Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 171-178

NINHIBIN: A SPERM FACTOR ATTENUATES THE ATRIAL NATRIURETIC FACTOR MEDIATED INHIBITION OF ADENYLATE CYCLASE: POSSIBLE INVOLVEMENT OF INHIBITORY GUANINE NUCLEOTIDE REGULATORY PROTEIN [1]

Madhu B. Anand-Srivastava[2], Roger A. Johnson*,
Sylvie Picard and Marc Cantin

Clinical Research Institute of Montreal, 110 Pine Avenue West,
Montreal, Quebec, Canada

Received March 25, 1985

Summary: The effect of ninhibin, a sperm factor extracted from bovine sperm, was studied on adenylate cyclase from rat aorta. Ninhibin treatment of the membranes activated adenylate cyclase in a concentration dependent manner. The maximal activation (≈4-fold) was obtained at 2μg ninhibin and at 10 min of treatment at 37°C. On the other hand, in untreated control membranes, ninhibin at 2 μg could stimulate adenylate cyclase by about 50-60% only. In addition, ninhibin potentiated the guanine nucleotide-, isoproterenol- and forskolin (FSK)-stimulated adenylate cyclase activities and also attenuated the GTPγs and atrial natriuretic factor (ANF)-mediated inhibition of enzyme activities. Furthermore the inhibition of isoproterenol- and FSK-stimulated adenylate cyclase activities by ANF was also abolished by ninhibin. These data indicate that ninhibin which has been suggested to inactivate or inhibit Ni-guanine nucleotide regulatory protein can also attenuate the ANF-receptor mediated inhibition of adenylate cyclase in rat aorta suggesting an involvement of Ni-guanine nucleotide regulatory protein in the coupling of ANF receptors to adenylate cyclase.

INTRODUCTION

The adenylate cyclase system is known to be composed of three components: receptor, catalytic subunit and guanine nucleotide binding protein that acts as a transducer and in the presence of guanine nucleotides, transmits the signal from the hormone occupied receptor to the catalytic sub-

1. This work was supported by grants from Quebec Heart Foundation and Medical Research Council of Canada to M.B.A-S., NIH grant AM 18185 to R.A.J. and from a group grant from the Medical Research Council of Canada to the Multidisciplinary Research Group on hypertension.

2. Canadian Heart Foundation Scholar. To whom all correspondence should be addressed.

* Department of Physiology, School of Medicine, Vanderbilt University, Nashville TN 37232

ABBREVIATIONS

ANF: Atrial natriuretic factor, GMP-P(NH)P:Guanyl-5'-yl(β-γ-imino)diphosphate, GTPγs:Guanosine 5'-O(thiotriphosphate).

0006-291X/85 $1.50

unit. The stimulation and inhibition of adenylate cyclase by hormones are mediated through stimulatory (Ns) and inhibitory (Ni) guanine nucleotide regulatory components of adenylate cyclase system respectively (1) Islet activating protein (IAP), a Bordetella Pertussis toxin, attenuates the GTP-dependent and receptor mediated inhibition of adenylate cyclase (2). The attenuation appears to result from IAP-catalyzed ADP-ribosylation of 41,000 Da membrane protein (3-4). Very recently, a sperm factor, also known as ninhibin isolated from bovine sperm (5) has been demonstrated to activate basal and hormone-stimulated adenylate cyclase activities in platelet and brain membranes (6-7). In addition, ninhibin has also been shown to attenuate α-adrenergic inhibition of platelet adenylate cyclase (7) by blocking Ni regulatory protein of adenylate cyclase.

We have recently reported that atrial natriuretic factor (ANF Arg-101 Tyr-126) inhibits adenylate cyclase activity in various target tissues (8-11), suggesting that ANF receptors are negatively coupled to adenylate cyclase system. In order to determine if the coupling of ANF receptors to adenylate cyclase is through Ni-guanine nucleotide regulatory protein, we used ninhibin as a tool to investigate if it could also attenuate the inhibitory effect of ANF on adenylate cyclase.

We report in the present studies that ninhibin activates basal, guanine nucleotide- and isoproterenol-stimulated enzyme activities and attenuates the GTP and ANF-mediated inhibition of adenylate cyclase in rat aorta. These data suggeest that ANF receptors are coupled to adenylate cyclase through Ni guanine regulatory protein.

