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ATRIAL NATRIURETIC FACTOR RECEPTORS ARE NEGATIVELY COUPLED TO ADENYLATE CYCLASE IN CULTURED ATRIAL AND VENTRICULAR CARDIOCYTES[1]

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SUMMARY: We have studied the effect of synthetic rat atrial natriuretic factor (ANF) on adenylate cyclase activity in cultured cardiocytes from atria (left and right) and ventricles from neonatal rats. ANF (Arg 101-Tyr 126) inhibited adenylate cyclase activity in a concentration dependent manner in cultured atrial (right and left atria) and ventricular cells. However the inhibition was greater in atrial cells as compared to ventricular cells. The maximal inhibition observed in ventricular cells was about 35% with an apparent Ki of about 10^{-10} M, whereas about 55% inhibition with an apparent Ki between 5 x 10^{-10} M and 65% inhibition with an apparent Ki of 10^{-9} M were observed in right and left atrial cardiocytes respectively. The inhibitory effect of ANF was dependent on the presence of guanine nucleotides. Various hormones and agents such as isoproterenol, prostaglandins, adenosine, forskolin and sodium fluoride stimulated adenylate cyclase activities to various degrees in these atrial and ventricular cardiocytes. ANF inhibited the stimulatory responses of all these agonists, however the degree of inhibition varied for each agent. In addition ANF also inhibited cAMP levels in these cells. These data indicate that ANF receptors are present in cardiocytes and are negatively coupled to adenylate cyclase.

INTRODUCTION Mammalian atria contain peptides which exhibit natriuretic, diuretic and vasorelaxant activities (1-3). These peptides are commonly known as ANF and have been purified and their amino acid sequence have been determined by several groups of investigators (4-6). ANF has also been shown to inhibit renin release from kidney slices (7), progesterone secretion in

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ABBREVIATIONS USED

ANF: Atrial natriuretic factor, GMP-P9NH)P: Guanyl-5'-yl(β-γ-imino)diphosphate, GTPγs: Guanosine 5'-O(thiotriphosphate, NECA: N-Ethylcarboxamide adenosine.

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murine leydig tumor cells (8), aldosterone secretion from zona glomerulosa cells and vasopressin release from posterior pituitary (9-10). The inhibition of these physiological responses by ANF appears to be mediated through the inhibition of adenylate cyclase/cAMP. We have recently shown the inhibition of adenylate cyclase activity by ANF in various target tissues such as vascular smooth muscle (11), anterior and posterior pituitary (12) and adrenal cortical membranes, and different nephron segments except proximal tubules (13,14). Since ANF is a circulating hormone and secreted from atria, we were interested to examine if ANF could also inhibit adenylate cyclase/cAMP system in atria, which may act as a signal/second messenger in the regulation of cardiac function and for release of this peptide from atria.

MATERIALS AND METHODS

Materials: Adenosine deaminase (EC 3.5.4.5), GTP, ATP, cyclic AMP were purchased from Sigma, St Louis, Missouri. Creatine kinase (EC 2.7.3.2) and myokinase (EC 2.7.4.3) were purchased from Boehringer Mannheim, Canada. [α-^{32}p]ATP was purchased from Amersham. Minimum Eagle's Medium (MEM) and calf serum were from Gibco and trypsin was from Difco Laboratories, Detroit, Michigan. N-Ethylcarboxamide adenosine (NECA) was from Research Biochemicals Inc., Wayland, MA and ANF (Arg 101-Tyr 126) was from Armand Frappier, Montreal.

Culture of cardiocytes: Cardiocytes were cultured as described previously (15). Each time a culture was started, the right atrium, left atrium, and the inferior part of both ventricles with the interventricular septum were dissected from 40 to 50 hearts of 2- to 3-day-old Spargue-Dawley rats. The tissues were pooled according to their anatomic origin in MEM, washed twice in Hanks' balanced salt solution (BSS) without calcium and magnesium, and dissociated at 37°C in 0.1% trypsin in Ca^{2+} and Mg^{2+}-free BSS and buffered with Hepes at pH 7.4. The complete dissociation procedure included seven steps, the first two steps each lasting 10 min, the third and fourth 15 min, and the fifth to seventh 20 min. After each step the minced tissues were aspirated 20 times in a 10-ml pipet to disperse the cells, placed in a 15-ml centrifugation tube, and mixed with 5 ml of fetal calf serum (FCS) to block enzyme activity. The cell suspensions were then centrifuged at 400xg for 5 min and each pellet resuspended in 2 ml of complete medium (CM) made up of MEM buffered with Hepes at pH 7.4 and supplemented with 10% FCS, 1% glutamine, and 1% penicillin-streptomycin.

