suggests that the CRH-mediated depolarization in mouse corticotropes may involve the closure of certain background conductance.

AA caused hyperpolarization via the activation of an outward current

In contrast to the depolarizing action of CRH, AA caused membrane hyperpolarization. As shown in Fig. 2A, application of AA (5 μM) resulted in strong hyperpolarization. The membrane potential was restored to basal level upon the removal of AA. Note that in the continued presence of AA, the membrane potential reached a plateau (*e.g.* see figure 8A). The resting potential of mouse corticotropes typically ranged from −40 to −50 mV. In 53 cells examined, the resting potential was -48 ± 3 mV. Figure 2B shows the average hyperpolarization caused by 5 or 10 μM AA. For cells challenged with 5 μM AA, their mean resting potential before and after AA was -40 ± 2 and -78 ± 3 mV (n = 21) (Fig. 2B). For cells challenged with 10 μM AA, their mean resting potential was hyperpolarized from -45 ± 2 to -82 ± 3 mV (n = 4) (Fig. 2B). To examine the ionic mechanisms underlying the AA-mediated hyperpolarization, we voltage-clamped individual corticotropes at −70 mV and applied voltage steps (200 msec in duration) to different potentials (10-mV increment) during the time course of the application of AA. An example of this experiment is shown in Fig. 3. In control condition (before AA application), outward currents that resembled the delayed rectifier could be evoked at potentials more than or equal to −20 mV (Fig. 3Ai). Application of AA (10 μM) resulted in a gradual increase in the holding current at −70 mV (Fig. 3B). About 2 min after the beginning of AA perfusion, the amplitude of the delayed rectifier-like current (activated at potentials ≥ −20 mV) was reduced, and there was an acceleration in the decay of this voltage-activated current (Fig. 3Aii). Subsequently (~5 min after the onset of AA perfusion), AA completely suppressed the delayed rectifier-like current and activated an outward current that exhibited little time dependence during the voltage step (Fig. 3Aiii). The removal of AA eliminated the time-independent outward

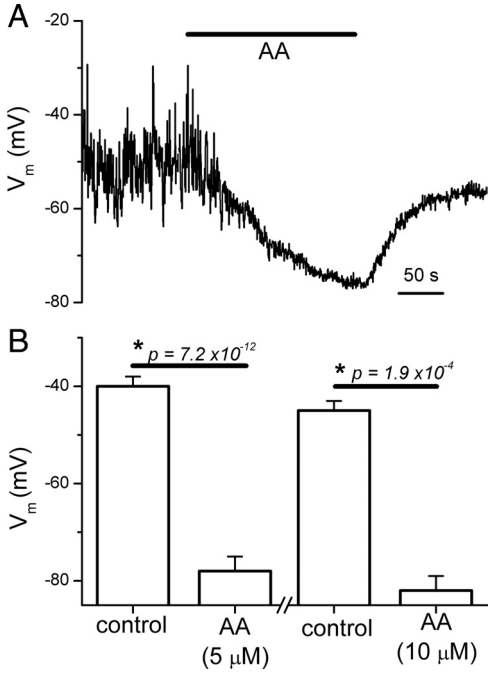

FIG. 2. AA caused hyperpolarization in corticotropes. A, Representative example of the changes in membrane potential (V_m) of a corticotrope when exposed to AA (5 μM). B, Mean hyperpolarization evoked by 5 or 10 μM AA. The resting V_m before (control) and in the presence of AA from the same group of cells were shown.

current (Fig. 3B) but failed to reverse the effects of AA on the delayed rectifier-like current (Fig. 3Aiv). As shown in Fig. 3C, AA initially suppressed the outward current evoked at potentials more than or equal to −20 mV (Fig. 3Cii) but subsequently activated a time-invariant outward current at all potentials examined (Fig. 3Ciii). The removal of AA reversed the activation of the time-invariant outward current but not the reduction in the amplitude or the acceleration in the decay kinetic of the delayed rectifier-like current (Fig. 3Civ). Because a change in the current level near the resting potential can strongly affect cellular excitability, we examined the change in current level by AA at −40 mV (near the mean resting potential of corticotropes) (Fig. 2B). In 53 cells examined, the mean membrane current at −40 mV before the application of AA (control condition) was −1.5 ± 0.7 pA, and this value increased to 102.6 ± 9.8 pA in the presence of 10 μM AA. For cells challenged with 5 μM AA, the increase in membrane current at −40 mV was approximately 3-fold smaller (30 ± 6 pA; n = 26), suggesting that the effect of AA on current activation is concentration dependent. Because the current response was more robust in 10 μM AA, we employed 10 μM AA in all subsequent experiments.