MATERIAL AND METHODS

Materials: ATP, cAMP and isoproterenol were purchased from Sigma (St. Louis, MO). Creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3), and GTPγs were purchased from Boehringer Mannheim, Canada. [α-^{32}P]ATP was purchased from Amersham. Forskolin was obtained from Calbiochem-Behring Corp. (San Diego, CA). Synthetic rat atrial natriuretic factor (Arg 101-Tyr 126) was a gift from Dr. R. Nutt of Merck, Sharp & Dohme Research Laboratories. Ninhibin was extracted from Bovine sperm as described previously (5).

Preparation of aorta washed particles: Aorta washed particles were prepared as described previously (8, 12). Aorta were dissected out and quickly frozen in liquid nitrogen. The frozen aorta were pulverized to a fine powder using a

percussion mortar cooled in liquid nitrogen. The powdered aorta were stored at -70°C until assayed. Aorta were homogenized using a motor-driven teflon glass homogenizer in a buffer containing 10 mM Tris-HCl and 1 mM EDTA pH 7.5. The homogenate was centrifuged at 16,000 x g for 10 min. The supernatant was discarded and the pellet was finally suspended in 10 mM Tris, 1 mM EDTA, pH 7.5 and used for adenylate cyclase activity determination.

Adenylate cyclase activity determination: Adenylate cyclase activity was determined by measuring [^{32}P] cAMP formation from [α-^{32}P]ATP as described previously (12, 13). Typical assay medium contained 50 mM glycyl-glycine, pH 7.5, 0.5 mM MgATP [^{32}P]ATP (1-1.5 x 10^6 CPM), 5mM $MgCl_2$ (in excess of the ATP concentration), 0.5 mM cAMP, 1 mM 3-isobutyl-1-methylxanthine, 1 mM EGTA, 100 mM NaCl, 10 µM GTPγS and ATP regenerating system consisting of 2 mM creatine phosphate, 0.1 mg creatine kinase per ml and 0.1 mg myokinase per ml in a final volume of 200 µl. Incubations were initiated by the addition of reaction mixture to the membranes (30-50 µg) which had been preincubated with ninhibin for 5 min at 37°C. In some experiments the incubations were initiated by the addition of the particulate fraction to the reaction mixture which had been thermally equilibrated for 2 min at 37°C. Reactions were conducted in triplicates for 10 min. at 37°C. Reactions were terminated by the addition of 0.5 ml of 144 mM Na_2CO_3 and subsequent chromatography by the double-column system as described by Salomon et al. (14). The unlabeled cAMP served to monitor recovery of the [^{32}P]-cAMP by measuring absorbance at 259 nm. Under the assay conditions used, the adenylate cyclase activity was linear with respect to protein concentration and time of incubation.

Protein was determined essentially as described by Lowry et al. (15) with crystalline bovine serum albumin as standard.

RESULTS

Effect of ninhibin on adenylate cyclase activity.

Fig. 1 shows the effect of various concentrations of ninhibin pretreatment on adenylate cyclase activity. Ninhibin stimulated adenylate cyclase activity in a concentration dependent manner, the maximal stimulation (≈3-fold) was observed at 2 µg of ninhibin with an apparent ka of 0.5-0.6 µg. However, in untreated control membranes ninhibin at 2 µg could stimulate adenylate cyclase by about 50-60% only (data not shown). The activation of adenylate cyclase by ninhibin was dependent on the time of pretreatment (Fig. 2). The maximal activation (≈4-fold) was observed at 10 min. of pretreatment at 37°C and thereafter, the extent of stimulation was decreased. However, the absolute enzyme activities (basal and ninhibin-sensitive) were decreased in a time dependent manner, basal was decreased to a greater extent as compared to ninhibin-sensitive enzyme activity (data not shown).

Effect of ninhibin on guanine-nucleotide-sensitive adenylate cyclase.

