ACTH inhibits the capsaicin-evoked release of CGRP from rat adrenal afferent nerves

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Ulrich-Lai, Yvonne M., Catherine A. Harding-Rose, Athena Guo, Walter R. Bowles, and William C. Engeland. ACTH inhibits the capsaicin-evoked release of CGRP from rat adrenal afferent nerves. Am J Physiol Regulatory Integrative Comp Physiol 280: R137–R142, 2001.—The adrenal cortex is innervated by afferent fibers that have been implicated in affecting cortical steroidogenesis. Modulation of neurotransmitter release from afferents may represent a regulatory system for the control of adrenal cortical function. The present studies validate an in vitro superfusion technique for adrenal capsules employing the drug capsaicin, which activates a subset of afferent fibers and induces the release of calcitonin gene-related peptide (CGRP). Capsaicin-evoked CGRP release from adrenal afferents was blocked by capsazepine, a competitive antagonist for the capsaicin receptor, or by removal of extracellular calcium. Exogenous ACTH prevented capsaicin-evoked CGRP release, elevated basal aldosterone release, and prevented capsaicin-induced reduction in aldosterone release. Immunolabeling for the recently cloned capsaicin vanilloid receptor 1 demonstrated its presence in adrenal nerves. These results show that in vitro superfusion of adrenal capsules can be used to characterize factors that modulate neurotransmitter release from adrenal afferents. Furthermore, the results suggest that activation of adrenal afferents in vivo may attenuate aldosterone steroidogenesis and that high levels of ACTH may prevent this phenomenon.

cortical cells do not express CGRP, the adrenal capsule contains a dense plexus of CGRP-positive nerve fibers (11, 15, 20). Neuronal retrograde tracing and double-immunolabeling experiments have shown that adrenal CGRP-positive fibers are of afferent origin and that many colabel for SP (11, 22). Moreover, there is evidence supporting a peripheral role for CGRP in the regulation of cortical steroidogenesis (1, 7, 13, 14). This suggests that factors modulating the release of CGRP from the peripheral terminals of sensory fibers may represent an additional regulatory system for the control of adrenal cortical function in vivo.

To examine factors modulating CGRP release from adrenal afferent fibers, an in vitro superfusion method was used; this superfusion method has been previously validated with peripheral tissues, such as rat paw skin and bovine dental pulp (6, 17). The technique takes advantage of the unique properties of the drug capsaicin, which is the pungent principle of hot peppers. Capsaicin has been shown to selectively act on a subset of primary afferent neurons (2, 8). Thus capsaicin can be applied to peripheral tissue in low, nontoxic concentrations to activate specifically a subset of afferent fibers, thereby resulting in the release of CGRP. Systemic treatment with high toxic doses of capsaicin removes CGRP-positive fibers from adrenal glands (15), suggesting that adrenal CGRP-positive nerve fibers are capsaicin-sensitive afferents. However, it is not known whether nontoxic doses of capsaicin can evoke neurotransmitter release from adrenal afferents, as occurs with afferents in other tissues. Thus the present studies employ in vitro superfusion of adrenal capsules to determine whether capsaicin can evoke the release of CGRP from adrenal afferents. The studies also address whether capsaicin-evoked release of CGRP is blocked by the competitive vanilloid receptor antagonist capsazepine, depends on extracellular calcium, or affects aldosterone steroidogenesis. After characterization of the capsaicin-evoked release of CGRP from adrenal capsular afferents, an additional experiment is performed to determine whether exogenous ACTH administration affects capsaicin-evoked release.
release of CGRP. Lastly, immunolabeling for vanilloid receptor 1 (VR1), the recently cloned capsaicin receptor (2), is performed to demonstrate its presence in rat adrenal glands.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (175–200 g; Harlan, Indianapolis, IN) were used in all experiments. The animals were housed on a 12:12-h light-dark cycle with free access to food and water. All procedures were approved by the University of Minnesota Animal Care and Use Committee.

