Losartan-sensitive All Receptors Linked to Depolarization-dependent Cortisol Secretion through a Novel Signaling Pathway*

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In bovine adrenal zona fasciculata (AZF) cells, angiotensin II (All) may stimulate depolarization-dependent Ca\(^{2+}\) entry and cortisol secretion through inhibition of a novel potassium channel (I\(_{AC}\)), which appears to set the resting potential of these cells. Aspects of the signaling pathway, which couples All receptors to membrane depolarization and secretion, were characterized in patch clamp and membrane potential recordings and in secretion studies. All-mediated inhibition of I\(_{AC}\) membrane depolarization, and cortisol secretion were all blocked by the All type 1 (AT\(_1\)) receptor antagonist losartan. These responses were also affected by the AT\(_1\) antagonist PD123319. Inhibition of I\(_{AC}\) by All was prevented by intracellular application of guanosine 5'-O-2',3'-dithiophosphate but was not affected by pre-incubation of cells with pertussis toxin. Although mediated through an AT\(_1\) receptor, several lines of evidence indicated that All inhibition of I\(_{AC}\) occurred through an unusual phospholipase C (PLC)-independent pathway. Acetylcholine, which activates PLC in AZF cells, did not inhibit I\(_{AC}\). Neither the PLC antagonist neomycin nor PLC-generated second messengers prevented I\(_{AC}\) expression or mimicked the inhibition of this current by All. I\(_{AC}\) expression and inhibition by All were insensitive to variations in intracellular or extracellular Ca\(^{2+}\) concentration. All-mediated inhibition of I\(_{AC}\) was markedly reduced by the non-hydrolyzable ATP analog adenosine 5’-[(\(\beta\),\(\gamma\)-imino)triphosphate and by the non-selective protein kinase inhibitor staurosporine. The protein phoshatase antagonist okadaic acid reversibly inhibited I\(_{AC}\) in whole cell recordings. These findings indicate that All-stimulated effects on I\(_{AC}\) current, membrane voltage, and cortisol secretion are linked through a common AT\(_1\) receptor. Inhibition of I\(_{AC}\) in AZF cells appears to occur through a novel signaling pathway, which may include a losartan-sensitive AT\(_1\) receptor coupled through a pertussis-insensitive G protein to a staurosporine-sensitive protein kinase. Apparently, the mechanism linking AT\(_1\) receptors to I\(_{AC}\) inhibition and Ca\(^{2+}\) influx in adrenocortical cells is separate from that involving inositol trisphosphate-stimulated Ca\(^{2+}\) release from intracellular stores. All-stimulated cortisol secretion may occur through distinct parallel signaling pathways.

All\(^1\) is a peptide that physiologically regulates the secretion of corticosteroid hormones. In most mammals, All stimulates the secretion of the mineralocorticoid aldosterone from the AZG, while in other species the peptide also enhances cortisol production by the AZF cell. All regulates corticosteroid production by Ca\(^{2+}\)-dependent mechanisms, which involve both Ca\(^{2+}\) release from an intracellular pool and Ca\(^{2+}\) influx across the plasma membrane (1, 2). The cellular mechanisms that underlie All-stimulated increases in Ca\(^{2+}\) are only partially understood and may involve multiple receptors and signaling pathways.

Two pharmacologically distinct types of All receptors have been identified in various cells, including those of the adrenal cortex (3–5). Losartan-sensitive AT\(_1\) receptors are coupled through separate G proteins to at least two signaling pathways. Most physiological responses mediated through AT\(_1\) receptors involve activation of PLC, which catalyzes the synthesis of inositol trisphosphate (IP\(_3\)) and diacylglycerol from phosphatidylinositol 4,5-bisphosphate (4, 6, 7). IP\(_3\) triggers the release of Ca\(^{2+}\) from intracellular stores. Other AT\(_1\) receptors are coupled to adenylyl cyclase inhibition through a PTX-sensitive G protein (8). AT\(_2\) receptors are losartan-insensitive but are blocked by PD123319 (3, 4). Although AT\(_2\) receptors comprise 20% of All receptors in rat adrenocortical cells, the signaling pathway and function of these receptors in corticosteroid secretion is unknown (9).

In adrenocortical cells, AT\(_1\) receptor activation leads to IP\(_3\) synthesis and Ca\(^{2+}\) release from intracellular stores (9). The signaling pathway that mediates All-stimulated Ca\(^{2+}\) influx remains to be identified. In many secretory cells, Ca\(^{2+}\) enters through specific voltage-gated channels activated in response to membrane depolarization. Recently, we identified a novel K\(^+\) permeable channel expressed in bovine AZF cells that appears to set the membrane potential (10). All inhibits this K\(^+\) current (I\(_{AC}\)) and depolarizes AZF cells at concentrations that stimulate cortisol secretion. Further, All-stimulated secretion is blocked by selective antagonists of T-type Ca\(^{2+}\) channels, the major Ca\(^{2+}\) channel expressed in these cells (11, 12). These findings appear to identify a specific cellular mechanism.

