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ATRIAL NATRIURETIC FACTOR INHIBITS ANGIOTENSIN-INDUCED ALDOSTERONE SECRETION: NOT THROUGH cGMP OR INTERFERENCE WITH PHOSPHOLIPASE C

Arunabha Ganguly, Shirley Chiou, Leigh A. West, and John S. Davis

Department of Medicine, University of South Florida College of Medicine
Tampa, Florida 33620

James A. Haley Veterans Hospital, Tampa, Florida 33612

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Summary: ANF did not prevent the formation of [^{3}H] inositol trisphosphate in response to AII but inhibited aldosterone secretion in calf adrenal glomerulosa cells. 8-bromo cGMP did not affect either inositol phosphate formation or aldosterone secretion. Changes in cytosolic Ca^{++} concentration induced by AII, as measured by Quin 2 fluorescence, were also unaffected by ANF. No difference in adrenal cell protein phosphorylation with AII or AII+ANF was observed. The results suggest that ANF may inhibit aldosterone secretion through a non-guanyl cyclase linked receptor system not involving the formation of phosphoinositide-derived second messengers. Interference with protein kinase C activity cannot be ruled out.

It is now known that ANF inhibits aldosterone secretion, especially when stimulated by its secretagogues, *in vivo* (1-3) and *in vitro* (2-10). However, the mechanism of this inhibition by ANF remains unknown. ANF is thought to produce some of its biological effects through the activation of guanyl cyclase and formation of cGMP (11-13). We have investigated the effect of ANF on angiotensin II (AII)-mediated aldosterone secretion, and the possible role of cGMP in this effect. Since AII-induced hydrolysis of phosphoinositides (14-16) liberates putative intracellular messengers, inositol 1,4,5-trisphosphate(IP_3) and 1,2-diacylglycerol(DAG), which initiate the rise of cytosolic free calcium (cCa^{++}) concentration (17,18) and activation of protein kinase C (14) respectively, we have examined the effects of ANF on IP_3 formation and cCa^{++} in calf adrenal glomerulosa cells.

Materials and Methods

Synthetic [^{Arg}101-^{Tyr}126]-ANF (26 amino acid rat sequence) was used in this study. Collagenase was obtained from Cooper Chemicals, Malvern, PA; myo-[2-^{3}H]-inositol was purchased from American Radiolabeled Chemicals, St. Louis, MO; tritiated (1,2,6,7-^{3}H)-aldosterone, and $^{32}PO_4$ were obtained from Amersham Co., Arlington Heights, IL. The columns for ion exchange chromatography and the anion exchange resin (AG-X 8) were purchased from Biorad, Inc., Richmond, CA. The rest of the chemicals were obtained from Sigma Chemicals Co., St. Louis, MO.

Calf adrenal glomerulosa cells were prepared as previously described (19). In brief, slices (0.5 mm) of the outer adrenal cortex dispersed for 1 hour using collagenase (3.0-3.5 mg/ml) and DNAase (50 µg/ml) in medium 199 containing 1.8 mM $CaCl_2$ and 5 mM KCl. The cells obtained after filtration (18) were washed several times and resuspended in the medium

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199. In all experiments, the adrenal cells were incubated in an atmosphere of 95% O_2 and 5% CO_2 in a shaking bath.

Adrenal glomerulosa cell inositol phospholipids were prelabeled with myo-[2-^{3}H]-inositol (50 μCi/ml) by incubating the cells for 2-3 hours (19,20). After this incorporation period, the cells were washed twice with unlabeled inositol (10 mM). After a further wash, the labeled cells were aliquoted into siliconized tubes in a water bath at 37°C and preincubated with LiCl (10 mM) for 15 min before initiating the experiments. The cells were then incubated (final volume 0.5 ml) in triplicate with control media, AII (10^{-8} M), ANF (10^{-8} M), 8-bromo cGMP (Br-cGMP) (2 mM), AII + ANF, and AII + Br-cGMP for 2, 10, or 30 min. After removing 0.2 ml of the medium from each tube for aldosterone measurement, 0.5 ml of 10% trichloroacetic acid was added to terminate the reaction. The inositol phosphates in this sample were isolated by ion exchange chromatography as described previously (20). The IP_3 fraction collected with this procedure contains inositol 1,4,5- and 1,3,4-trisphosphates. These experiments were performed 3 times.

