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Negative regulation of atrial natriuretic factor receptor coupled membrane guanylate cyclase by phorbol ester

Potential protein kinase C regulation of cyclic GMP signal in isolated adrenocortical carcinoma cells of rat

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Rat adrenocortical carcinoma cells possess a high density of atrial natriuretic factor (ANF) receptors which are coupled with membrane guanylate cyclase and corticosterone production. Herein we show that pretreatment of these cells with phorbol 12-myristate 13-acetate (PMA), a known activator of protein kinase C, attenuates the ANF-stimulated cyclic GMP accumulation in a dose-dependent manner. The half maximum inhibitory concentration of PMA was 10^{-10} M. When these cells were incubated with PMA in the presence of 1-(5-isoquinolinyl-sulfonyl)-2-methyl piperazine, a protein kinase C inhibitor, the PMA-mediated attenuation of ANF-stimulated cyclic GMP formation is blocked. These results suggest that protein kinase C negatively regulates the ANF-receptor coupled membrane guanylate cyclase system in these cells.

Atrial nutriuretic factor; cyclic GMP; Guanylate cyclase; Protein kinase C; Phorbol ester

1. INTRODUCTION

Original studies with rat adrenocortical carcinoma indicated a mediatory role of cyclic guanosine monophosphate (cyclic GMP) in ACTH and in α_2-adrenergic receptor mediated signal transduction as reviewed in [1]. Until recently, this hypothesis was not widely accepted since there was a general disbelief in the existence of a distinct hormone-dependent membrane guanylate cyclase in any endocrine or nonendocrine tissue [2,3]. The reservations to this concept were overcome when two distinct types of guanylate cyclase – membrane and soluble – were demonstrated in rat adrenocortical carcinoma. Only the membrane enzyme is hormone specific [4–6], but both α_2-adrenergic receptor signals [7] and ACTH [4–6] are positively coupled to the membrane guanylate cyclase. The studies with isolated fasciculata cells of rat adrenal cortex [8], in vivo studies with rat adrenal gland [9] and in situ studies with mouse interstitial [10] and Leydig cells [11,12], all of which indicate the second messenger role of cyclic GMP in ANF steroidogenic signal transduction may be of exceptional importance. Consistent with this concept is the isolation, biochemical and immunological characterization of a homogeneous 180 kDa membrane guanylate cyclase coupled ANF receptor from the steroid secreting adrenocortical carcinoma cells [13], which provides a direct transmembrane biochemical link between the receptor and cyclic GMP formation. One of the regulatory mechanisms of cyclic GMP-mediated ANF signal transduction has now been offered by our demonstrating that phorbol ester signal, probably through protein kinase C, negatively regulates the ANF receptor-coupled membrane guanylate cyclase.

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2. MATERIALS AND METHODS

The isolated rat adrenocortical carcinoma cells were prepared by the trypsin digestion method [14,15]. The cells have been thoroughly characterized, morphologically and biochemically [15,16]. The isolated adrenocortical carcinoma cells were suspended in Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 4% albumin and 0.2% glucose. To study the influence of PMA on ANF-dependent cyclic GMP formation, the cells (5×10^5) were incubated at 37°C under 5% CO_2/95% O_2 with varying concentrations of PMA in the presence or absence of 1 μM ANF in a total volume of 1.0 ml of Krebs-Ringer bicarbonate buffer. Every incubation experiment was conducted in triplicate and repeated at least three times. Unless otherwise noted, the cells were preincubated for 10 min in the presence of PMA prior to initiation of the appropriate assay; PMA was present throughout the subsequent assay incubation period. The assay for cyclic GMP was conducted at 20 min. At the end of the incubation time, 1.0 ml of 1 M perchloric acid was added to terminate the reaction. The reaction mixture was neutralized by the addition of 10 M KOH and the tubes were centrifuged at 500 × g for 10 min. The cGMP levels were determined in the supernatant by radioimmunoassay [17]. To determine the particulate guanylate cyclase activity changes in response to the PMA, the adrenocortical carcinoma cells (1×10^6) were preincubated with 1 μM PMA for 10 min and then another 20 min with 1 μM ANF at 37°C in the continuous presence of PMA. The cells were isolated by centrifugation at 600 × g, and were washed two times with Krebs-Ringer bicarbonate buffer. The washed cells were suspended in 1.0 ml of ice-cold 40 mM Tris-HCl buffer (pH 7.5), containing 10 mM $MgCl_2$, 0.5 mM EDTA and 10 mM β-mercaptoethanol (buffer A), and sonicated for 15 s. The homogenate was centrifuged at 105000 × g for 60 min and the pellet was resuspended in the buffer A. Particulate guanylate cyclase activity was essentially assessed as described earlier [13]. The reaction mixture (0.1 ml) contained 10 mM theophylline, 15 mM creatine phosphate, 20 μg creatine kinase, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 4 mM GTP in 50 mM Tris buffer (pH 7.5) and 20 μl of enzyme suspension. The reaction was initiated by the addition of the substrate solution containing $MgCl_2$ and GTP. Incubation was stopped after 20 min at 37°C by the addition of 0.9 ml of ice-cold 50 mM sodium phosphate buffer (pH 6.2). After centrifugation, the cGMP formed was determined by radioimmunoassay [17].