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§ Losartan-sensitive AII Receptors Linked to Depolarization-dependent Cortisol Secretion through a Novel Signaling Pathway*.

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1 The abbreviations used are: All, angiotensin II; DMEM, Dulbecco’s modified Eagle’s medium; AT\(_1\), All type 1; AT\(_2\), All type 2; PMA, phorbol 12-myristate 13-acetate; IP\(_3\), inositol trisphosphate; PLC, phospholipase C; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; ACTH, adrenocorticotropic hormone; PTX, pertussis toxin; Ach, acetylcholine; AMP-PNP, adenosine 5’-(\(\beta\),\(\gamma\)-imino)triphosphate; GDP\(_S\), guanyl-5’-yri thiophosphate; GTP\(_\gamma\)S, guanosine 5’-3-O-(thiodi)phosphate; AA, arachidonic acid.
whereby the binding of AII to a specific receptor on AZF membranes is coupled to depolarization-dependent Ca**2+** entry and cortisol secretion. The present study was done to characterize components of this signal transduction pathway in bovine adrenal cortical cells.

**MATERIALS AND METHODS**

Tissue culture media, antibiotics, fibronectin, and fetal calf serum were obtained from Life Technologies, Inc. Culture dishes were purchased from Corning. Coverslips were from Bellco (Vineland, NJ.). Enzymes, AII, GTP, MgATP, AMP-PNP, GDPsS, GTP-β-S, pertussis toxin, IP3, hemoglobin, arachidonic acid, acodilin, phenidone, phorbol 12-myristate 13-acetate (PMA), and indomethacin were obtained from Sigma. Receptor antagonists losartan and Du 532 were kindly provided by Dr. Ronald Smith (Dupont-Merck Pharmaceutical Co.).

Isolation and Culture of AZF Cells—Bovine adrenal glands were obtained from steers (age range 1–3 years) within 15 min of slaughter at a local slaughterhouse. Fatty tissue was removed immediately, and the glands were transported to the laboratory in ice-cold phosphate-buffered saline containing 0.2% dextrose. Isolated AZF and AZG cells were prepared as previously described (13) with some modifications. In a sterile, aseptic environment, the adrenals were cut in half longitudinally, and the lighter medulla tissue was trimmed away from the cortex and discarded. The capsule with attached glucomerula and thicker fascicula-reticularis layer was then dissected into large pieces approximately 1.0 × 1.0 × 0.5 cm. A Stadie-Riggs tissue slicer (Thomas Scientific) was used to slice fascicula-reticularis tissue from the glomerula and resuspended in DMEM/F12 (1:1). All solutions were filtered through 0.22-μm cellulose acetate filters.

Isolation and Culture of AZG Cells—Adrenocortical cells were obtained from steers (age range 1–3 years) within 15 min of slaughter at a local slaughterhouse. Fatty tissue was removed immediately, and the glands were transported to the laboratory in ice-cold phosphate-buffered saline immediately before adding 0.2% dextrose. Isolated AZF and AZG cells. Results reported in this study were obtained from isolated AZF cells.

**RESULTS**

**AT1 Responses**—In previous studies, we identified two components of K**"**+ current in bovine AZF cells. These include a rapidly inactivating A-type current and a novel K**"**+ current (IAC) (10, 16). IAC is a non-inactivating, outwardly rectifying, weakly voltage-dependent, high unitary conductance K**"**+ current that develops over a period of many minutes in whole cell recordings (10). We now report that IAC is also expressed by AZG cells where, as in AZF cells, it is inhibited by both ACTH and AII. No differences in IAC were observed between AZF and AZG cells. Results reported in this study were obtained from isolated AZF cells.

Previously, it was shown that AII inhibits IAC by a maximum of approximately 77% with an IC**50** of 145 pM (10). We have explored the mechanism of AII-mediated inhibition of IAC in adrenocortical cells. Cells were clamped in the whole cell configuration, and K**"**+ currents were recorded at 30-s intervals until IAC grew to a stable amplitude. In voltage protocols designed to activate both IAC and the transient A-type K**"**+ current, IAC was measured as the non-inactivating K**"**+ current present at the end of a 300-ms test pulse to +20 mV. A second voltage protocol was designed to isolate IAC by elimination of the rapidly inactivating A-type K**"**+ current (Fig. 1B), which inactivated completely at −20 mV (16).