To obtain cultures enriched in cardiocytes, a modification of Kasten's original technique (16) of selective plating was used (17). The final cells suspension was mixed and placed in a gelatinized 25-cm culture flask at 37°C for 30 min. This was repeated three times to allow as many nonmyogenic cells as possible to attach to the gelatinized surface. The final supernatant was centrifuged at 400xg, resuspended in CM, counted in a hemocytometer, and cultured at an initial density 2 x 10^6 to 3 x 10^6 cells in 6 ml of CM renewed after 24 h and then every second day. Cultures were incubated at 37°C with air as the gas phase, in gelatinized 25-cm flasks. The cells, as visualized by phase-contrast microscopy, as already described (17), started to form confluent monolayers of synchronously beating cells between the third and fourth day of culture. Between the first and third day, large islands of

cells were found beating synchronously. The ventricular cardiocytes started beating after 18 to 20 h of culturing whereas the atrial cardiocytes did so only after 24 h. While the ventricular cardiocytes were characterized by a forceful contraction that displaced cells and organelles, the atrial cardiocyte contractions were more like a ripple on their surface.

Preparation of crude homogenates: The cells (after 5 to 9 days in culture) were rinsed twice with a buffer containing 10 mM Tris, 1 mM EDTA, and 1 mM DTT, pH 7.5, to remove culture medium. The cells were then removed from the flasks with a rubber policeman. The cells were suspended and homogenized in a Dounce homogenizer in a buffer containing 10 mM Tris, 1 mM EDTA, and 1 mM DTT, pH 7.5. This preparation was used for the adenylate cyclase assay.

Adenylate cyclase activity determination: Adenylate cyclase activity was determined by measuring [^{32}p]cAMP formation from [α-^{32}p]ATP as described previously (13). Typical assay medium contained 50 mM glycylglycine, pH 7.4, 0.5 mM MgATP, [α-^{32}p]ATP (1-1.5 x 10^6 cpm), 5 mM $MgCl_2$ (in excess of the ATP concentration), 0.5 mM cAMP, 5 U adenosine deaminase/ml, 10 µM GTP, and ATP-regenerating system consisting of 2 mM creatine phosphate, 0.1 mg creatine kinase/ml, and 0.1 mg myokinase/ml in a final volume of 200 µl. Incubations were initiated by the addition of the crude homogenate (25-50 µg of protein) to the reaction mixture which had been thermally equilibrated for 2 min at 37°C. Reactions were conducted in triplicate for 10 min at 37°C. Reactions were terminated by the addition of 0.6 ml of 120 mM zinc acetate. cAMP was purified by coprecipitation of other nucleotides with $ZnCO_3$ by the addition of 144 mM Na_2CO_3 and subsequent chromatography by the double-column system as described by Salomon et al. (18).

Cyclic AMP levels were determined by using RIA kit from New England Nuclear. Protein was determined as described by Lowry et al (19), with crystalline bovine serum albumin as standard.

RESULTS

As reported earlier (15,17) virtually all cells in culture were cardiocytes as evidenced by the presence of myofilaments with Z bands, and in the case of atrial cardiocytes, by the presence of typical secretory granules.

Effect of ANF on adenylate cyclase activity: In order to demonstrate the presence of ANF receptors coupled to adenylate cyclase in cardiocytes, the effect of ANF on adenylate cyclase activity was studied in ventricular, left and right atrial cardiocytes and the results are shown in Fig. 1. ANF inhibited adenylate cyclase activity in a concentration dependent manner in cultured ventricular, right and left atrial cardiocytes. However, the inhibition was greater in atrial cells as compared to ventricular cells. The maximal inhibition observed in ventricular cells was about 35% with an apparent Ki of about 10^{-10} M, whereas about 55% inhibition with an apparent Ki between 5 x 10^{-10} - 10^{-10} M and 65% inhibition with an apparent Ki of 10^{-9} M were observed in right and left atrial cardiocytes respectively. These data