The AA-activated current did not require AA metabolism and was carried primarily by K^+

The result in Fig. 3 suggests that the initial effect of AA was a suppression of the delayed rectifier. Consistent with this notion, in the presence of tetraethylammonium (TEA) (an inhibitor of the delayed rectifier), only the enhancing effect of AA on the time-invariant outward current was observed (Fig. 4). As shown in Fig. 4A, application of TEA eliminated most of the delayed rectifier-like current evoked at potentials more than or equal to −20 mV. In the continued presence of TEA, AA activated an outward current at potentials more than −80 mV (Fig. 4B), and this current exhibited little time dependence during the individual voltage step (Fig. 4A). Because AA is metabolized in cells into a variety of eicosanoid products, we tested whether the effect of AA on the outward current requires AA metabolism. We examined whether the actions of AA can be prevented by eicosatetraynoic acid (ETYA), a nonmetabolizable analog of AA that is a blocker of lipoxygenase, cycloxygenase, and P450 pathways for AA metabolism (17). As shown in the example in Fig. 4C, application of ETYA (30 μM) partially reduced the current evoked at potentials more than 0 mV, suggesting that ETYA partially mimicked the inhibitory action of AA on the delayed rectifier. However, in the continued presence of ETYA, AA (10 μM) could still enhance the time-invariant outward current at all potentials. In the presence of extracellular ETYA (30 μM), the mean increase in outward current at −40 mV by AA (10 μM) was 81.2 ± 29.4 pA (n = 3). In separate experiments, we included ETYA (30 μM) in the whole-cell pipette solution. Under this condition, exogenous AA (10 μM) could still activate an outward current, and the mean increase in current at −40 mV was 104.7 ± 35.0 pA (n = 4). These values are similar to the AA-mediated enhancement of current described above in control cells (102.6 ± 9.8 pA; n = 53). Thus, AA metabolism is not essential for the activation of outward current by AA. To examine whether the AA-activated current is a K^+ current, we voltage-clamped individual corticotropes at −70 mV in an extracellular solution containing 9, 19, 36, or 87 mM [K^+]. Voltage steps (200 msec in duration) were then applied to different potentials (10-mV increment) before and after the application of AA (10 μM). Because AA inhibited the delayed rectifier current, the difference between the current evoked in AA and the control (before AA application) comprised primarily the AA-activated current. Figure 5A shows examples of IV plot of the AA-activated current when cells were bathed in different extracellular [K^+]. Note that the reversal potential of the AA-activated current shifted toward more positive potentials with increasing extracellular [K^+]. Figure 5B summarizes the reversal potential of the AA-activated current obtained from various cells in the presence of the four different extracellular [K^+]. The slope of the linear regression is 45 mV (*cf.* 57 mV expected for K^+), suggesting that the AA-mediated current is carried primarily by K^+.