The selective AT1 receptor antagonist losartan and the AT2 receptor antagonist PD123319 (4, 5) were used to identify the AT1 receptor subtype mediating inhibition of IAC. In the experiment illustrated in Fig. 1, the cell was pre-exposed to losartan (500 nm) for 5 min before superfusing saline containing both this antagonist and AII (2 nm). In the presence of losartan, AII had no effect on IAC (Fig. 1A and C). When the same cell was re-exposed to AII after losartan was removed by a 5-min wash in control saline, IAC was inhibited by 70%. Similar results were obtained in each of three experiments (Fig. 2B). AII-mediated inhibition of IAC was not easily reversed, even after prolonged washing with saline containing losartan.

The AT2-antagonist PD123319 did not blunt or prevent AII-mediated inhibition of IAC (Fig. 2A). In the presence of 500 nm PD123319, 2 nm AII inhibited IAC by 83.5 ± 12.5% (n = 3). By computer and PCLAMP software with an Axolab interface (Axon Instruments, Inc., Burlingame, CA). Currents were digitized at 1–50 kHz after filtering with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records using scaled hyperpolarizing steps of 5 to 1 μA.

Data were analyzed and plotted using PCLAMP (CLAMPAN and CLAMPFIT) and GraphPad Inplot. Drugs were applied by bath perfusion, controlled manually by a six-way rotary valve.

Intracellular Voltage Recordings—Intracellular recordings of membrane potential were made at 35–37°C using a WPI model FD-223 electrometer and glass electrodes that, when filled with 1 M KCl, had resistances of 100–150 megohms. Cells were continuously superfused with bath solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 10 HEPEs, and 5 glucose, pH 7.4.

Secretion Experiments—AZF cells were cultured on fibronectin-treated 35-mm plates at a density of about 4 × 10**4** per dish in DMEM/F12 (1:1) containing 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin and the antioxidants 1 μM tocopherol, 20 μM selenium, and 100 μM ascorbic acid. After 24 h, the media were aspirated and changed to defined media consisting of DMEM/F12 (1:1), 50 μg/ml bovine serum albumin, 100 μM ascorbic acid, 1 μM tocopherol, 100 μM insulin, and 10 μg/ml transferrin. Peptide hormones and Ca**2+** antagonists were also added at this time. Cells were then returned to the incubator for the duration of the experiment. 200–μl samples of media were collected at selected times and frozen at −20°C and later assayed for cortisol using a solid-phase radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA). All experiments were performed on triplicate 35-mm dishes, and hormone assays were performed in duplicate at several dilutions.
comparison, AII alone at concentrations of 1 and 5 nM inhibited $I_{AC}$ by $65.7 \pm 4.9\%$ ($n = 7$) and $71.6 \pm 5.1\%$ ($n = 5$) (Fig. 2B). These results show clearly that AII-mediated inhibition of $I_{AC}$ in AZF cells occurs exclusively through activation of a losartan-sensitive receptor.

Membrane Potential—If $I_{AC}$ sets the membrane potential of AZF cells, then AII-stimulated depolarization of AZF cells should show the same sensitivity to AII as $I_{AC}$ inhibition. Intracellular voltage recordings from AZF cells demonstrated that AII-stimulated membrane depolarization is also mediated through a losartan-sensitive AT$_1$ receptor. In this regard, the resting potential of AZF cells ($-72 \pm 1.4$ mV, $n = 16$) varied upon increasing external K$^+$ from $5-15$ mV nearly as predicted from the Nernst equation for a membrane that is selectively permeable to this ion ($\Delta V_m = +21.3 \pm 0.53$ mV, $n = 42$). Losartan, but not PD 123319, effectively inhibited AII-stimulated depolarization of AZF cells. In the experiment illustrated in Fig. 3, AII (2 nM) depolarized the AZF cell by only 7 mV in the presence of 500 nM losartan. After removal of losartan, superfusion of this same cell with AII resulted in a reversible 51-mV depolarization. In the presence of PD123319 (500 nM), AII elicited a 47-mV depolarization that was again reversible. Overall in three cells, 2 nM AII depolarized AZF cells by only $9.0 \pm 1.5$ mV in the presence of 500 nM losartan. Subsequent superfusion of these cells by AII in normal saline caused a 38 $\pm$ 17.0 mV depolarization. In other cells, AII alone (2 nM) depolarized AZF cells by $46.7 \pm 2.7$ mV ($n = 3$).