Guanine nucleotides play an important role in the regulation of adenylate cyclase activity (1). In order to determine if ninhibin could mbdulate the

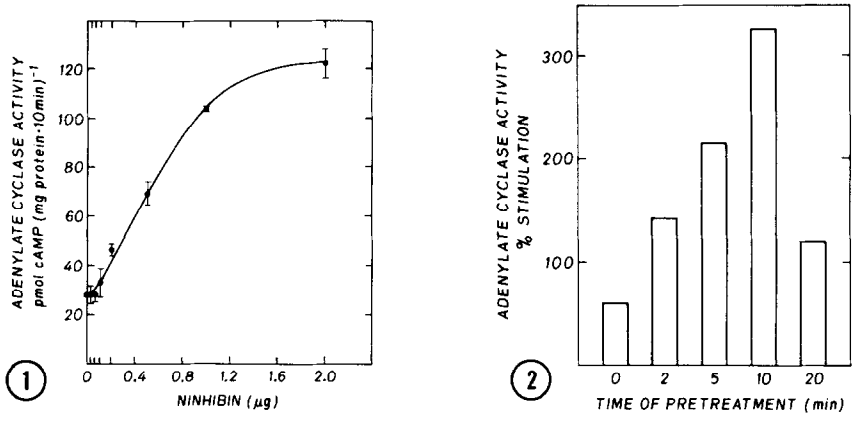

Figure 1. Stimulation of adenylate cyclase activity by ninhibin in rat aorta. Aorta particulate fraction was preincubated with various concentrations of ninhibin at 37°C for 5 min. The determination of enzyme activity was initiated by the addition of cyclase reaction mixture. The reaction was terminated after 10 min. as given under "Methods". The values are means ± S.E.M. of triplicate determinations from one of three separate experiments.

Figure 2. Time course of ninhibin pretreatment on adenylate cyclase activity in rat aorta. Aorta particulate fraction was pretreated with ninhibin at a concentration of 2 µg for various time intervals at 37°C. The determination of enzyme activity was initiated by the addition of cyclase reaction mixture. The reaction was terminated after 10 min as given under "Methods". Adenylate cyclase activity is expressed as percent stimulation. Values are means of triplicate determinations from one of two experiments.

sensitivity of guanine nucleotides to stimulate adenylate cyclase, the effect of ninhibin was studied on GMP-P(NH)P and GTPγs-stimulated enzyme activities and the results are shown in Table 1. GMP-P(NH)P and GTPγs stimulated adenylate cyclase to various degrees, GTPγs was more potent and stimulated enzyme activity by about 11-fold where as about 38% stimulation was observed with GMP-P(NH)P. Ninhibin potentiated the stimulatory effects of these guanine nucleotides on adenylate cyclase.

The effect of ninhibin on the entire dose response curve of GTPγs was also studied and the results are shown in Fig. 3, GTPγs showed biphasic effect on adenylate cyclase, stimulation at lower concentrations and inhibition at higher concentrations. Ninhibin potentiated the stimulatory effect of GTPγs and blocked the inhibitory phase of GTPγs action.

Since ninhibin was able to stimulate basal and guanine nucleotide-sensitive adenylate cyclase activities, it was interesting to know, whether

Table I

Effect of ninhibin on guanine nucleotide-stimulated adenylate cyclase activity from rat aorta particulate fraction

Additions	Adenylate cyclase activity pmol cAMP (mg protein . 10 min)$^{-1}$	
	Basal	Ninhibin (2 µg)
None	37 ± 4	111 ± 5
GMP-P(NH)P (10 µM)	51 ± 1	130 ± 3
GTPγs (10 µM)	421 ± 20	669 ± 11

The aorta particulate fraction was preincubated with nihibin for 5 min at 37°C. The determination of enzyme activity was initiated by the addition of cyclase reaction mixture. The reaction was terminated after 10 min as given under "Methods". The values represent the mean ± S.E.M. of triplicate determinations from one of three separate experiments.

ninhibin can also potentiate the receptor mediated and receptor non-mediated stimulation of adenylate cyclase. Table II shows that isoproterenol and forskolin, stimulated enzyme activity to various degrees and when the effect of these agents was studied in the ninhibin-treated membranes, the effect was potentiated. The similar potentiating effect of ninhibin on PGE_1-stimulated adenylate cyclase activity in platelet membranes has also been reported previously (7).