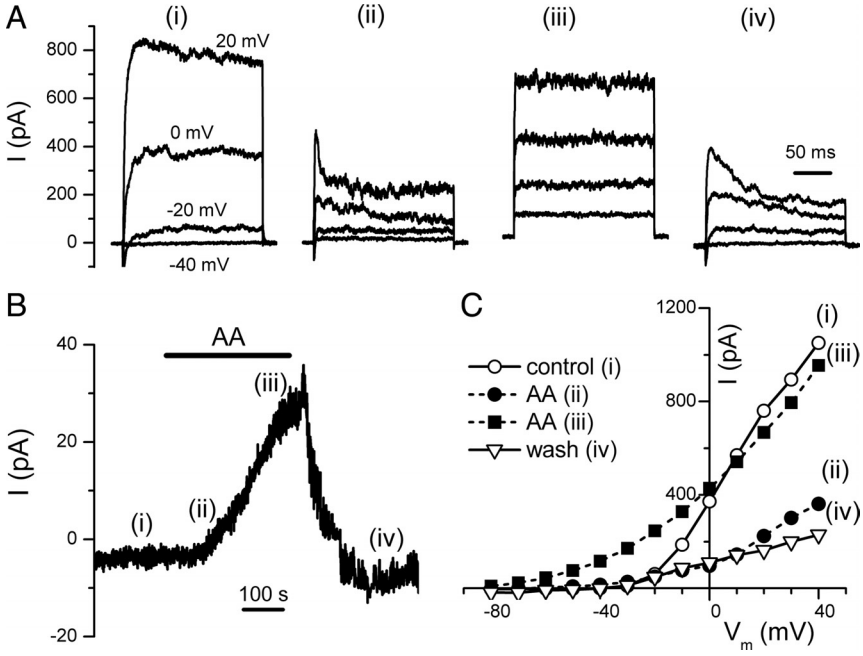

FIG. 3. Dual actions of AA on membrane current. A, Current traces evoked from voltage steps (200 msec) to −40, −20, 0, and 20 mV from a holding potential of −70 mV in control condition (i), in the presence of 10 μM AA (ii and iii), and after AA removal (iv). At approximately 2 min after starting the perfusion of AA, there was a reduction and a change in the decay kinetics of the delayed rectifier-like current evoked at potentials more than or equal to −20 mV (ii). After approximately 5 min of perfusion, AA increased an outward current that exhibited little time dependence during the voltage step (iii). The increase in the time-invariant outward current (but not the inhibition of the delayed rectifier-like current) was reversed upon the removal of AA (iv). B, Changes in the holding current at −70 mV during AA application. Same cell as in A. At the different times indicated (i–iv), the cell was voltage stepped to different potentials (−80 to 40 mV in 10-mV increment). C, IV plots at different time course of AA application. The current at different potentials was measured from the mean of the last 40 msec of the voltage step (same cell as in A and B). Note that the delayed rectifier-like outward current evoked at potentials more positive than −20 mV was initially suppressed by AA (ii). Subsequently, AA enhanced the time-invariant outward current (iii) at all potentials examined.

The AA-mediated current was due to activation of TREK-1 channels

Because AA was reported to activate the TREK family of K^+ channels (8), we examined the involvement of the TREK and TRAAK channels using pharmacological blockers. We first tested the antidepressant, fluoxetine, which was reported to be a potent inhibitor of human TREK-1 channels (18, 19). As shown in the example in Fig. 6A, application of fluoxetine (50 μM) in the continued presence of AA (10 μM) reduced the outward current over a wide range of potentials. In six cells examined, the mean reduction of the AA-mediated current by fluoxetine (50 μM) at −40 mV was 82 ± 7% (Fig. 6E). We found that the AA-mediated current could also be reduced by the antipsychotic drug, chlorpromazine, which was shown to inhibit both human TREK-1 and TREK-2 channels but not TRAAK channels (20). Figure 6B shows that application of chlorpromazine (10 μM) in the continued presence of AA suppressed the current at all potentials examined. On average, chlorpromazine (10 μM) reduced the AA-mediated current at −40 mV by 57 ± 7% (n = 6) (Fig. 6E). The sensitivity of the AA-mediated K^+ current to fluoxetine and chlorpromazine suggests that the AA response in corticotropes involves primarily TREK-1 or TREK-2 but not TRAAK channels. Consistent with this, Fig. 6C shows that ruthenium red (10 μM), which was reported to inhibit approximately 80% of the TRAAK channels (21), had only a minor effect (33 ± 12%; n = 8) on the AA-mediated current in corticotropes (Fig. 6E). Extracellular acidification was reported to inhibit TREK-1 channels but enhance TREK-2 channels (22). To further distinguish between the involvement of TREK-1 and TREK-2 channels, we lowered the pH in the extracellular solution (pH_{ext}) from 7.4 to 6.4. Figure 6D shows that extracellular acidification caused a partial inhibition of the AA-mediated current. In eight cells examined, extracellular acidification reduced the AA-mediated current at −40 mV by 44 ± 6% (Fig. 6E). Thus, the pharmacological profile of the AA-activated K^+ current in corticotropes resembles that of TREK-1 channels. The diphenylbutylpiperidine (DPBP) antipsychotics, such as penfluridol and pimozide, have been reported to be highly potent inhibitors of the TREK-1 channels that are basally expressed (*i.e.* in the absence AA) in bovine adrenal zona fasciculata (AZF) cells (23, 24). Figure 6F shows that penfluridol at 2 and 10 μM suppressed the current activated by 10 μM AA in mouse corticotropes by 30 ± 5% (n = 6) and 88 ± 4% (n = 6), respectively. A similar response was obtained with pimozide (Fig. 6F); pimozide at 2 and 10 μM reduced the current activated by 10 μM AA by 45 ± 10% (n = 5) and 86 ± 1% (n = 3), respectively. Interestingly, when the concentration of AA was lowered to 2 μM, pimozide at 2 μM reduced the AA-mediated current by 82 ± 6% (n = 3) (Fig. 6F). This raises the possibility that the potency of DPBP in suppressing TREK-1 channels may be related to the concentration of AA employed to activate the channels.

The AA-activated TREK current was negatively regulated by cAMP and CRH

The TREK, but not TRAAK, channels are sensitive to cAMP (16). Consistent with the activation of TREK-1 channels by AA, we found that the membrane permeable cAMP analog, 8-(4-chlorophenylthio)-cAMP (8CPT-cAMP), strongly reduced the AA-mediated outward current in corticotropes. Figure 7A shows that the increase in outward cur-