Cortisol Secretion—Patch clamp and membrane potential recordings with AII receptor antagonists demonstrate that AII-mediated inhibition of $I_{AC}$ occurs through activation of a losartan-sensitive AT$_1$ receptor. If $I_{AC}$ inhibition leads to depolarization-dependent Ca$^{2+}$ entry and cortisol secretion, then losartan but not PD123319 should block AII-stimulated cortisol secretion. Unstimulated AZF cells produce little cortisol. AII (2 nM) triggered large (>50-fold) increases in cortisol production. The AT$_1$-receptor antagonist losartan (500 nM) completely inhibited AII-stimulated cortisol production. In contrast, the AT$_2$-receptor antagonist PD123319 (500 nM) was ineffective, inhibiting cortisol secretion by approximately 10% (Fig. 4A). PD123319 was also ineffective at inhibiting AII-stimulated cortisol secretion measured over 4 h, while losartan again produced complete inhibition (data not shown). Complete inhibition of cortisol secretion by the competitive antagonist losartan could be overcome by increasing the AII concentration to 200 nM (Fig. 4B). In contrast, the non-competitive AT$_1$ antagonist, Du 532, at an equivalent concentration (relative to reported K$_i$ values) completely inhibited cortisol secretion stimulated by 200 nM AII (Fig. 4B).

$I_{AC}$ Inhibition

The above results are consistent with a model where losartan-sensitive AT$_1$ receptors are coupled to membrane depolar-
Novel Angiotensin II Mechanism

Voltage Dependence and Specificity—Peptide-mediated modulation of ion channels frequently involves a shift in voltage-dependent channel activity. Fig. 5 shows that AII-mediated inhibition of I\textsubscript{AC} is specific and independent of membrane potential. In the experiment illustrated, I\textsubscript{AC} was permitted to grow to a stable amplitude as monitored by test pulses applied to ±20 mV from a holding potential of −80 mV (inset). A current-voltage relationship for I\textsubscript{AC} was obtained by applying a voltage ramp between +60 and −140 mV from a holding voltage of 0 mV. The outwardly rectifying I\textsubscript{AC} current reversed from outward to inward at −90 mV, as predicted by the Nernst equation for a channel that is selectively permeable to K\textsuperscript{+}. 10 nM AII selectively inhibited the I\textsubscript{AC} current but had no effect on the rapidly inactivating A-type K\textsuperscript{+} current (inset). The current-voltage relationship showed that I\textsubscript{AC} was inhibited by approximately 80% over a wide range of potentials.

Guanine Nucleotides and GTP-binding Proteins—AII receptors are coupled to enzymes including phospholipase C and adenylyl cyclase through G protein intermediates. To determine if AII-mediated inhibition of I\textsubscript{AC} channels requires an activated G protein, we studied inhibition of I\textsubscript{AC} in whole cell recordings using pipette solutions containing the inactive guanine nucleotide GDP\textsubscript{S} rather than GTP. In the experiment illustrated in Fig. 6A, the cell was patch clamped in the whole cell mode, using a patch electrode containing 1 mM GDP\textsubscript{S}. Once I\textsubscript{AC} had grown to a stable amplitude, the cell was superfused with external solutions containing AII at concentrations ranging from 0.4 to 50 nM. At the highest AII concentration, I\textsubscript{AC} was inhibited by only 12%. No significant inhibition of I\textsubscript{AC} was observed at AII concentrations below 10 nM. With standard pipette solution (200 μM GTP), 10 nM AII inhibited I\textsubscript{AC} by 76.5 ± 4.6% (n = 6) (Fig. 6C). When GDP\textsubscript{S} replaced GTP in the pipette, AII (10 nM) inhibited I\textsubscript{AC} by only 12.8 ± 10.4% (n = 7). Excluding GTP from the pipette solution without the addition of GDP\textsubscript{S} was not effective in preventing AII-mediated inhibition. With no guanine nucleotide in the pipette, 10 nM AII inhibited I\textsubscript{AC} by a total of 79 ± 7.0% (n = 2).

Experiments with GDP\textsubscript{S} indicated that AII-mediated inhibition of I\textsubscript{AC} occurred through a G protein intermediate. To determine if either G\textsubscript{i} or G\textsubscript{q} mediated this inhibition, adrenal cortical cells were pre-incubated prior to patch clamping with PTx, which suppresses activation of G\textsubscript{i} and G\textsubscript{q}. PTx had no significant effect on the time-dependent growth of I\textsubscript{AC} or its inhibition by AII (Fig. 6, B and C). In cells pretreated for 6–12 h with PTx (200 ng/ml) (Fig. 6C), AII (2 nM) inhibited I\textsubscript{AC} by 73.
Results with guanine nucleotides and PTx indicated that AII-mediated inhibition of IAC requires a G protein other than Gi or Go.

Inhibition of Single K⁺ Channels by AII—The delay of one to several minutes required for AII-mediated inhibition of IAC to occur in whole cell recordings suggests a mechanism more complicated than a membrane-delimited coupling between AII receptors and IAC channels through a G protein intermediate. Inhibition of unitary K⁺ channel activity by AII in cell-attached patch recordings indicated that a diffusible second messenger was involved in the inhibitory process. Superfusing the cell membrane surrounding the area circumscribed by the patch electrode with AII inhibited unitary K⁺ channel activity with a delay similar to that observed in whole cell recordings (Fig. 7). At a concentration of 10–100 nM, AII completely inhibited single channel currents in three of seven membrane patches.