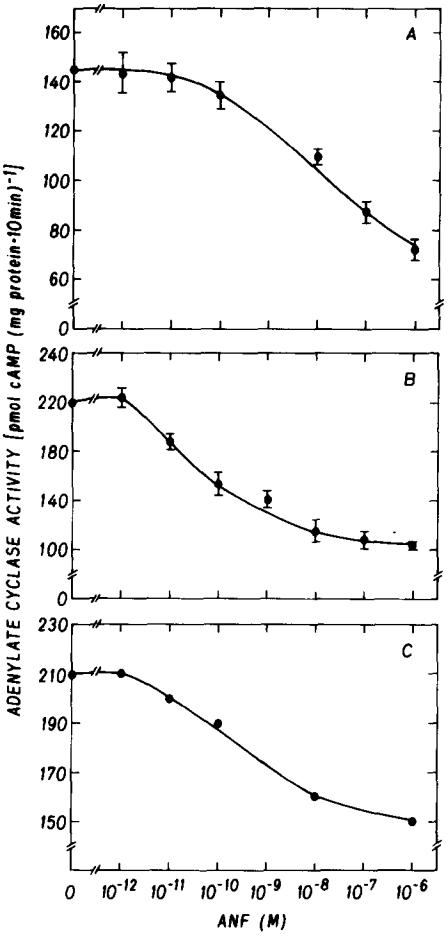

Figure 1. Effect of various concentrations of synthetic ANF on adenylate cyclase activity in cultured left atrial (A), right atrial (B) and ventricular cardiocytes (C). Adenylate cyclase activity was determined as under "Methods". Values are means ± S.E.M. of triplicate determinations from one of three experiments.

suggest that both these cardiocytes, atrial and ventricular, contain ANF receptors which are coupled to adenylate cyclase in a negative manner.

In order to investigate if ANF could also decrease cAMP levels in these cells, the effect of ANF on cAMP levels was determined and the results are shown in Table I. ANF at 10^{-8} M decreased cAMP levels in left atrial, right atrial and ventricular cardiocytes by about 55, 60 and 45% respectively. This decrease in intracellular cAMP levels by ANF may be very well correlated with the inhibition of adenylate cyclase activity.

Table I

Effect of ANF on cAMP levels in atrial and ventricular cardiocytes

	cAMP levels pmol/mg protein		
	Basal Control	ANF (10^{-8} M)	% decrease
Left atrial cardiocytes	179	82	54
Right atrial cardiocytes	241	91	62
Ventricular cardiocytes	190	107	44

cAMP levels were determined in atrial and ventricular cardiocytes as described in "Methods". The cells were incubated at 37°C with and without ANF (10^{-8} M) for 3 min. After the incubation, the cells were washed three times with the buffer containing 10 mM Tris, 4 mM EDTA, pH 7.4, homogenized and boiled for 5 min. The homogenate was centrifuged at 1000xg for 10 min. The supernatant was collected and cAMP levels were determined by using RIA kit from New England Nuclear.

Guanine nucleotide requirement for ANF-mediated inhibition of adenylate cyclase

We have recently shown the dependence of ANF-mediated inhibition of adenylate cyclase on guanine nucleotides in glomeruli from dog kidney (14). In order to examine if guanine nucleotides are also required for inhibitory effect of ANF on adenylate cyclase in cultured cardiocytes, the effect of various guanine nucleotides on ANF-mediated inhibition was studied and the results are shown in Table II. GTP, GMP-P(NH)P and GTPγS all stimulated adenylate cyclase activity to various degrees. ANF in the absence of guanine nucleotides did not show any inhibitory effect on adenylate cyclase activity, however in the presence of guanine nucleotides, ANF inhibited adenylate cyclase activity, the maximal inhibition ($\sim$25-30%) was observed at 10 μM of all these guanine nucleotides. These data suggest that ANF receptors like other hormone receptors are coupled to adenylate cyclase by guanine nucleotide regulatory protein in cardiocytes also. The involvement of inhibitory guanine nucleotide regulatory protein (Ni) has been shown previously (20).