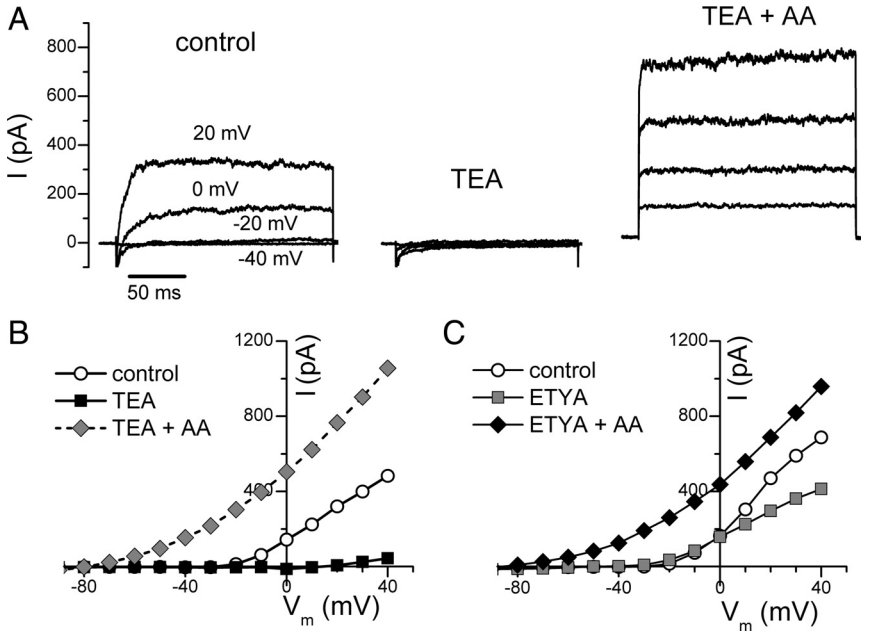

FIG. 4. The AA-mediated enhancement of the time-invariant outward current was not affected by TEA or ETYA. A, After the inhibition of the delayed rectifier by TEA, AA increased the time-invariant outward current. Current traces evoked from voltage steps (200 msec) to −40, −20, 0, and 20 mV from a holding potential of −70 mV in control, in TEA (10 mM), and in the presence of 10 μM AA + 10 mM TEA. Note that TEA alone eliminated most of the delayed rectifier evoked at positive potentials. In the presence of TEA, AA increased an outward current, which exhibited little time dependence. B, IV plots of the same cell (as in A) in control, in TEA, or in TEA + 10 μM AA. Note that in control condition, most of the delayed rectifier evoked at potentials more than or equal to −20 mV was suppressed by TEA. In the presence of TEA, AA enhanced the time-invariant outward current at all potentials examined. C, The AA-mediated enhancement of the time-invariant outward current did not require AA metabolism. ETYA, an inhibitor of AA metabolism, caused a small reduction in the delayed rectifier but could not prevent the enhancing effect of AA on the time-invariant outward current. IV plots of a cell in control, in ETYA (30 μM), and in ETYA + 10 μM AA.

rent at −70 mV by AA (10 μM) was essentially abolished by the application of 8CPT-cAMP (500 μM). As shown in Fig. 7B, 8CPT-cAMP suppressed the AA-activated current at a wide range of potential. In seven cells examined, 8CPT-cAMP (500 μM) reduced the AA-mediated current at −40 mV by 76 ± 7%. Because CRH receptors are coupled to adenylate cyclase, we examined whether the activation of CRH receptors also modulates the TREK current. Figure 7C shows that the AA-mediated increase in outward current at −70 mV was strongly suppressed by CRH (20 nM). The IV plot in Fig. 7D shows that CRH suppressed the AA-mediated current at a wide range of potentials. On average, CRH (20 nM) suppressed the AA-mediated current at −40 mV by 76 ± 7% (n = 7). Figure 8A shows that CRH also reversed the AA-mediated hyperpolarization. In sixteen cells examined, the AA-mediated hyperpolarization was −38 ± 3 mV, and the application of CRH (in the presence of AA) depolarized the cells by 26 ± 3 mV (n = 12) (Fig. 8B). Thus, in the presence of AA, the CRH-mediated depolarization could only partially restore the potential to the basal values (before AA). Application of the TREK-1 channel inhibitor, fluoxetine (50 μM), to corticotropes under basal condition (without AA) depolarized the cells by 23 ± 3 mV (n = 23) (Fig. 8B), similar to the depolarization evoked by CRH (~21 mV) (Fig. 1C). In the presence of fluoxetine, the CRH-evoked depolarization (ΔV_m = 11 ± 2 mV; n = 10) (Fig. 8B) was significantly smaller (P = 0.04) than that evoked by CRH in control cells (ΔV_m = 21 ± 5 mV) (Fig. 1C), suggesting that the CRH response was attenuated by the inhibition of TREK-1 channels.