IAC Inhibition and Phospholipases—In many cells, the binding of AII to AT₁ receptors is coupled to the activation of PLC through a PTx-insensitive G protein. Cellular responses are mediated by second messengers including diacylglycerol and IP₃ produced from PLC-catalyzed cleavage of phosphatidylinositol 4,5-bisphosphate. Surprisingly, several lines of evidence indicate that AII-mediated inhibition of IAC appears to occur through a novel PLC-independent mechanism.

Acetylcholine (Ach) binds to muscarinic receptors on bovine AZF cells, activating PLC and triggering cortisol secretion (6, 17). However, Ach at concentrations that produce maximal stimulation of PLC and cortisol secretion produced no inhibition of IAC in whole cell patch clamp recordings. In the experiment illustrated in Fig. 8A, a cell was sequentially superfused with Ach (10 μM) and AII (10 nM) for 10 min each. At the end of the exposure to Ach, IAC (measured as the non-inactivating component of current present at the end of the 300-ms voltage step) had not changed. AII selectively reduced IAC by 60% from 110 to 44 pA. Similar results were obtained in each of six separate cells.

PLC-generated second messengers also failed to mimic AII-mediated inhibition of IAC. The membrane-permeable phorbol
IAC to approximately the same extent with each of these pipette solutions (Fig. 9) in Ca<sup>2+</sup> ester PMA (200 nM), which mimics diacylglycerol in activating kinase C, when superfused for times ranging from 5 to 9 min, did not reduce I<sub>AC</sub> in each of four experiments similar to the one illustrated in Fig. 8C. When the membrane-impermeant phospholipid IP<sub>3</sub> (5 μM) was included in the patch electrode, the time-dependent expression of I<sub>AC</sub> was not affected, and I<sub>AC</sub> was inhibited to the same extent by AII as under control conditions (Fig. 8B, Fig. 9, A and B).

As in most cells, PLC-generated IP<sub>3</sub> in adrenal cortical cells acts to release Ca<sup>2+</sup> from intracellular stores (2). However, changes in [Ca<sup>2+</sup>]<sub>i</sub> were not involved in the modulation of I<sub>AC</sub> in our studies. In unstimulated cells, I<sub>AC</sub> expression was not affected by pipette solutions in which Ca<sup>2+</sup> was buffered over a range of nominally 0–230 nM (Fig. 9A). Further, AII inhibited I<sub>AC</sub> to approximately the same extent with each of these pipette solutions (Fig. 9B). In particular, AII effectively inhibited I<sub>AC</sub> in Ca<sup>2+</sup>-free pipette solutions where Ca<sup>2+</sup> was strongly buffered with 20 mM BAPTA (Fig. 9B). AII also effectively reduced I<sub>AC</sub> in Ca<sup>2+</sup>-free external solutions containing 1 mM EGTA (data not shown).

The antibiotic neomycin prevents activation of PLC in a variety of cell types (18). The inclusion of neomycin in the recording pipette failed to significantly alter AII-mediated inhibition of I<sub>AC</sub> (Fig. 9B). In a total of six experiments with either 200 or 500 μM neomycin in the pipette, AII (10 nM) inhibited I<sub>AC</sub> by 61.4 ± 10.6%.

The phospholipase A<sub>2</sub> pathway is another source of second messengers that have been shown to behave as ion channel modulators. Arachidonic acid (AA) liberated from cell membranes following activation of phospholipases as well as biologically active arachidonic acid metabolites modulate K<sup>+</sup> channels in some cells (19, 20). Neither AA nor its metabolites were responsible for AII-mediated inhibition of I<sub>AC</sub>. The inclusion of AA (10 μM) in the pipette solution did not prevent the typical growth of I<sub>AC</sub> in whole cell recordings or inhibition of this current by AII (Fig. 9, A and B). Also, including the lipooxygenase inhibitor phenidone (1 mM) or the cyclo-oxygenase inhibitor indomethacin (100 μM) in the pipette to block, respectively, the formation of leukotrienes and prostaglandins from AA did not alter AII-mediated inhibition of I<sub>AC</sub> (Fig. 9B). Manoalide (10 μM in the recording pipette), which inhibits both phospholipase A<sub>2</sub> and phospholipase C (21, 22), was also ineffective in preventing AII inhibition of I<sub>AC</sub> (data not shown).