ANF consisting of residues 8–33, was a kind gift from Dr Ruth F. Nutt, Merck Sharp and Dohme Research Laboratories; trypsin and soyabean trypsin inhibitors were from Cooper Biomedical and phorbol 12-myristate 13-acetate, 4β-phorbol and phorbol 12,13-didecanoate were purchased from Sigma. All other reagents were of analytical grade from commercial sources.

3. RESULTS AND DISCUSSION

Consistent with our previous results [8], ANF stimulated both the membrane guanylate cyclase and cyclic GMP formation in isolated rat adrenocortical carcinoma cells (fig.1A). Sodium nitroprusside, an agent known to stimulate soluble

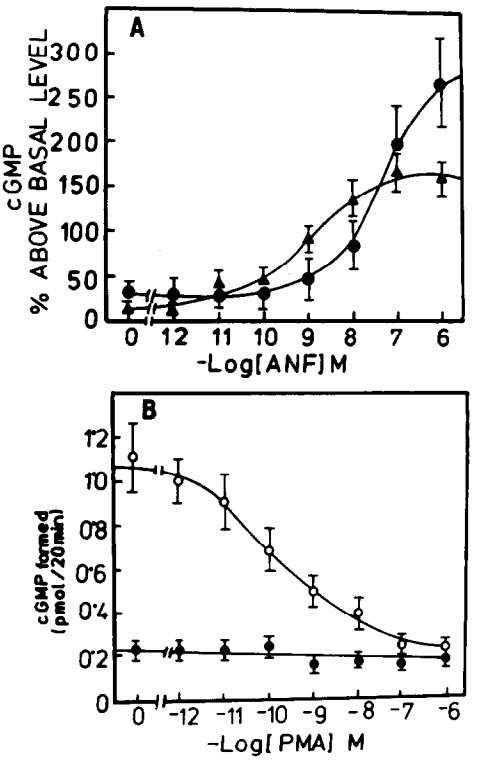

Fig.1. (A) Dose dependence of cyclic GMP (•) and membrane guanylate cyclase (▲) formation by ANF in rat adrenocortical carcinoma cells. Incubation system: 0.8 ml adrenocortical carcinoma cells suspension (5×10^{-5}); reagents dissolved in 0.2 ml Krebs-Ringer bicarbonate buffer with glucose and albumin; total volume of incubation mixture, 1.0 ml. The data summarize the results of 3 separate experiments, each of which contain triplicate cell incubation mixtures. The basal value of cyclic GMP (0.25 × 0.04 pmol) has been subtracted. Guanylate cyclase was assayed for 20 min as described [17]. Results are shown as the mean ± SE (n = 3). Basal guanylate cyclase activity is 2.6 pmol/20 min per 10^6 cells. (B) Concentration dependence of PMA inhibition of ANF-stimulated cyclic GMP formation. The rat adrenocortical carcinoma cells were treated with various concentrations of PMA for 10 min, and then exposed to 1 μM AMF for 20 min. Conditions were similar to those in A. Results are expressed as the mean values of three separate determinations ± SE. (•) Without ANF, (○) with 1 μM ANF.

guanylate cyclase [3] but ineffective toward particulate guanylate cyclase [5], did not stimulate cyclic GMP formation in the adrenocortical carcinoma cells [6], thus indicating the selective coupling of particulate guanylate cyclase with the ANF receptor. To determine the interaction of phorbol ester signal transduction with basal and ANF-dependent cyclic GMP levels, the adrenocor-

tical carcinoma cells were incubated with varying concentrations of PMA (the active analog of phorbol ester) in the presence or absence of 1 μM ANF, the concentration which causes the maximal formation of cyclic GMP. There was no effect on the basal level of cyclic GMP, but the ANF-dependent cyclic GMP rise was blocked by PMA in a concentration-dependent manner, with a K_i of 10^{-10} M and with a complete block by 5×10^{-7} M (fig.1B). PMA also inhibited the hormonally dependent membrane guanylate cyclase (table 2). Since the phorbol ester receptor is protein kinase C [18,19], the results imply that this protein kinase is the mediator of the inhibitory signal. This interpretation is further supported by two types of observations. First, the two phorbol ester analogs, 4β phorbol and phorbol 12,13-didecanoate, which do not cause stimulation of protein kinase C, did not interfere with the ANF-dependent elevation of cyclic GMP levels (table 1). Second, a non-specific inhibitor of protein kinase C, 1-(5-isoquinolinylsulfonyl)-2-methyl piperazine, [H-7], which has been shown to inhibit various cellular responses stimulated by protein kinase C activating phorbol esters [20,21], released the inhibition caused by PMA on the ANF-dependent increment of cyclic GMP levels (fig.2).