In all the experiments described above, the intracellular pH (pH_{int}) was buffered to pH 7.4 with the whole-cell pipette solution. Because intracellular acidification was known to activate TREK-1 channels (16), we examined whether lowering the pH_{int} to 7.0 affected the basal activity of TREK-1 channels in corticotropes. Figure 8C shows that in cells recorded with a whole-cell pipette solution of pH 7.0, the amplitude of the background TREK-1 current at −40 mV was 24 ± 4 pA (n = 21). This value is comparable with the current recorded from cells with pH_{int} of 7.4 when stimulated by 5 μM AA (30 ± 6 pA; n = 26) but much larger than the basal TREK-1 current at pH_{int} 7.4 (−1.5 ± 0.7 pA; n = 53). As shown in Fig. 8C, application of CRH (20 nM) or fluoxetine (50 μM) essentially eliminated the background TREK-1 current in cells with intracellular acidification. The increase in basal TREK-1 current with intracellular acidification was accompanied by hyperpolarization. Figure 8D shows that the mean resting potential in corticotropes with pH_{int} 7.0 (−82 ± 2 mV; n = 22) was more negative (ΔV_m of 34 ± 5 mV) than that recorded from cells with pH_{int} of 7.4 (−48 ± 3 mV; n = 53) but comparable with corticotropes (with pH_{int} 7.4) that were stimulated with AA (Fig. 2). Application of CRH (20 nM) or fluoxetine (50 μM) caused a similar depolarization in these cells (ΔV_m of 35 ± 4 mV for CRH and 36 ± 4 mV for fluoxetine) (Fig. 8D). Thus, the activity of TREK-1 channel is responsible for setting the resting potential of corticotropes.

Discussion

AA inhibited the delayed rectifier but increased the TREK-1 current

Our results show that in GFP-labeled mouse corticotropes, exogenous application of AA caused an initial sup-

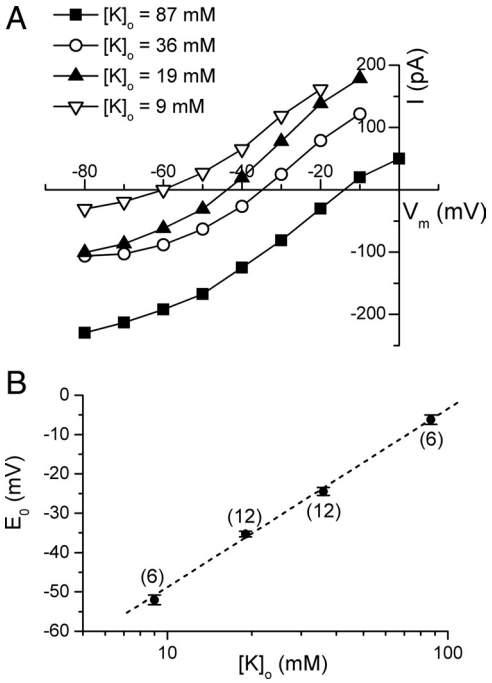

FIG. 5. The AA-mediated current was carried primarily by K^+. A, IV plots of the AA-mediated current (difference between the current evoked before and in the presence of 10 μM AA) when cells were bathed in different $[K^+]$ ($[K^+]_o$). B, Plot of the reversal potential (E_o) of the AA-mediated current *vs.* extracellular $[K^+]$. The slope of the linear regression was 45 mV. Each data point was the average of the reversal potential obtained from six or 12 cells.