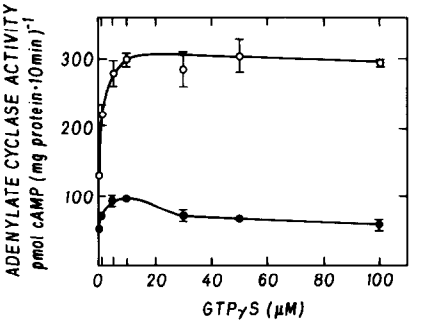

Figure 3. Effect of Ninhibin on GTPγs-sensitive adenylate cyclase activity in rat aorta. Aorta particulate fraction was preincubated with various concentrations of GTPγs alone (●-●) or in combination with 2µg ninhibin (o-o) at 37°C for 5 min. The determination of adenylate cyclase activity was initiated by the addition of cyclase reaction mixture. The reaction was terminated after 10 min as given under "Methods". The values are means ± S.E.M. of triplicate determinations from one of two experiments.

Table II

Effect of ninhibin on isoproterenol and FSK-stimulated adenylate cyclase activity from aorta particulate fraction

Additions	Adenylate cyclase activity pmol cAMP (mg protein . 10 min)$^{-1}$	
	Basal	Ninhibin
None	28 ± 2	122 ± 6
Isoproterenol (50 μM)	41 ± 2	230 ± 7
Forskolin (50 μM)	243 ± 4	866 ± 10

The aorta particulate fraction was preincubated with isoproterenol or forskolin in the absence or presence of ninhibin (2μg) for 5 minutes at 37°C. The determination of enzyme activity was initiated by the addition of cyclase reaction mixture. The reaction was terminated after 10 min as given under "Methods". The values are means ± S.E.M. of triplicate determinations from one of three separate experiments.

Since ninhibin stimulates adenylate cyclase and also attenuates the guanine nucleotide mediated inhibition, it is possible that these effects may be mediated through the inhibition or inactivation of Ni-regulatory protein (7). If this is the case, then the receptor mediated inhibition should also be attenuated by ninhibin. Fig. 4 shows the effect of ninhibin on ANF-

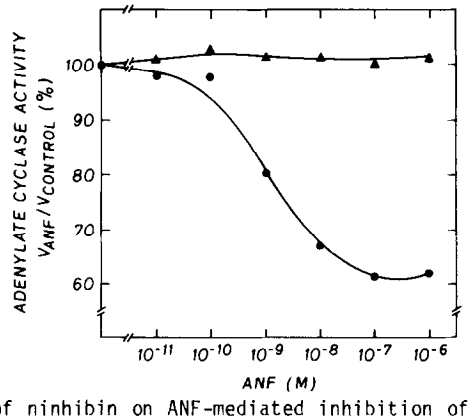

Figure 4. Effect of ninhibin on ANF-mediated inhibition of adenylate cyclase in rat aorta particulate fraction. Adenylate cyclase activity was determined in the presence of various concentrations of ANF alone (●-●) or in combination with 2 μg ninhibin (▲-▲). The preincubation of aorta particulate fraction for 5 min at 37°C with ninhibin was omitted in this experiment. Indicated on the ordinate is the adenylate cyclase activity as % of control activity measured in the absence of ANF. Control activities were 208 and 243 pmol cAMP formed per mg protein per 10 min in the absence and presence of 2 μg ninhibin respectively. The values are means of triplicate determinations from one of two experiments.

mediated inhibition of adenylate cyclase activity. ANF inhibited adenylate cyclase in a concentration dependent manner, and ninhibin (2 μg) attenuated the inhibitory effect of ANF.

In order to see, whether ninhibin can also attenuate the ANF-mediated inhibition of isoproterenol and forskolin-responsive activities, the effect of ninhibin was studied on isoproterenol- and forskolin-stimulated adenylate cyclase activities in the presence or absence of ANF and results are shown in Table III. ANF inhibited basal, isoproterenol- and forskolin-stimulated activities but when the activities were monitored in the presence of ninhibin (2 μg), the inhibition was completely blocked. The smaller stimulation of cyclase by ninhibin in this experiment was due to the fact that membranes were not pretreated with ninhibin but ninhibin was added in the assay tubes.