Effect of ANF on hormone-responsive adenylate cyclase activity

Since ANF inhibited adenylate cyclase activity in cultured atrial and ventricular cardiocytes, it was of interest to investigate, if ANF was also

Table II

Dependence on guanine nucleotides of inhibition of adenylate cyclase by ANF

Addition (μM)	Adenylate cyclase activity pmol cAMP (mg protein 10 min)$^{-1}$ Control	ANF (10^{-8} M)	% inhibition
None	282 ± 15	299 ± 15	-
GTP			
1	347 ± 11	318 ± 5	9
10	372 ± 14	301 ± 9	20
100	293 ± 24	232 ± 4	21
GMP-P(NH)P			
1	347 ± 40	285 ± 22	18
10	381 ± 46	260 ± 17	32
100	425 ± 18	295 ± 23	31
GTPγs			
1	2453 ± 29	1880 ± 76	23
10	2525 ± 112	1964 ± 173	23
100	2391 ± 29	1822 ± 29	24

Adenylate cyclase activity was determined in the absence and presence of ANF and various concentrations of GTP, GMP-P(NH)P and GTPγs as given under "Methods". The values represent the mean ± S.E.M. of triplicate determinations from one of two experiments.

able to inhibit hormone-responsive adenylate cyclase activities. Fig. 2 shows the effect of ANF on adenylate cyclase activities stimulated by various hormones and forskolin in cultured left atrial, right atrial and ventricular cardiocytes. Isoproterenol, dopamine, N-Ethylcarboxamide adenosine (NECA), and forskolin, stimulated adenylate cyclase activities to various degrees in cultured left atrial and right atrial cells (Fig. 2a, b) and ANF inhibited the stimulatory responses of all these hormones and forskolin, however, the stimulations were never completely abolished. In the case of ventricular cardiocytes (Fig. 2c), isoproterenol, dopamine, NECA, prostaglandins (PGE_1) and forskolin also stimulated adenylate cyclase activities and ANF inhibited the stimulatory responses of all these agonists, however the extent of inhibition was different for each agonist. For example, dopamine- and PGE_1-stimulated enzyme activities were completely abolished by ANF, whereas isoproterenol-, NECA- and forskolin-sensitive enzyme activities were inhibited slightly (10-20%). The effect of ANF was also studied on the entire dose response curves of NECA in left and right atrial and ventricular cardiocytes