I<sub>AC</sub> Inhibition and Protein Kinases—The activity of many ion channels appears to be regulated by protein kinases, including those activated by cAMP, Ca<sup>2+</sup>, and Ca<sup>2+</sup>/calmodulin (23, 24). We used the potent non-selective protein kinase antagonist staurosporine (25) to determine whether AII-mediated inhibition of I<sub>AC</sub> might be mediated by such a kinase. Pre-exposing cells to 100 nM staurosporine reduced inhibition of I<sub>AC</sub> by 10 nM AII from 76.5 ± 4.6% (n = 6) to 27.9 ± 5.6% (n = 12) (Fig. 10). This effect of staurosporine was specific. ACTH-mediated inhibition of I<sub>AC</sub> was not reduced by staurosporine (Fig. 10A). Experiments such as those illustrated in Fig. 10A also demonstrate that individual AZF cells express both AII and ACTH receptors, which are coupled to I<sub>AC</sub> channels.

MgATP is the phosphate donor for most protein kinases. To determine whether ATP hydrolysis is necessary for AII-mediated inhibition of I<sub>AC</sub>, the non-hydrolyzable ATP analog AMP-PNP (1 mM) was substituted for ATP in the recording pipette. With this ATP analog in the pipette, AII (10 nM) inhibited I<sub>AC</sub> by only 18.1 ± 8.4% (n = 6) (Fig. 10B). The relative ineffectiveness of AII in the presence of staurosporine and AMP-PNP suggests that a protein kinase mediates inhibition of I<sub>AC</sub> channels.

The activity of many ion channels appears to be regulated by opposing phosphorylation/dephosphorylation mediated by kinase and phosphatases. If AII inhibits K<sup>+</sup> current through a kinase-stimulated phosphorylation, the potent phosphatase inhibitor okadaic acid (26) might be expected to produce a similar
inhibition of I\textsubscript{AC} by enhancing steady state phosphorylation of the channel. At a concentration of 200 nM, okadaic acid reversibly inhibited I\textsubscript{AC} in AZF cells by a maximum of 84\% (Fig. 11). Similar results were obtained in three of four cells exposed to okadaic acid at this concentration.

Although our results indicate that all\textsubscript{-}mediated inhibition of I\textsubscript{AC} is mediated by a protein kinase, it does not appear to be one typically activated through AT\textsubscript{1} receptor stimulation of PLC. Specifically, the inability of PMA to mimic AII inhibition of I\textsubscript{AC} indicates that protein kinase C does not mediate the response. The Ca\textsuperscript{2+}/calmodulin kinase inhibitor KN-62 (5 \textmu M) failed to prevent AII-mediated inhibition of I\textsubscript{AC} in three cells where I\textsubscript{AC} was reduced by 68 \pm 12\%.

**DISCUSSION**

We have demonstrated that in bovine AZF cells, AII inhibits I\textsubscript{AC} K\textsuperscript{+} current, depolarizes cells, and stimulates cortisol secretion through a losartan-sensitive AT\textsubscript{1} receptor. I\textsubscript{AC} inhibition appears to occur through a novel PLC-independent pathway, which includes a PTx-insensitive G protein and a staurosporine-sensitive protein kinase. The results indicate that in AZF cells, AT\textsubscript{1} receptors may regulate cortisol secretion by two parallel, Ca\textsuperscript{2+}-dependent pathways: a PLC-dependent release of Ca\textsuperscript{2+} from intracellular stores and a PLC-independent influx of Ca\textsuperscript{2+} secondary to membrane depolarization.

**AII Receptor Subtypes**—Of the two pharmacologically distinguishable AII receptor subtypes, only losartan-sensitive AT\textsubscript{1} receptors appear to mediate I\textsubscript{AC} inhibition, membrane depolarization, and steroid hormone secretion in bovine adrenal cortical cells. According to our model, AII-mediated depolarization of AZF cells is tightly coupled to I\textsubscript{AC} inhibition because these K\textsuperscript{+} channels set the resting membrane potential. In this regard, we have observed that in whole cell patch clamp recordings, all\textsubscript{-}mediated inhibition of I\textsubscript{AC} was not reversible, while in membrane potential recordings all\textsubscript{-}mediated depolarization was readily reversed. This apparent inconsistency may be due to the markedly different recording conditions. I\textsubscript{AC} recordings were made from thoroughly dialyzed cells at room temperature. Membrane potential recordings were made with sharp microelectrodes from metabolically intact cells at 35–37 \degree C. In a dialyzed cell, molecules required to restore inhibited I\textsubscript{AC} channels to a functional pool could be lost. The signaling pathway and cellular functions associated with AT\textsubscript{2} receptors are yet to be described. Our results clearly demonstrate that AT\textsubscript{2} receptors do not mediate AII-stimulated cortisol secretion, depolarization, or I\textsubscript{AC} inhibition in bovine adrenal cortical cells. The slight (<10\%) inhibition of cortisol secretion observed with 0.5 \mu M PD123319 may have been due to the interaction of this antagonist with an AT\textsubscript{1} receptor at this concentration (4).