Superfusion technique. Rats were killed by decapitation, and their adrenal glands were quickly removed, cleaned, and decapsulated. Adrenal capsules, consisting of the complete zona glomerulosa with portions of the zona intermedia and zona fasciculata (personal observation), were placed into superfusion chambers within 5 min of collection; nine capsules were placed in each chamber for all experiments, except for the high K+ experiment in which 18 capsules were placed in the chambers. Chambers were perfused with oxygenated Krebs buffer (in mM: 135 NaCl, 3.5 KCl, 1 MgCl2, 2.5 CaCl2, 0.1% BSA, 3.3 dextrose, 0.1 ascorbic acid, 10 HEPES, and 16 μM thiorphan; 36°C, pH 7.4) at a rate of 0.23 ml/min. After a period of baseline collection, tissue was treated with either high K+ (50 mM) buffer, which results in nontropic activation of neurons, or capsaicin (100 μM), a drug that selectively activates a subset of nociceptive primary afferent neurons, followed by a washout period. Superfusate was collected every 10 min for 150 min (15 fractions) and assayed for CGRP via RIA; in some experiments, superfusates were also assayed for aldosterone via RIA.

To determine whether the capsaicin-evoked release of CGRP from adrenal capsules was receptor mediated, the competitive vanilloid receptor antagonist capsazepine (RBI, Natick, MA) from adrenal capsules was receptor mediated, the competitive vanilloid receptor antagonist capsazepine (RBI, Natick, MA) was applied to both treatment groups. On fraction 8, one group was pretreated with capsazepine (300 μM, 10 min) to adrenal capsules while the other group was treated with the capsaicine vehicle (0.29% EtOH, 10 min, n = 7 chambers). On fraction 13, capsaicin (100 μM, 10 min) was coapplied to each group.

To determine whether the capsaicin-evoked release of CGRP from adrenal capsules was receptor mediated, the competitive vanilloid receptor antagonist capsazepine (RBI, Natick, MA) was employed. In this experiment, chambers were divided into two treatment groups. On fraction 12, one group was pretreated with capsazepine (300 μM, 10 min, n = 4 chambers), while the other group was treated with the capsaicine vehicle (0.29% EtOH, 10 min, n = 7 chambers). On fraction 13, capsaicin (100 μM, 10 min) was applied to both treatment groups.

To determine whether ACTH could affect basal and capsaicin-evoked release of CGRP from adrenal capsules, the effects of exogenous human ACTH-(1–39) (Bachem, Torrance, CA) were assessed. Chambers were divided into two treatment groups. One group was perfused with buffer in which Ca2+ was omitted and 10 mM EGTA was added (n = 4 chambers). On fraction 13, capsaicin (100 μM, 10 min) was applied to both treatment groups.

CGRP RIA. The CGRP RIA consisted of incubating superfusate samples for 48 h at 4°C with CGRP antisera (kindly donated by Dr. M. Iadarola) at a final dilution of 1:4,000,000. Then 100 μl of 125I-labeled [Tyr]CGRP (∼20,000 cpm) and 50 μl of goat anti-rabbit antisera coupled to ferric beads (PerSepBio Systems, Framingham, MA) were added and allowed to incubate for an additional 48 h at 4°C. The RIA was stopped by immunomagnetic separation. The minimum detection of the assay is 2 fmol/tube with a 50% displacement of 18 fmol/tube. All drugs were tested for interference in the RIA.

Adrenal capsule superfusion. Stable basal levels of CGRP release from adrenal capsules were generally obtained within 1 h of the initiation of superfusion. The application of high K+ (50 mM; fraction 8) to adrenal capsules evoked an increase in CGRP release in fraction 9 that returned to basal levels in fraction 10 (Fig.)
The application of capsaicin (100 \mu M; fraction 12) evoked increased CGRP release in fraction 13 that returned to basal levels over the next two fractions (Fig. 2A). Moreover, capsaicin treatment resulted in attenuated aldosterone release in fractions 14 and 15 (Fig. 2B). In a separate control experiment, the basal release of CGRP (6.33 \pm 1.22 fmol/fraction) was not affected \((P = 0.50)\) by application of the capsaicin vehicle (0.17\% EtOH; fraction 12; \(n = 4\) chambers); in this same experiment, basal aldosterone release (2.40 \pm 0.13 ng\:ml\^{-1}\:fraction\^{-1}) was also not affected \((P = 0.52)\) by vehicle application.