DISCUSSION The results reported here demonstrate that ninhibin treatment of the rat aorta membranes activated adenylate cyclase in a concentration and time dependent manner. Ninhibin treatment also potentiated the guanine nucleotide-, isoproterenol- and forskolin- stimulated enzyme activities.

Table III

Effect of ninhibin on ANF mediated attenuation of basal, isoproterenol- and forskolin-stimulated adenylate cyclase activities in rat aorta particulate fraction

Additions	Adenylate cyclase activity pmol cAMP (mg protein . 10 min)$^{-1}$	
	Basal	ANF (10^{-7}M)
None	372 ± 7	270 ± 24
Isoproterenol (50 μM)	462 ± 20	377 ± 31
Forskolin (50 μM)	1217 ± 70	1165 ± 47
Ninhibin (2 μg)	446 ± 4	458 ± 60
Isoproterenol + Ninhibin	592 ± 3	608 ± 36
Forskolin + Ninhibin	1615 ± 20	1615 ± 10

The determination of adenylate cyclase activity was initiated by the addition of aorta particulate fraction to the reaction mixture as given under "Methods". The pretreatment of aorta particulate fraction with ninhibin was omitted in this experiment but ninhibin was added in the assay tubes where indicated. The values represent the mean ± S.E.M. of triplicate determinations from one of three separate experiments.

These results are consistent with the previous studies (7) where ninhibin has been shown to activate basal and PGE_1-stimulated adenylate cyclase activities in platelet membranes. The observed potentiation of adenylate cyclase activities by ninhibin may be due to the inactivation or inhibition of Ni-guanine nucleotide regulatory protein (7). This notion is further supported by the fact that ninhibin treatment was also able to attenuate the inhibitory phase of $GTP\gamma S$ action on adenylate cyclase which appears to be medited via Ni-protein. These data indicate that aorta contains a functional Ni-component of adenylate cyclase system. In addition, ninhibin attenuated the ANF receptor-mediated inhibition of adenylate cyclase and also blocked the inhibitory effects of ANF on isoproterenol- and forskolin-stimulated adenylate cyclase activities. These data further suggest the involvment of Ni-guanine nucleotide regualtory protein in the coupling of ANF receptors to adenylate cyclase. Although the mechanism(s) by which ninhibin modifies the functions of Ni-protein is not known yet, it can also be used as a useful tool like pertusis toxin to investigate the involvement of Ni-protein in the receptor-cyclase interactions.

ACKNOWLEDGEMENT We wish to thank Lyne Guindon for secretarial help.

REFERENCES

1. Gilman, A.G. (1984) J. Clin. Invest. 73, 1-4
2. Hazeki, O. and Ui, M. (1981) J. Biol. Chem. 256, 2856-2862
3. Katada, T. and Ui, M. (1982) Proc. Natl. Acad. Sci. USA 79, 3129-3133
4. Katada, T. and Ui, M. (1982) J. Biol. Chem. 257, 7210-7216.
5. Johnson, R.A., Jakobs, K.H. and Schultz, G. (1985) J. Biol. Chem. 114-121.
6. Johnson, R.A., Awad, J.A., Jakobs, K.H. and Schultz, G. (1983) FEBS Lett 152, 11-16.
7. Jakobs, K.H., Johnson, JR.A. and Schultz, G. (1983) Biochem. Biophys. Acta. 756, 369-375.
8. Anand-Srivastava, M.B., Franks, D.J., Cantin, M. and Genest, J. (1984) Biochem. Biophys. Res. Commun. 121, 855-862.
9. Anand-Srivastava, M.B., Cantin, M. and Genest, J. (1985) Life Sciences (In Press).
10. Anand-Srivastava, M.B., Genest, J. and Cantin, M. (1985) FEBS Letter 181, 199-202.
11. Anand-Srivastava, M.B., Vinay, P., Genest, J. and Cantin, M. (1985) Am. J. Physiol. (Submitted).
12. Anand-Srivastava, M.B. (1983) Biochem. Biophys. Res. Commun. 117, 420-428
13. Anand-Srivastava, M.B. and Cantin, M. (1983) Arch. Biochem. Biophys. 223, 468-476.
14. Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541-548.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.