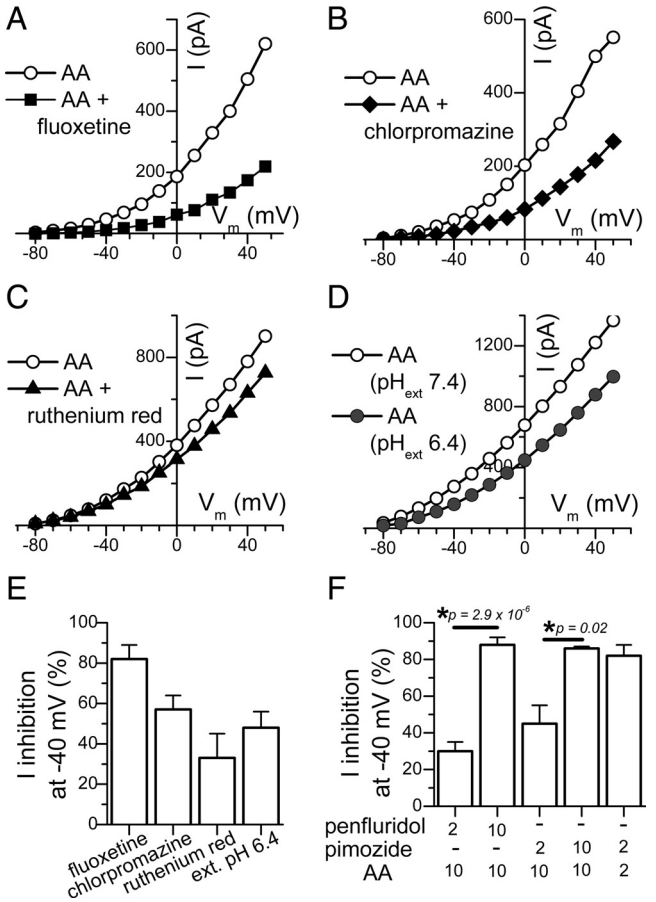

FIG. 6. The AA-activated current involves TREK-1 channels. A, Application of the TREK-1 channel inhibitor, fluoxetine (50 μM), strongly suppressed the AA-mediated current at a wide range of potentials. B, The AA-mediated current was also reduced by chlorpromazine (10 μM), an inhibitor of TREK-1 and TREK-2 channels. C, Application of ruthenium red (10 μM), an inhibitor of TRAAK channels, had only minor inhibitory effect on the current. D, External acidification (pH_{ext} 6.4) caused a partial inhibition of the current. A–D, Representative IV plots from four different cells when exposed to 10 μM AA and subsequently to the various inhibitors (in the continued presence of AA). E, Summary of the average reduction of AA-mediated current at −40 mV by fluoxetine (n = 6), chlorpromazine (n = 6), ruthenium red (n = 8), and pH_{ext} 6.4 (n = 8). F, The effect of penfluridol and pimozide (2 or 10 μM) on the amplitude of AA-mediated current at −40 mV. Values in each group were averaged from three to six cells.

pression and acceleration in the current decay of the delayed rectifier (Fig. 3A). Similar actions of AA on the delayed rectifier have been described in other cell types, including the rat pulmonary arterial myocytes (25) and bovine AZF cells (26). The AA-mediated inhibition of the delayed rectifier was shown to cause depolarization in pulmonary arterial myocytes (25). However, there was no activation of the delayed rectifier near the resting potential of corticotropes. Instead, the dominant effect of AA in corticotropes at the resting potential was the activation of a background current. The shift in the reversal potential of the AA-activated background current with changes in extracellular $[K^+]$ (Fig. 5) was similar to that predicted by the Nernst potential of K^+, indicating that the AA-activated current was primarily carried by K^+. At −40 mV, the mean increase in membrane current was approximately 100 pA. Because the mean input resistance of corticotrope is approximately 1.3 GΩ (Fig. 1C), the large increase in background K^+ current by AA near the resting potential resulted in a strong hyperpolarization (Fig. 2B).

Several lines of evidence indicate that the AA-activated current in corticotropes is due to an increase in the activities of TREK-1 channels. First, the AA-mediated increase in current did not require AA metabolism. Similar to that described in bovine AZF cells (26), inhibition of AA metabolism by ETYA failed to block the AA-mediated activation of the TREK-1 current in corticotropes (Fig. 4C). Second, the AA-activated current was strongly suppressed by pharmacological blockers of TREK channels but not that of TRAAK channels. We found that the AA-activated K^+ current (at −40 mV) in corticotropes was reduced approximately 80% and approximately 60%, respectively, by fluoxetine (50 μM) and chlorpromazine (10 μM) (Fig. 6E). These values closely resembled the reported values of inhibition of human TREK-1 channels by fluoxetine (19) and human TREK-1 and TREK-2 channels by cholorpromazine (20). Our results ruled out the involvement of TRAAK channels, because TRAAK channels are insensitive to chlorpromazine (20). Furthermore, ruthenium

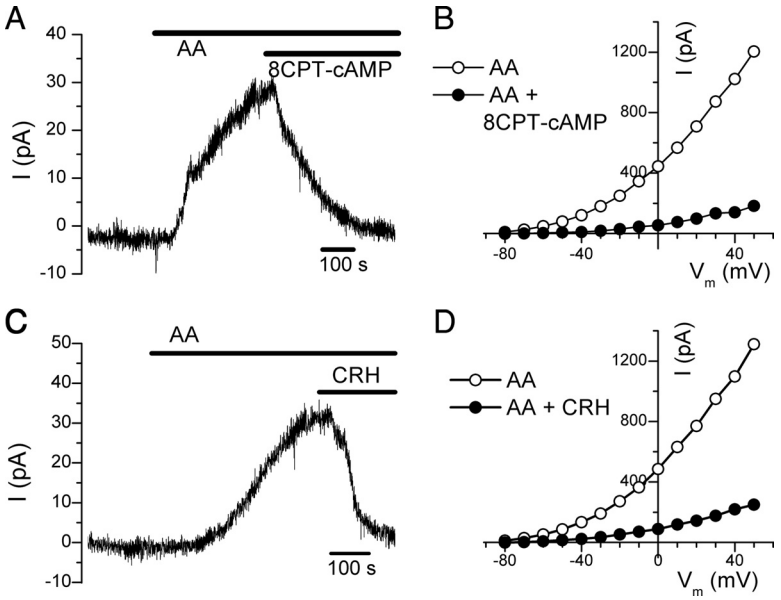

FIG. 7. The AA-mediated current was negatively regulated by cAMP and CRH. A, The cell permeable cAMP analog, 8CPT-cAMP (0.5 mM), strongly reduced the AA (10 μM)-mediated increase in outward current. The cell was voltage clamped at −70 mV. B, IV plots of the current in AA and in AA + 8CPT-cAMP. Same cell as in A. C, The AA (10 μM)-mediated increase in outward current was strongly suppressed by CRH (20 nM). The cell was voltage clamped at −70 mV. D, IV plots of the current in the presence of AA and in AA + CRH. Same cell as in C.