Coadministration of the competitive vanilloid receptor antagonist capsazepine (300 \mu M) prevented the capsaicin-evoked release of CGRP from adrenal capsules (Fig. 3). The blockade of capsaicin’s effects by capsazepine is consistent with capsaicin acting via a specific vanilloid receptor-mediated mechanism.

The removal of calcium and addition of EGTA (10 mM) to the perfusion buffer reduced basal levels of CGRP release by \(\sim 50\%\) (Fig. 4). Furthermore, capsaicin treatment did not evoke CGRP release in the absence of extracellular calcium (Fig. 4). These results are consistent with the release of CGRP from adrenal capsules occurring via calcium-mediated exocytosis.

The addition of ACTH (1 nM) to the perfusion buffer did not affect basal levels of CGRP release (Fig. 5A). However, the capsaicin-evoked release of CGRP was
blocked in the presence of ACTH (Fig. 5A). Aldosterone release was decreased by capsaicin treatment in the absence of exogenous ACTH (Fig. 5B). In the presence of exogenous ACTH, basal levels of aldosterone release were elevated, and capsaicin treatment did not alter aldosterone steroidogenesis (Fig. 5B).

**VR1 and CGRP immunolabeling.** Adrenal glands contained several VR1-positive nerve fibers; specific VR1 immunoreactivity was not observed on cortical or medullary cells. VR1-positive nerve fibers were often observed in the adrenal capsule (Fig. 6A). Several radially oriented VR1-positive fibers were observed in the cortex (Fig. 6B). In addition, VR1-positive fibers were often seen in the medulla (Fig. 6C).

Double-labeling studies demonstrated that VR1-positive nerve fibers were generally located near CGRP-positive fibers and were frequently intertwined with them (Fig. 7, A and B). However, the VR1 and CGRP immunoreactivities did not colocalize to the same nerve fibers (Fig. 7, A and B).

**DISCUSSION**

The present studies demonstrate that the application of either high K⁺ (50 mM) buffer or capsaicin (100 μM) to in vitro superfused adrenal capsules results in the release of CGRP. The capsaicin-evoked release of CGRP from adrenal capsules was prevented by cotreat-
ment with the vanilloid receptor antagonist capsazepine, suggesting a receptor-mediated mechanism for capsaicin's actions. The capsaicin-evoked release of CGRP from adrenal capsules was also eliminated in the absence of extracellular calcium, consistent with an exocytotic mechanism for CGRP release. The present results from visceral, adrenal afferents are similar to those previously reported for CGRP release from somatic afferents in response to capsaicin (6, 10), suggesting a similar mechanism for capsaicin-evoked release of CGRP from these fibers.

Because CGRP has been implicated in modulating cortical steroidogenesis (1, 7, 13, 14), factors that affect the release of CGRP from the peripheral terminals of sensory fibers may represent an additional regulatory system for the control of adrenal cortical function in vivo. Specifically, it is of interest to determine whether hormones important to the endocrine function of the gland, such as ACTH, can alter the release of neurotransmitter from adrenal afferent fibers. The addition of ACTH to the perfusion buffer did not affect basal release of CGRP from adrenal capsules, whereas the increased CGRP release from capsaicin was not seen. These studies are the first to show that neurotransmitter release from adrenal afferents can be modulated. Importantly, this is also the first demonstration that ACTH can modulate capsaicin-evoked neurotransmitter release from afferent fibers; further studies are required to determine whether modulation by ACTH is a common attribute of afferent fibers or is unique to adrenal afferents.