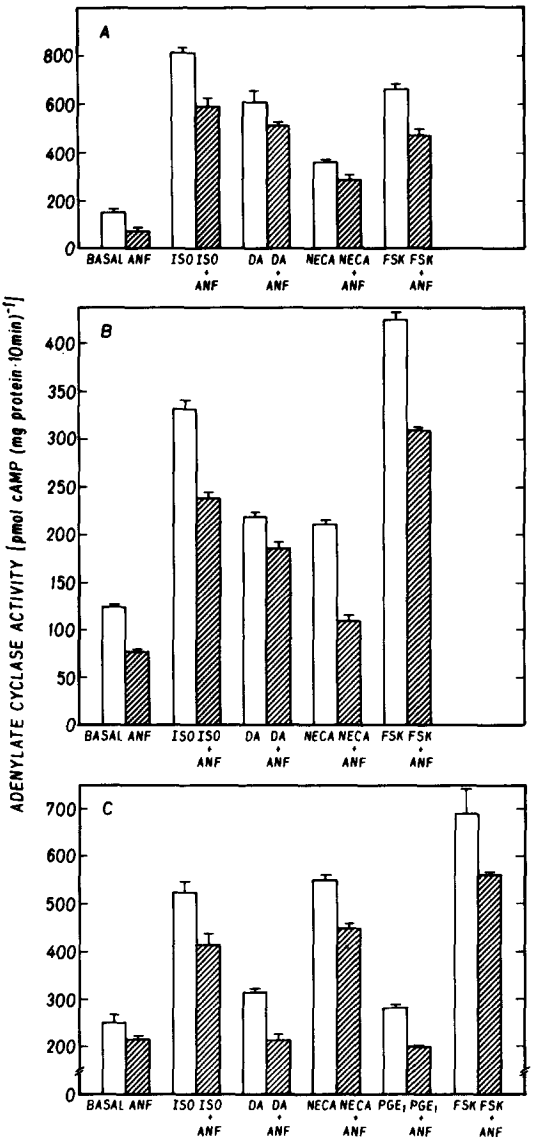

Figure 2. Effect of ANF on basal or stimulated adenylate cyclase activity by various agonists in cultured left atrial (A), right atrial (B) and ventricular cardiocytes. Adenylate cyclase activity was determined in the absence or the presence of 50 μM isoporterenol (ISO), 100 μM dopamine (DA), 10 μM N-Ethylcarboxamide adenosine (NECA), 1 μM prostaglandins (PGE_1) and 50 μM forskolin (FSK) alone or in combination with 10^{-8} M atrial natriuretic factor (ANF). Values are means ± S.E.M. of triplicate determinations from one of four experiments.

and the results are shown in Fig. 3. ANF inhibited the stimulatory effect of NECA on adenylate cyclase activity at all the concentrations used in the present studies and this inhibition appears to be associated with a decrease

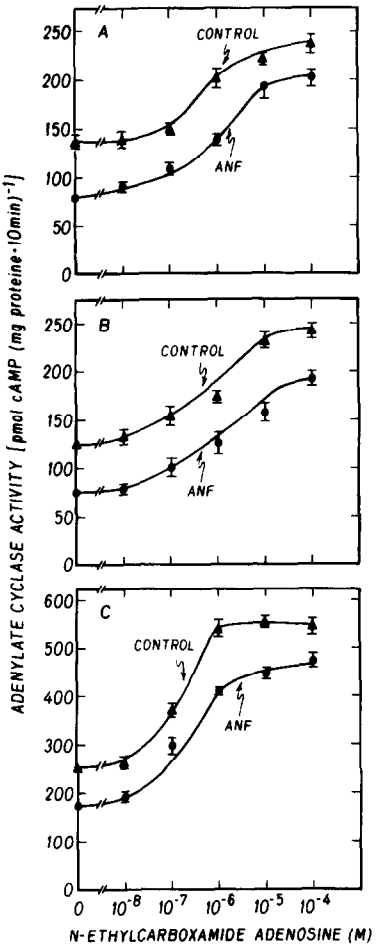

Figure 3. Effect of ANF on NECA-stimulated adenylate cyclase activity in cultured left atrial (A), right atrial (B) and ventricular cardiocytes (C). Adenylate cyclase activity was determined in the absence or presence of NECA alone (▲-▲) or in combination with 10^{-8} M. ANF (●-●) as under "Methods". Values are means ± S.E.M. of triplicate determinations from one of two experiments.

in Vmax and not an increase in Ka. Similar results have also been reported in other target tissues recently (11-14).

DISCUSSION The present studies demonstrate that ANF inhibited adenylate cyclase activity and cAMP levels in cultured atrial and ventricular cardiocytes from neonatal rats. These data suggest that ANF receptors are present in atrial and ventricular cardiocytes and are negatively coupled to adenylate cyclase. The similar inhibition of adenylate cyclase by ANF has

also been reported previously in other tissues (11-14). The requirement of guanine nucleotides to elicit the inhibitory effect of ANF on adenylate cyclase, suggests that ANF receptors, like other stimulatory or inhibitory receptors are also coupled to adenylate cyclase through the guanine nucleotide regulatory protein. The dependence of ANF inhibition of adenylate cyclase on guanine nucleotide has recently been shown in glomeruli from dog kidney (14). Recently, we have also shown the involvement of guanine nucleotide regulatory protein (Ni) in the coupling of ANF receptors to adenylate cyclase in rat aorta (20). The apparent Ki observed in the present studies is comparable with the values observed in other tissues. In addition, ANF also decreased cAMP levels in these cells, which may be due to the inhibition of adenylate cyclase by this peptide. The similar decrease in cAMP levels by ANF has been reported in other tissues (7,10). The degree of inhibition of stimulatory responses of hormones by ANF was different for different hormones, for example, ANF in ventricular cardiocytes completley abolished the stimulation exerted by dopamine and PGE_1, whereas isoproterenol, NECA and forskolin-sensitive enzyme activities were inhibited to a small extent, which suggest that ANF interacts differentially with different hormones. The differential interaction of ANF with PGE_1 and PTH in glomeruli from kidney has recently been shown (14).