The aldosterone in the incubation media of various experiments was measured by radioimmunoassay as reported (19) after extracting 0.1-0.15 ml of the media with 20 volumes of methylene chloride. In experiments involving incubation of unlabeled cells, aldosterone was measured directly from the medium.

In other experiments, we incubated adrenal glomerulosa cells for 2 hours with 8-bromo cGMP (Br-cGMP), calcium ionophore, A23187, phorbol ester,12-O-tetradecanoyl phorbol13-acetate, (TPA) or analogues of DAG (1-oleoyl-2-acetylglycerol and 1,2-dioctanoylglycerol) in various combinations to determine the effects on aldosterone secretion by AII or AII+ANF.

In other experiments, adrenal glomerulosa cells were loaded with Quin 2 AM according to the method described by Capponi et al. (18). Such Quin 2 loaded cells in triplicates were then treated with AII (10^{-8} M) alone or with AII + ANF (10^{-8} M). The fluorescence produced by Quin 2 was then monitored and the changes in cytosolic free Ca^{++} concentration produced in these experiments were then calculated as previously reported (18).

Phosphorylation of adrenal glomerulosa cell proteins was performed essentially as described by Elliott and Goodfriend (21). Glomerulosa cells (3.2 x 10^6 cells) were incubated for 60 min in medium 199 containing $^{32}PO_4$ (1mCi/ml) with AII (10^{-8} M), ANF (10^{-8} M) or AII+ANF in a final volume of 0.25 ml. Hormone treatments were added at the beginning of incubation and again at 30 min. The incubations were terminated at 60 min by the addition of an ice-cold buffer containing 100mM NaF, 80mM sucrose, 10mM EDTA and 10mM Tris-HCl, pH 7.4. After washing, the cells of each incubate were resuspended in a homogenizing buffer (10 mM NaF, 50 mM 2-mercaptoethanol, 2 mM EDTA, 5 mM EGTA, 250 mM sucrose, and 20 mM Tris-HCl, pH 7.5) and sonicated. The homogenate was centrifuged at 130,000 x g for 20 min. The supernatant (cytosol fraction) was removed and the pellet (membrane fraction) was resupended in the homogenizing buffer. Aliquots were removed for estimation of protein concentration. Vertical slab SDS-PAGE was carried out after loading 0.08 mg of either membrane or cytosol proteins from each sample onto 1.5 mm gel consisting of a 4% acrylamide stacking gel and 12% acrylamide separating gel. After electrophoresis the gels were stained and dried. Autoradiography was performed to determine the location of the labeled protein bands.

Results

AII significantly increased [^{3}H]-IP_3 in the glomerulosa cells during the timed incubation at 2, 10, and 30 min (Fig. 1) and other [^{3}H]-inositol phosphates (not shown). The amount of IP_3 formed in response to AII plus ANF was similar to that produced in its absence but aldosterone secretion was markedly reduced in the presence of ANF (Fig. 1 and 2). The findings were consistent since all 3 experiments showed the same results.

When Br-cGMP was added with AII, again the magnitude of increase of IP_3 in response to AII was unaffected (Fig. I). Aldosterone produced in the presence or absence of Br-cGMP was similar (Fig. 2). Other experiments also showed (Table 1) that Br-cGMP did not inhibit either basal or AII-induced aldosterone secretion over a wide range of concentrations (0.1 to 5.0 mM). If anything, Br-cGMP tended to increase basal aldosterone secretion (by about 34%) with the highest dose used.