Aldosterone release from in vitro superfused adrenal capsules was decreased after capsaicin treatment in the absence of exogenous ACTH. Because CGRP receptors are present on cells of the zona glomerulosa (16), it suggests that CGRP released from adrenal afferents in response to capsaicin may affect aldosterone steroidogenesis via a direct action on zona glomerulosa cells. Previous studies examining the effects of CGRP on aldosterone release from adrenal cells have been inconsistent. Aldosterone release from dispersed rabbit glomerulosa cells was inhibited in the presence and absence of angiotensin II (14). However, aldosterone release from dispersed rat zona glomerulosa cells was not affected by CGRP either in the presence or absence of ACTH (7). In the present studies, capsaicin treatment evoked CGRP release and reduced aldosterone release. Notably, administration of ACTH prevented both these effects. These results are consistent with the hypothesis that CGRP released from adrenal afferents in response to capsaicin acts on cells of the zona glomerulosa, either directly or indirectly, to attenuate aldosterone steroidogenesis. Additionally, it is also possible that other factors known to be released from afferent fibers, such as SP or glutamate (21), may be released from adrenal afferents in response to capsaicin then to affect aldosterone steroidogenesis. Studies employing neurotransmitter-specific receptor antagonists are required to determine the relative contribution of these various factors to the capsaicin-evoked decrease in aldosterone biosynthesis.

Determination of the mechanism by which capsaicin selectively activates a subset of afferent fibers is currently an active area of research. It is generally believed that capsaicin acts via a specific membrane receptor that is expressed exclusively on a subset of afferent fibers; this receptor, VR1, was recently cloned (2). Activation of the receptor by capsaicin opens a nonselective cation channel with high calcium permeability to produce depolarization and exocytotic release of neurotransmitters (reviewed in Ref. 9). Presumably, capsaicin would act directly on adrenal afferents to evoke CGRP release. The present studies demonstrate VR1-positive nerve fibers in the adrenal capsule, cortex, and medulla, but these fibers did not colabel for CGRP. These results are identical to those found previously for rat dermis and cornea, in which few nerve fibers contained both VR1 and CGRP (5). This may indicate that capsaicin's action on VR1 is to induce the release of an excitatory neurotransmitter(s) that may in turn evoke the release of CGRP from nearby fibers (5). However, this idea is difficult to reconcile with the fact that systemic treatment with high, toxic doses of
capsaicin removes CGRP-positive fibers in the adrenal gland (15), implying that adrenal CGRP-positive fibers are sensitive to capsaicin. Alternatively, the results may indicate that there are additional capsaicin receptors, as suggested by others (5, 18), and that these other receptors colocalize with CGRP and are responsible for its release in response to capsaicin.

The primary goal of the current studies was to determine whether the pharmacological agent capsaicin, a drug that selectively activates a subset of afferent fibers (2, 8), evokes the release of CGRP from adrenal afferents as described previously for other tissues. The studies employ capsaicin as a pharmacological tool to stimulate adrenal afferents in vitro to determine whether endocrine factors, such as ACTH, modulate activation of these fibers. Capsaicin was used to mimic an in vivo situation in which these afferent fibers could be activated (such as nociception, inflammation, etc.). However, it should be noted that VR1 is a heat-responsive channel whose responses are sensitized by low pH (19), suggesting that beyond the pharmacological use of capsaicin, the VR1 receptor may also play important roles in adrenal physiology.

Perspectives

Traditionally, the adrenal cortex has been viewed as a tissue strictly under endocrine control, but more recently innervation of the cortex has been implicated in modulating the function of the gland (reviewed in Refs. 3, 4). The present studies demonstrate that activation of adrenal afferent nerve fibers with capsaicin evokes CGRP release and attenuates aldosterone release in the absence of ACTH, but not in its presence. These results support the potential for neural modulation of adrenal steroid production. Moreover, the current work suggests that the converse may also occur; endocrine factors, such as ACTH, may modulate adrenal neural function. Collectively, the data suggest that complex neuroendocrine interactions may be involved in the control of adrenal cortex steroidogenesis. For instance, when plasma ACTH is low, such as during nonstress conditions, activation of adrenal afferents would result in the release of neurotransmitter(s) that could then act to attenuate basal steroid release. In contrast, when plasma ACTH is high, such as during a stress response, ACTH may prevent the release of neurotransmitter(s) from activated adrenal afferents, thereby maintaining high levels of steroid release. Additional experiments evaluating the contribution of adrenal afferents to cortical responses in vivo are required to evaluate these predictions.

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