Atrial extracts have also been reported to depress cardiac performance in whole animal (21). Since adenylate cyclase/cAMP system is believed to be one of the biochemical mechanisms participating in the regulation of cardiovascular functions (22) it may be possible that the inhibition of adenylate cyclase/cAMP system by ANF is responsible for the observed cardio inhibitory action of ANF. Nevertheless, it is concluded from these studies that ANF receptors are present in atrial and ventricular cardiocytes and are negatively coupled to adenylate cyclase. The inhibition of adenylate cyclase and cAMP levels by ANF in the cardiocytes may play an important physiological role in regulating cardiac function, and/or may play a feed back regulator in the release of ANF from atria.

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REFERENCES

1. De Bold, A.J., Borenstein, H.B., Veress, A.T. and Sonnenberg, H. (1981) Life Sci. 28, 89-94.
2. Misono, K.S., fukumi, H., Grammer, R.T. and Inagami, T. (1984) Biochem. Biophys. Res. Commun. 119, 524-529.
3. Garcia, M.G., Thibault, G., Cantin, M. and Genest, J. (1984) Am. J. Physiol. 247, R34-R39.
4. Seidah, N.G., Lazure, C., Chrétien, M., Thibault, G., Garcia, R., Cantin, M., Genest, J., Nutt, R.F., Brady, S.F., Lyle, T.A., Paleveda, W.J., Colton, C.D., Ciccarone, T.M. and Veber, D.F. (1984) Proc. Natl. Acad. Sci. USA 81, 2640-2644.
5. Flynn, T.C., De Bold, M.L. and De Bold, A.J. (1983) Biochem. Biophys. Res. Commun. 117, 859-865.
6. Currie, M.G., Geller, D.M., Cole, B.R., Siegel, N.R., Fok, K.F., Adams, S.P., Eubanks, S.R., Gallupi, G.R. and Needleman, P. (1984) Science 223, 67-69.
7. Obana, K., Naruse, M., Naruse, K., Sakurai, H., Demura, H., Inagami, T. and Kazuo, S. (1985) Endocrinology 117, 1282-1284.
8. Pandey, KN.N, Kovacs, W.J. and Inagami, T. (1985) Biochem. Biophys. Res. Commun. 133, 800-806.
9. Matsuoka, H., Ishii, M., Sugimoto, T., Hirata, Y., Sugimoto, T., Kangawa, K. and Matsuo H. (1985) Biochem. Biophys. Res. Commun. 127, 1052-1056.
10. Obana, K., Naruse, M., Inagami, T., Brown, A.B., Naruse, K., Kurimoto, F., Sakurai, H., Demura, H. and Shizmu, K. (1985) Biochem. Biophys. Res. Commun. 132, 1088-1094.
11. Anand-Srivastava, M.B., Franks, D.J., Cantin, M. and Genest, J. (1984) Biochem. Biophys. Res. Commun. 121, 855-862.
12. Anand-Srivastava, M.B., Cantin, M. and Genest, J. (1985) Life Sci. 36, 1873-1879.
13. Anand-Srivastava, M.B., Genest, J. and Cantin, M. (1985) FEBS Letter 181, 199-202.
14. Anand-Srivastava, M.B., Vinay, P., Genest, J. and Cantin, M. (1986) Am. J. Physiol. (In press).
15. Anand-Srivastava, M.B. and Cantin, M. (1983) Arch. Biochem. Biophys. 223, 468-476.
16. Kasten, F.H. (1973) in Tissue Culture. Methods and Application (Kruse, P.F., Jr. and Patterson, M.K., Jr. Eds.) pp. 72-81, Academic Press, New York.
17. Cantin, M., Ballak, M., Beuzeron-Mangina, J., Anand-Srivastava, M.B. and Tautu, C. (1981) Science 214, 569-570.
18. Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541-548.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
20. Anand-Srivastava, M.B., Johnson, R.A., Picard, S. and Cantin, M. (1985) Biochem. Biophys. Res. Commun. 129, 171-178.
21. Ackermann, U., Irizawa, T.G., Milojevic, S. and Sonnenberg, H. (1984) Can. J. Physiol. Pharmacol. 62, 819-826.
22. Drummond, G.I. and Severson, D.L. (1979) Circulation Res. 44, 145-153.