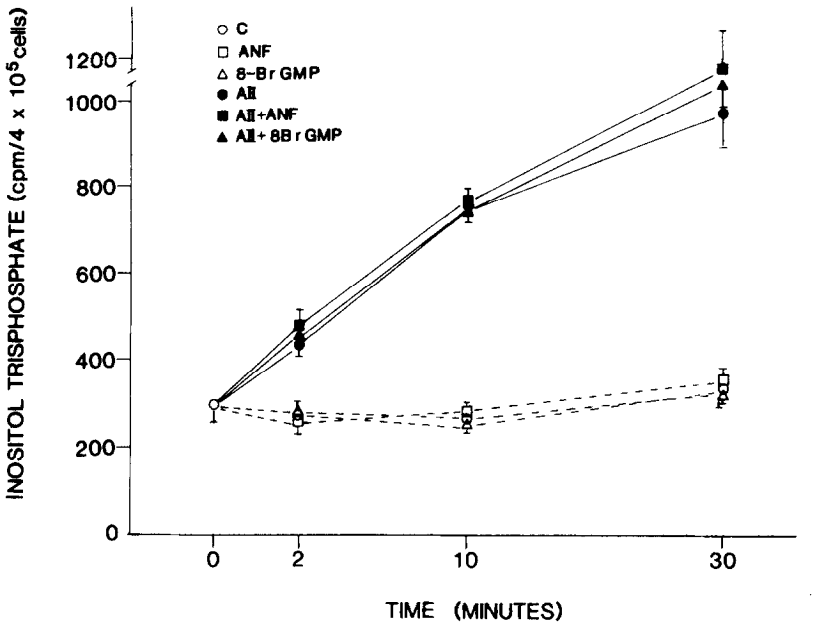


Fig. 1 IP_3 (cpm/4x10^5 cells) responses in labeled calf adrenal glomerulosa cells to AII (10^{-8}M), ANF (10^{-8}M), Br-cGMP (2mM), AII + ANF and AII + Br-cGMP as compared with control in timed incubation for 2, 10 and 30 minutes (mean ± sem of triplicates) of one representative experiment.

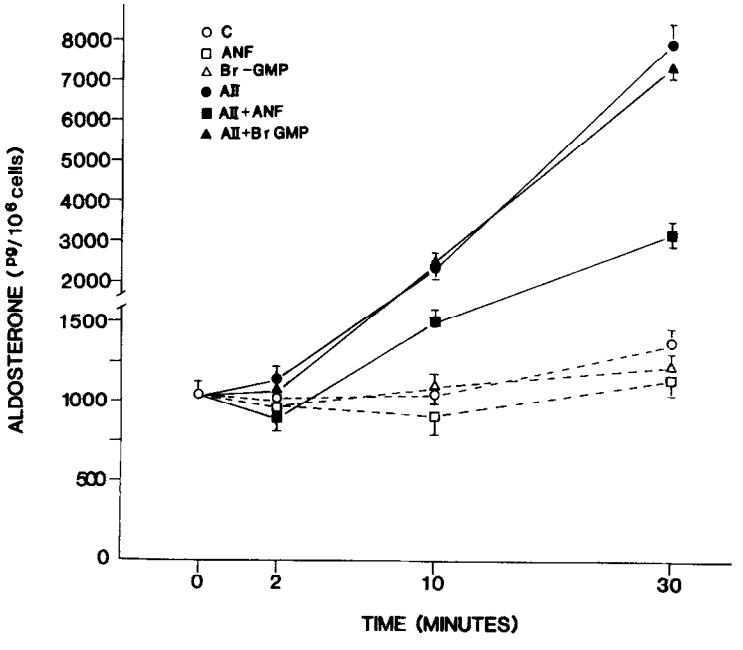


Fig. 2 Aldosterone production from calf adrenal glomerulosa cells in timed incubation for 2,10 and 30 minutes in response to AII (10^{-8}M), ANF (10^{-8}M), Br-cGMP (2mM), AII + ANF and AII + Br-cGMP as compared with control. The results are mean ± sem of triplicates.

TABLE 1. DOSE RESPONSE OF BROMO GMP ON ALDOSTERONE SECRETION [MEAN(SEM) NG/10^6 CELLS]

Control	AII (10^{-8}M)	ANF (10^{-8}M)	Br GMP 0.1mM	Br GMP 5mM	AII (10^{-8}M) + ANF (10^{-8}M)	AII (10^{-8}M) + Br GMP 0.01	0.1	1.0	2.5	5.0
14.2 (0.9)	57.4 (1.2)	10.2 * (0.4)	14.3 (0.4)	19.1** (0.2)	34.6 *** (1.9)	59.6 (3.3)	60.4 (0.4)	64.8 (2.4)	60.0 (3.6)	63.2 (1.5)