red, a potent inhibitor of TRAAK channels (21), had only a minor effect on the AA-activated current in corticotropes (*e.g.* Fig. 6C). The AA-activated current was also sensitive to DPBP; the current activated by 10 μM AA was largely inhibited by penfluridol or pimozide at 10 μM (Fig. 6F). However, penfluridol and pimozide were reported to inhibit the basal TREK-1 current (without AA) in bovine AZF cells with IC_{50} values of 0.19 and 0.35 μM, respectively (23). This discrepancy may be related to the high concentration of AA (10 μM) used to activate the TREK-1 channels in corticotropes. As shown in Fig. 6F, a 5-fold lower concentration of pimozide (2 μM) was able to largely suppress the current activated by 2 μM AA. Third, consistent with the activation of TREK channels by intracellular acidification (16), the lowering of pH_{int} (from 7.4 to 7.0) dramatically increased the amplitude of the basal current in corticotropes. Fourth, the TREK, but not TRAAK, channels are known to be negatively regulated by cAMP-dependent signaling pathways, including protein kinase A (27) and the cAMP-activated guanine nucleotide exchange factors-mediated pathway (28). Consistent with this, the cell permeable cAMP analog, 8CPT-cAMP (0.5 mM), strongly suppressed the AA-activated K^+ current in corticotropes (*e.g.* Fig. 7B). Fifth, among the members of the TREK family, only the TREK-1 channel exhibits a strong outward rectification due to its sensitivity to extracellular divalent ions (16). Consistent with the involvement of TREK-1 channels, the AA-mediated current in corticotropes in the normal bath solution (containing Ca^{2+} and Mg^{2+}) exhibited outward rectification (Fig. 4B). In corticotropes, the AA-activated current during each voltage step (*e.g.* at 20 mV) was essentially a square wave that exhibited little time dependence (Fig. 3Aiii). Similarly, the AA-activated TREK-1 current in bovine AZF did not exhibit any time dependence at 20 mV (26). In contrast, in cells with expressed human (19) or mouse TREK-1 channels (29), the TREK-1 current evoked at positive test potentials (>0 mV) comprised an instantaneous and a sustained component. It is not clear whether this discrepancy is due to the difference between native and expressed TREK-1 channels. Lastly, extracellular acidification (pH 6.4) reduced the AA-activated K^+ current in corticotropes (Fig. 6E). Extracellular acidification was reported to have inhibitory

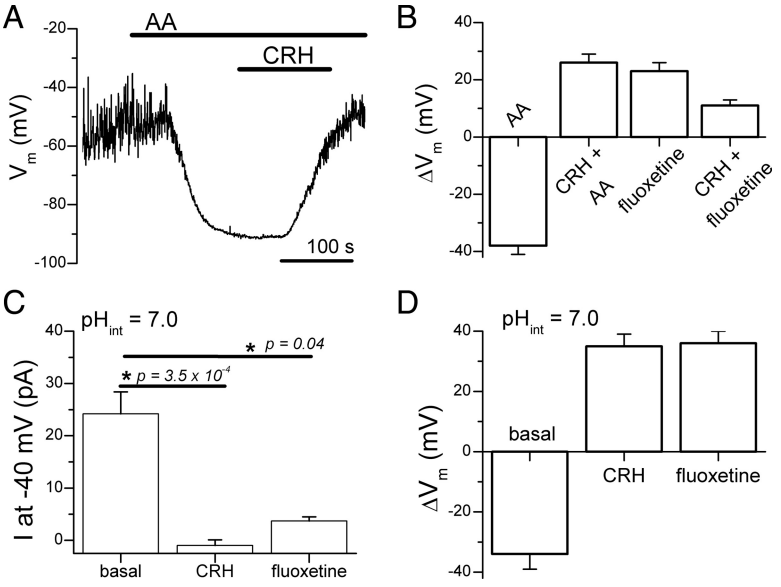

FIG. 8. Effects of CRH and intracellular acidification on membrane potentials. A, The AA (10 μM)-mediated hyperpolarization was reversed by CRH (20 nM). B, Summary of the effects of AA (10 μM), CRH (20 nM; in the presence of AA), fluoxetine (50 μM; in the absence of AA), and CRH (20 nM; in the presence of fluoxetine) on membrane potentials. Values averaged from 10 to 23 cells (pH_{int} 7.4) for each treatment. C, Intracellular acidification (pH_{int} 7.0) increased the basal current at −40 mV (n = 22), and this current could be inhibited by CRH (20 nM; n = 9) or fluoxetine (50 μM; n = 5). D, Hyperpolarization induced by intracellular acidification and its reversal by CRH or fluoxetine. Same cells as in C.

action on human TREK-1 channels but enhancing actions on TREK-2 channels (22). Our observation that approximately 48% of the AA-activated current was inhibited with pH_{ext} 6.4 was similar to that reported for murine TREK-1 channels (22).