Several distinct AT\textsubscript{1} receptor cDNAs, each coding for losartan-sensitive receptors have been cloned including two from rat adrenal cortex (7, 27–29). Possibly, one of these AT\textsubscript{1} receptor subtypes is specifically coupled to I\textsubscript{AC} inhibition through a
mechanism that does not involve PLC. However, both adrenal AT₁ receptor subtypes cloned thus far are coupled to PLC-mediated Ca²⁺ mobilization. Perhaps identical AT₁ receptors are linked to several signaling pathways through one or more G proteins.

In addition to activating PLC, losartan-sensitive AT₁ receptors in some cells are also coupled to adenylate cyclase inhibition through a PTx-sensitive G protein (8). This second AT₁-coupled pathway appears to be unrelated to IAC inhibition and cortisol secretion in AZF cells since the associated G protein is PTx insensitive. Inhibition of cAMP synthesis would suppress rather than stimulate cortisol secretion.

Phospholipase C-independent Inhibition of IAC—Several lines of evidence indicated that AII-mediated inhibition of IAC was independent of PLC. The inability of acetylcholine to inhibit IAC at concentrations that produce maximal activation of PLC and cortisol secretion (6, 17) in bovine AZF cells argues against a role for this enzyme in IAC inhibition, at least under the conditions of our patch clamp experiments. The failure of PLC antagonists neomycin (18, 30, 31) and manoalide (22) to significantly blunt IAC inhibition by AII, even though applied directly to the cell interior by patch pipette, leads to a similar conclusion.

Second messengers generated in response to PLC activation (or the biochemical equivalent) were ineffective in suppressing the expression of IAC or mimicking the action of AII on this current. PMA was applied after recording IAC in the whole cell mode for a period of 10–35 min. Failure of this phorbol ester to suppress IAC under these conditions might be due to "washout" of the target enzyme, protein kinase C. Even large macromolecules such as enzymes may reach diffusional equilibrium within minutes after initiating whole cell recording (32). However, since AII effectively inhibits IAC under identical conditions, it remains unlikely that diacylglycerol is the second messenger mediating inhibition of IAC by AII.

The failure of IP₃ to prevent the time-dependent expression of IAC in whole cell recordings or to alter the inhibition of this current by AII demonstrates that AII-stimulated IP₃ synthesis is not necessary for IAC inhibition by this peptide and that this phospholipid does not directly inhibit IAC channels. However,
in most of our experiments, intracellular Ca\(^{2+}\) was strongly buffered by pipette solutions containing 11 mM BAPTA. Under these conditions, IP\(_3\)-stimulated increases in intracellular Ca\(^{2+}\) would be eliminated or strongly suppressed (33, 34). Our findings then do not exclude the possibility that under physiological conditions, AII might also inhibit IAC through IP\(_3\)-stimulated release of Ca\(^{2+}\) where \([\text{Ca}^{2+}]_i\) may reach micromolar levels. Several types of Ca\(^{2+}\)-inhibited K\(^+\) channels have been identified (33, 35). However, our results do not indicate a major role for Ca\(^{2+}\) in IAC expression and modulation, since varying pipette Ca\(^{2+}\) concentration from 0 to 220 nM did not alter IAC expression or inhibition by AII. The observation that buffering [Ca\(^{2+}\)] to nominally zero with 20 mM IP\(_3\) (n = 2), neomycin (200 or 500 \(\mu\)M, n = 6), 10 \(\mu\)M AA (n = 3), 1 mM phenidone (n = 2) and 100 \(\mu\)M indomethacin (n = 2). Results are mean ± S.E.

We conclude that AII-mediated inhibition of IAC occurs through a phospholipase C-independent pathway. A similar phospholipase C-independent parallel pathway appears to function in muscarinic inhibition of K\(^+\) current in neuronal cells (37).

Protein Kinases and IAC Inhibition—Experiments with AMP-PNP and staurosporine indicated that AII may inhibit IAC through activation of a protein kinase. It is unlikely that this kinase is either of the serine-threonine kinases that are activated by PLC-generated second messengers. Specifically, the failure of PMA to mimic AII appears to exclude kinase C. The insensitivity of AII-mediated inhibition to varying [Ca\(^{2+}\)], to strong Ca\(^{2+}\) buffering by BAPTA, and to calmodulin kinase inhibitor KN-62 indicates that Ca\(^{2+}\)/calmodulin kinase is not involved.