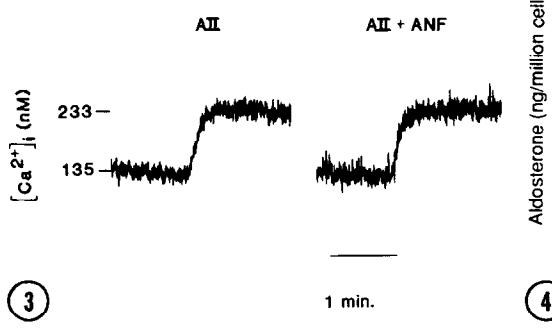
* $p<0.02$ (Control vs. ANF) ** $p< 0.01$ (Control vs. Br GMP 5mM) *** $p< 0.001$ (AII vs. ANF)

Monitoring of Quin 2 signals in response to AII (10^{-8} M) and AII with ANF (10^{-8} M) revealed identical changes in intracellular fluorescence (Fig. 3). In three separate trials ANF did not alter basal cCa^{++} (109 ± 13 vs 111 ± 14 nM for control and ANF, respectively) or AII-stimulated levels (186 ± 24 vs 182 ± 17 nM for AII and AII ± ANF, respectively). At these doses, AII-induced aldosterone secretion was consistently reduced by ANF as shown earlier. Calcium ionophore, A23187, or TPA failed to reverse the effect of ANF on the AII-evoked aldosterone secretion (Fig. 4). The same agents as well as DAG analogues were also not able to prevent the inhibitory effect of ANF on basal aldosterone secretion (not shown) in the concentrations used.

Phosphorylation of membrane and cytosolic proteins in ^{32}P-labeled adrenal cells was similar in the presence and absence of AII, ANF or AII + ANF (Fig. 5).

Discussion

Our present studies showing that ANF consistently reduced aldosterone secretion in vitro from the calf adrenal glomerulosa cells are in agreement with a number of other studies (2-



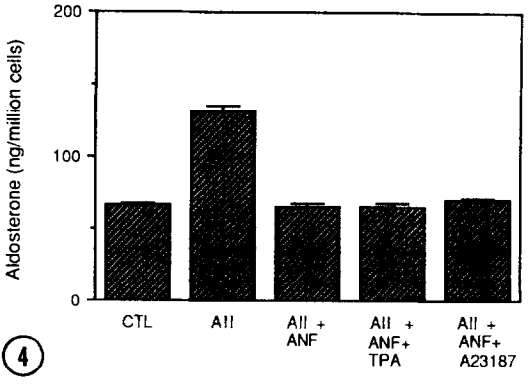


Fig. 3 Changes in cytosolic free Ca^{++} levels (nM) in calf adrenal glomerulosa cells in response to AII (10^{-8}M) with or without ANF (10^{-8}M) in one of three representative experiments.

Fig. 4 Aldosterone response from calf adrenal glomerulosa cells incubated for 2 hours with AII (10^{-8}M), AII + ANF (10^{-8}M), latter with or without TPA (10nM) or A23187 (10nM). Mean ± sem of triplicates is shown.

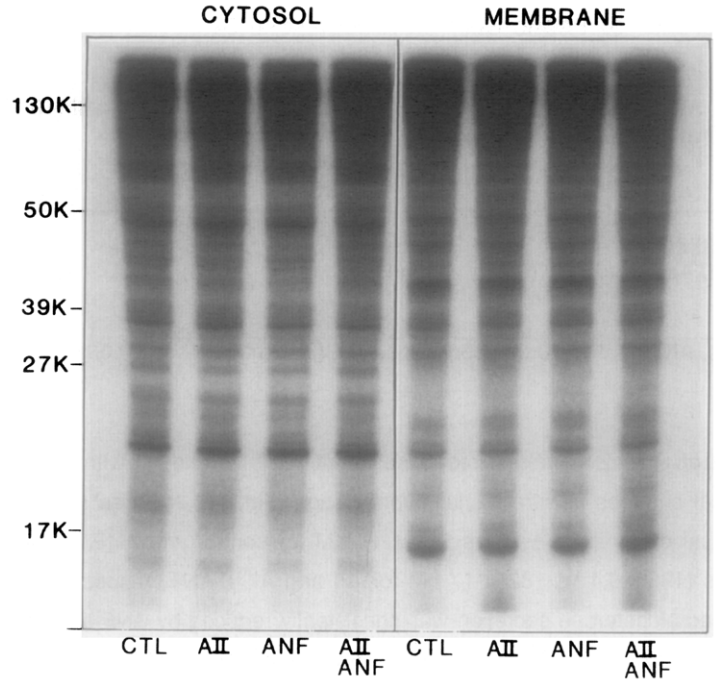


Fig. 5 Autoradiographs of phosphoproteins in the cytosolic and membrane fractions of calf adrenal glomerulosa cells incubated for 60 minutes with AII (10^{-8}M), ANF (10^{-8}M), Br-cGMP (2mM) or AII + ANF in the presence of $^{32}PO_4$. Further details are in the Methods Section.