The TREK-1 channels set the resting potential and were targets of CRH

Our previous study showed that CRH evoked depolarization via a cAMP-dependent closure of a background K^+ conductance in rat corticotropes, but the identity of the CRH-sensitive background K^+ current remains elusive (2). Here, we show that CRH exerted a similar effect on the membrane potential of GFP-labeled mouse corticotropes. The CRH evoked membrane potential depolarization (Fig. 1C) was accompanied by an increase in cell input resistance (Fig. 1D), reflecting the closure of a background conductance. We also found that application of the TREK-1 channel inhibitor, fluoxetine, to mouse corticotropes caused a similar depolarization (Fig. 8B). Although fluoxetine was also reported to inhibit the delayed rectifier (30), there was no significant activation of the delayed rectifier in corticotropes at potentials less than −20 mV (Figs. 3 and 4). Thus, the fluoxetine-evoked depolarization in corticotropes is primarily due to the inhibition of basal activity of TREK-1 channels. This finding suggests that the resting potential of corticotropes is primarily determined by the basal activity of TREK-1 channels. Consistent with this, an increase in TREK-1 current by intracellular acidification resulted in a strong hyperpolarization that could be reversed by fluoxetine (Fig. 8D). The activities of TREK-1 channels are strongly regulated by phosphorylation, and some TREK-1 channels are phosphorylated under basal condition (16). This may contribute to the variability of resting potential among corticotropes (ranging from −40 to −50 mV).

Our results also indicate that the CRH-sensitive background K^+ current is the TREK-1 current. As shown in Fig. 8C, the basal TREK-1 current induced by intracellular acidification was largely abolished by CRH, resulting in a strong depolarization (Fig. 8D). CRH also suppressed the TREK-1 current activated by AA and reversed the AA-mediated hyperpolarization (Figs. 7C and 8A). In the presence of fluoxetine, CRH evoked only a small depolarization (~11 mV) (Fig. 8B). Fluoxetine inhibited only approximately 80% of the TREK-1 current (Fig. 6E); thus, it is possible that the small depolarization was due to a suppression of the remaining TREK-1 current by CRH. Because the CRH receptor is coupled to adenylate cyclase, the inhibitory action of CRH is likely to be mediated via the elevation of cellular cAMP (Fig. 7, A and B).

Potential role of AA as a negative feedback regulator in ACTH release

Upon stimulation with CRH or AVP, AA is generated in the pituitary gland (4, 5). Because both CRH and AVP trigger $[Ca^{2+}]_i$ rise in corticotropes (2, 15), it is likely that the $[Ca^{2+}]_i$ elevation activates the Ca^{2+}-dependent cytosolic form of phospholipase A_2, which in turn cleaves the membrane phospholipids to generate AA. The concentration of AA near the corticotropes during CRH stimulation is unclear, but in most tissues, the concentration of AA is estimated to be at 2–10 μM (7). Note that AA can activate TREK-1 channels from either side of the plasma membrane (16). It is conceivable that with a prolonged CRH stimulation (*e.g.* during a chronic stress), the concentration of AA near the plasma membrane (inside or outside) of a corticotrope could reach several micromolar. In our experiments, 5 or 10 μM AA was able to evoke strong hyperpolarization (Fig. 2B). There was a 3-fold increase in the amplitude of the TREK-1 current (at −40 mV) in corticotropes when the concentration of AA was increased from 5 to 10 μM. A similar increase in TREK-1 current by the same concentrations of AA had been reported in bovine AZF (26). Therefore, it is likely that during a prolonged CRH stimulation, the concentration of AA near the plasma membrane of corticotropes could cause activation of the TREK-1 current and hyperpolarization. This will in turn limit the depolarization evoked by CRH and thus reduce voltage-gated Ca^{2+} channels activation and ACTH release. Interestingly, a previous study has shown that AA at high concentration (100 μM) increased basal ACTH release but had no effect on the CRH-stimulated ACTH secretion from rat pituitary cells (4). We found that when corticotropes were exposed to high concentrations of AA (*e.g.* 30 μM), the patch clamp recording became unstable (possibly due to changes in membrane fluidity), and there was an increase in the leakage of extracellular Ca^{2+} into the cell (Lee, A. K. and A. Tse, unpublished observations). Thus, it is possible that with very high concentrations of AA, the increase in extracellular Ca^{2+} leak into corticotropes in turn triggers ACTH release. Such effect may offset the inhibitory action of AA on the electrical excitability of the corticotropes. Overall, our result raises the possibility that AA at physiological concentrations (5 to 10 μM) may act as a negative regulator of ACTH release. We suggest that other than the negative feedback action of glucocorticoids, the activation of TREK-1 channels in corticotropes by AA may provide an additional protective mechanism to prevent excessive ACTH release during chronic stress.

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