Staurosporine (100 nM) reduced AII-mediated inhibition of IAC by approximately 64%. Since this drug inhibits the identified serine-threonine kinases with IC\(_{50}\) values ranging from <1 to 20 nM (25), the incomplete block of AII effects on IAC may suggest that a different protein kinase is involved. Staurosporine also inhibits tyrosine kinases, although at somewhat

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**Fig. 9.** Effect of Ca\(^{2+}\) and phospholipases on IAC expression and inhibition by AII. Whole cell IAC currents were recorded using the voltage protocol shown in Fig. 1B and a variety of pipette solutions. IAC was allowed to reach a stable maximum value before superfusing the cell with 10 nM AII. A, maximum IAC current density (pA/pF) observed with pipette solutions where free Ca\(^{2+}\) was buffered to nominally 0 (Ca\(^{2+}\) = 0, n = 5), 22 nM (control, n = 6), or 220 nM (Ca\(^{2+}\) = 10, n = 2) using the Bound and Determined program (14). Current densities are also shown for standard pipette solutions supplemented with 5 mM IP\(_3\) (n = 2) or 10 \(\mu\)M AA (n = 3). B, the maximum inhibition of IAC by AII (10 nM) was measured using pipette solutions containing 0 (Ca\(^{2+}\) = 0, n = 5), 22 nM (control, n = 6), or 220 nM (Ca\(^{2+}\) = 10, n = 2) free [Ca\(^{2+}\)] as described above. IAC block by AII was also measured when standard pipette solution was supplemented with 5 \(\mu\)M IP\(_3\) (n = 2), neomycin (200 or 500 \(\mu\)M, n = 6), 10 \(\mu\)M AA (n = 3), 1 mM phenidone (n = 2) and 100 \(\mu\)M indomethacin (n = 2). Results are mean ± S.E.

**Fig. 10.** IAC block and kinase inhibition by staurosporine and AMP-PNP. A, staurosporine. K\(^+\) current was activated by voltage steps to +20 mV from a holding potential of −80 mV. Cell was superfused with 100 nM staurosporine for 10 min before switching to solutions containing this antagonist as well as AII (10 nM) and then ACTH (100 pM) as indicated. IAC amplitude (measured at the end of 300-ms voltage step) is plotted against time. B, AMP-PNP and staurosporine. The inhibition of IAC by 10 nM AII was studied in experiments similar to those described in A. Staurosporine (100 nM) was applied by bath perfusion, and AMP-PNP (1 mM) was substituted for ATP in the pipette solution. Fraction of IAC inhibited expressed as mean ± S.E. for AII, AII + staurosporine, and AII + AMP-PNP as indicated.
higher concentrations (25). In this regard, AII has been shown to induce losartan-sensitive tyrosine phosphorylation in rat aortic smooth muscle cells. This phosphorylation is inhibited by staurosporine (38). Perhaps a similar tyrosine kinase is involved in AII-mediated inhibition of IAC.

The ability of okadaic acid to reversibly inhibit IAC raises the possibility that AII might act through the inhibition of a protein phosphatase. Our results with staurosporine and AMP-PNP suggest that this phosphatase inhibition might require prior activation of a protein kinase. The delay of up to several minutes that precedes AII’s effect on IAC and membrane potential is consistent with such a multi-step process. Complex pathways requiring both kinases and phosphatases in peptide-mediated ion channel modulation have been described (39, 40).

Major components of the pathway that links AT1 receptors to IAC are yet to be specifically identified. The delay of 1 to several minutes that precedes AII-mediated inhibition of the current is inconsistent with a response that involves a tight coupling between AII receptor, G protein, and IAC channels. A delay of this duration is excessive, even for responses that require the synthesis of a cytoplasmic diffusible secondary messenger. Further, since AII-mediated inhibition was observed in cells that had been held in whole cell patch clamp for periods in excess of 30 min, it seems doubtful that a freely diffusible cytoplasmic messenger is involved. Protein kinases tightly associated with rat brain K+ channels have been shown to modulate these channels after reconstitution into lipid bilayers (24). Our results suggest that a similar membrane-associated kinase may couple AT1 receptors in AZF cells to phosphorylation and inhibition of IAC channels. The successful inhibition of unitary IAC currents in cell-attached patches upon superfusion of the cell membrane outside the pipette with AII is consistent with the suggestion that diffusible, lipid-soluble intramembranous second messengers may be functioning.

Secretion studies showed that AII-stimulated cortisol production, like membrane depolarization and IAC inhibition, occurs through losartan-sensitive AT1 receptors. Apparently, AT1 receptors are coupled through PTx-insensitive G proteins to cortisol secretion by two parallel Ca2+-dependent signaling pathways. In addition to the previously described PLC- and IP3-mediated release of Ca2+, we have identified a novel PLC-independent path that couples losartan-sensitive receptors to membrane depolarization and Ca2+ entry through voltage-gated T-type Ca2+ channels (10, 12). Because components of this signaling mechanism appear to be identical in AZF and AZG cells, AII-mediated inhibition of IAC should function as a stimulus for both cortisol and aldosterone secretion.

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Fig. 11. Inhibition by okadaic acid (OKAA). IAC was activated in an AZF cell by voltage steps to + 20 mV as described in the legend of Fig. 1. The cell was then superfused with 100 nM okadaic acid for the indicated durations. IAC amplitude is plotted against time.