In molecular terms the obvious mechanism by which protein kinase C could regulate the membrane guanylate cyclase activity is via the process of phosphorylation and dephosphorylation. In such a mechanism, protein kinase C would terminate the cyclic GMP-mediated signal transduction by phosphorylation of the receptor-coupled guanylate cyclase and dephosphorylation of the enzyme which would cause the signal to be propagated.

Membrane guanylate cyclase and protein kinase C interaction raises other interesting points. The two limbs of the phosphatidylinositol signal pathway are turned on by the activation of phosphatidylinositol 4,5-bisphosphate phospho-

Table 1

Effects of various analogs of phorbol esters on the ANF-stimulated cyclic GMP level in isolated rat adrenocortical carcinoma cells

Pretreatment	cyclic GMP formed (pmol/20 min per 5×10^5 cells)	
	− ANF	+ ANF
Basal	0.25 ± 0.04	1.20 ± 0.06
PMA	0.21 ± 0.03	0.30 ± 0.02
4α-Phorbol 12,13-didecanoate	0.24 ± 0.04	1.05 ± 0.04
4β-Phorbol	0.20 ± 0.02	0.90 ± 0.04

Cells (5×10^5) were incubated with various analogs of phorbol esters (1 μM) for 10 min and then another 20 min with or without 1 μM ANF in a total volume of 1.0 ml, and were then assayed for cyclic GMP formation as described in section 2. Values are means ± SE (n = 3)

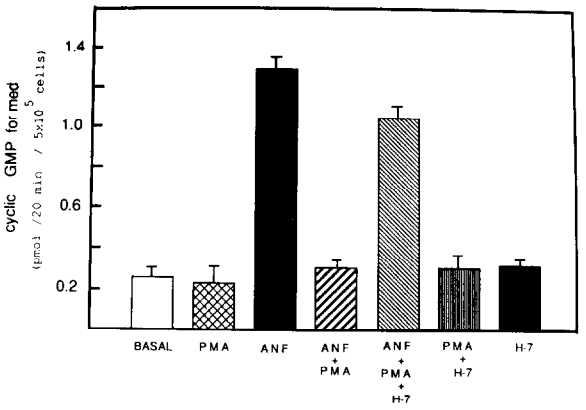

Fig.2. Effect of PMA, ANF and H-7 on the cyclic GMP formation in isolated adrenocortical carcinoma cells. The conditions of the experiments were identical to those in fig.1, except the tubes containing H-7 (100 μM final) were preincubated together with PMA for 10 min and then stimulated with 1 μM ANF for another 20 min. Results are shown as the mean ± SE (n = 3).

Table 2

Effect of PMA on membrane guanylate cyclase activity in response to ANF in isolated adrenocorticl carcinoma cells

Treatment	Guanylate cyclase activity (pmol/20 min per 10^6 cells)	
	− ANF	+ ANF
Cells treated without PMA	2.6 ± 0.10	6.0 ± 0.21
Cells treated with PMA	2.4 ± 0.06	2.5 ± 0.12

The cells (1×10^6) were preincubated with or without PMA (1 μM) for 10 min and then with or without 1 μM ANF for another 20 min in a total volume of 1.0 ml as described in section 2. The tubes were then centrifuged at 600 × g in an IEC centrifuge, model PR-1. The cells were washed twice with 40 mM Tris-HCl buffer, pH 7.4; containing 10 mM $MgCl_2$, 0.5 mM EDTA and 10.0 mM β-mercaptoethanol. The cells were sonicated for 15 s by a Branson sonifier cell disruptor, model 185, and the homogenate was centrifuged at 105000 × g for 60 min. The pellet was resuspended in the above buffer and guanylate cyclase activity was assayed as described [17]

diesterase which catalyzes the cleavage of phosphatidylinositol 4,5-bisphosphate into inositol triphosphate (IP_3) which in turn regulates the levels of intracellular calcium, and 1,2-diacylglycerol which activates protein kinase C. Analogous to the situation with the phorbol ester receptor, the vasopressin receptor signal also negatively regulates the ANF-dependent formation of cyclic GMP and positively regulates the phosphatidylinositol turnover [21]. The link between vasopressin and ANF receptor signals might also be protein kinase C. The α_2-adrenergic signal transduction positively regulates membrane guanylate cyclase and negatively regulates the adenylate cyclase [7]. Together, the results indicate that these transmembrane receptor signals in which cyclic GMP plays a bona fide second messenger role are intertwined.

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