10). We have extended these studies by exploring the possible mechanism of this ANF-mediated inhibition of aldosterone secretion. ANF could potentially affect one of different intracellular mediators involved in aldosterone secretion. ANF does not affect the binding of AII to its adrenal cell surface receptor (22). Specific ANF receptors have been identified in the adrenal glomerulosa cells (23), one of which is linked to guanyl cyclase. An increase in cGMP levels has been observed in response to ANF in the adrenal glomerulosa cells in association with the inhibition of aldosterone secretion (24). On the basis of this and the alleged involvement of cGMP in the vasodilatory effect of ANF, cGMP has been suspected in the ANF-mediated inhibition of aldosterone secretion. We, however, did not observe any inhibitory effect of 8-bromo cGMP on aldosterone secretion. ANF as well as 8-bromo cGMP also did not inhibit the hydrolysis of phosphatidylinositol 4,5-bisphosphate, as judged by the generation of IP_3 in response to AII, while aldosterone secretion from the same cells was markedly inhibited only by ANF. Thus, a known intracellular messenger produced by ANF could not account for the inhibitory effects of ANF in glomerulosa cells.

Since the rise in the cytosolic free Ca^{++} is postulated to be an important concomitant of AII-induced aldosterone secretion, we compared the Quin 2 fluorescence signals in the adrenal cells in response to AII in the presence or absence of ANF. Again, these signals were unaffected by ANF. Capponi et al. (8) have observed similar results suggesting at least that the initial rise of the cytosolic free Ca^{++} produced by AII in the presence of ANF was normal. This suggestion is

in agreement with our data on IP_3, since IP_3 is thought to be responsible for the intracellular mobilization of Ca^{++} producing the early Quin 2 signal.

ANF has been shown to inhibit phosphorylation of some proteins in the adrenal cells (25). Elliott and Goodfriend (21) using one-dimensional electrophoresis, have observed inhibition of phosphorylation of a 17.6Kd protein by ANF in the adrenal cells stimulated by AII. Takagi et al. (26), however, were unable to demonstrate specific alterations in a 17.6Kd protein phosphorylation in response to ANF. In our experiments, we did not observe any difference in the patterns of phosphorylation of cytosolic or membrane proteins in adrenal glomerulosa cells stimulated by AII in the presence or absence of ANF. Whether two-dimensional electrophoresis may show a different pattern of phosphorylation of proteins induced by AII (27) in the presence or absence of ANF is presently unknown.

We have also examined the effects of Ca^{++}-ionophore, A23187 on ANF-mediated inhibition of basal and AII-provoked aldosterone secretion. A23187 failed to reverse the influence of ANF on aldosterone secretion as did TPA and diacylglycerol analogues. ANF may (28) or may not (29) inhibit Ca^{++}-influx into the adrenal glomerulosa cells. There is evidence of inhibition by ANF of Ca^{++}-influx into vascular tissues induced by angiotensin and vasopressin (30-32). We have not examined the effect of ANF on Ca^{++}-influx. In the vascular tissue, however, ANF has not been able to alter the calcium transient (8,33), thought to be due to intracellular mobilization of Ca^{++} induced by AII through the mediation of IP_3, a finding consistent with our results in the adrenal cells.

Thus, our investigations suggest that ANF does not interfere with the formation of IP_3 and initial mobilization of cellular Ca^{++}. ANF conceivably may affect either Ca^{++}-influx since we have recently demonstrated that Ca^{++}-influx is crucial throughout the period of stimulation of aldosterone secretion by AII (34) or ANF may influence a later step involving the action of protein kinase C. Alternatively, ANF might inhibit aldosterone secretion by a non-guanyl cyclase (35) but as yet unidentified mechanism.

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