Effects of vasoactive substances and cAMP related compounds on adrenomedullin production in cultured vascular smooth muscle cells

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Abstract To elucidate the regulation mechanism of adrenomedullin (AM) production in blood vessels, we examined the effects of 30 substances on AM production in cultured rat vascular smooth muscle cells (VSMCs). Forskolin and 8-bromo-cAMP suppressed production and gene transcription of AM. Since VSMC expresses AM receptors coupled with adenylate cyclase, AM production may be regulated by intracellular cAMP concentration. Thrombin, vasoactive intestinal polypeptide and interferon-γ also inhibited AM production, while angiotensin II, endothelin-1, bradykinin, substance P, adrenaline, phorbol ester and fetal calf serum stimulated AM production in VSMC. These results suggest that AM production is regulated by a variety of substances, indicating complex systems regulating AM production.

Key words: Adrenomedullin; cAMP; Vasoactive peptide; Blood vessel; Thrombin; Rat vascular smooth muscle cell

1. Introduction

Vascular tone is regulated by many factors coming from the blood stream, endothelial cells (ECs) and perivascular nerves [1–3]. Recent studies have verified that VSMCs are also active sources of bioactive substances, such as interleukin-1 (IL-1) and endothelin-1 (ET-1), which in turn act on VSMCs, ECs and other surrounding cells and regulate their function [4,5].

AM is a potent vasorelaxant peptide recently isolated from pheochromocytoma tissue [6,7]. We have demonstrated that AM is produced in VSMC and that AM mRNA level in VSMC is about 4 times higher than that in AM-producing tissue, such as adrenal gland [8]. Furthermore, AM specific receptors coupled with adenylate cyclase have been shown to be expressed on VSMC [9,10]. These data suggest that AM functions as an autocrine or paracrine factor regulating vascular tone. We have recently reported that AM production in VSMC is highly augmented by tumor necrosis factor (TNF), IL-1 and lipopolysaccharide (LPS) [11]. However, there should be more substances regulating AM production, since VSMC are known to be regulated by many vasoactive substances. Thus, we systematically applied 30 substances to cultured rat VSMC and measured the AM production levels.

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Abbreviations: AM, adrenomedullin; EC, endothelial cell; VSMC, vascular smooth muscle cell; ET, endothelin; ir, immunoreactive; RIA, radioimmunoassay; DMEM, Dulbecco's Modified Eagle Medium; ED_{50}, half-maximal effective dose; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; AVP, arginine vasopressin; Ang II, angiotensin II; CGRP, calcitonin gene-related peptide; INF, interferon; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; PGI2, prostaglandin I2; PAF, platelet activating factor; VIP, vasoactive intestinal polypeptide; FCS, fetal calf serum; TPA, 12-O-tetradecanoylphorbol-13-acetate; 8-Br-cAMP, 8-bromo-cAMP; 8-Br-cGMP, 8-bromo-cGMP; iNOS, inducible NO synthase.

2. Materials and methods

2.1. Materials

The following materials were used: murine interferon-α (INF-α) and INF-β (Calbiochem); rat INF-γ and fetal calf serum (FCS) (Gibco); adrenaline, thrombin, 8-bromo-cAMP (8-Br-cAMP) and 8-bromo-cGMP (8-Br-cGMP) (Sigma); noradrenaline, carbachol, serotonin, histamine, prostaglandin I2 (PGI2), adenosine, 12-O-tetradecanoyl phorbol-13-acetate (TPA) and forskolin (Wako Pure Chemicals); dopamine (Nakarai Tesque); platelet activating factor C16 (PAF C16) (Cascade Biochem). Human AM[40–52]-NH2 and its N-Tyr derivative were synthesized by a peptide synthesizer (Applied Biosystems, 431A). All other peptides were of rat origin and were purchased from Peptide Institute. TPA, PGI2 and forskolin were first dissolved in ethanol and diluted with an incubation medium (Dulbecco's Modified Eagle Medium (DMEM) containing 0.1% bovine serum albumin).

2.2. Cell culture

VSMC-1 and VSMC-2 were prepared from Sprague–Dawley rat thoracic aorta by the explant method and VSMC-3 by the enzyme dispersion method [8,11]. VSMC-2 was kindly donated from Dr. M. Furuya (Suntory Institute for Biomedical Research) [12]. VSMC-1 was used for all AM production assays except for those in Fig. 3. VSMC was maintained in DMEM containing 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2 and was used at passages 8 20.

2.3. Preparation of conditioned medium

VSMCs, grown to confluence in 6-well plates, were washed twice with DMEM and preincubated in the incubation media (serum-free DMEM containing 0.1% bovine serum albumin) for 2 h. The incubation media were then replaced with those containing stimulants (1 ml) and incubated for 16 or 24 h at 37°C. Conditioned media thus obtained were acidified with acetic acid, heated at 100°C for 10 min and lyophilized. The media incubated with catecholamines and FCS were treated with Sep-Pak C18 cartridges (Millipore, Waters Division) and adsorbed materials on the cartridges were eluted with 60% CH_3CN in 0.1% trifluoroacetic acid. The eluate was lyophilized, dissolved in a radioimmunoassay (RIA) buffer and submitted to RIA for AM.

2.4. RIA for AM

Details of the RIA system using antiserum #172CI-7 against human AM[40–52]-NH_2, which recognizes the C-terminal amide structure common to human and rat AMs, have been reported [13].

2.5. RNA blot analysis

RNA blot analysis for AM was performed as reported [14]. Total RNA (25 μg) was electrophoresed and transferred to nylon membrane. *Eco*R1-*Bgl*I fragment of rat AM cDNA (nucleotide 153-422) was used as a probe [15]. The membrane was re-hybridized to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Band intensity was estimated by a Bioimage analyser (Fuji Film, BAS 2000) and was compared after correction with GAPDH band intensity.

2.6. Statistics

Values were expressed as mean ± S.E.M. Statistical analysis of data was performed by Student's t test and $P<0.01$ was considered statistically significant.

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3. Results

The concentration of immunoreactive (ir-) AM accumulated in the incubation medium of VSMC was measured, since AM was shown to be produced and constitutively secreted from VSMC into the culture medium [8]. As shown in Table 1, 12 substances significantly influenced AM production in cultured rat VSMC. In the three INFs, only INF-γ showed a weak inhibitory effect, decreasing ir-AM concentration to 72% of the control. Forskolin and thrombin markedly suppressed AM production and AM concentration in the medium was decreased to 30 and 33% of the control. These two substances were found to be the most potent inhibitors of AM production in VSMC. 8-Br-cAMP also lowered ir-AM concentration to 68% of the control. Among 12 vasoactive peptides examined, angiotensin II (Ang II), bradykinin, substance P and ET-1 increased ir-AM concentration to 156, 116, 128 and 123% of the control, while only vasoactive intestinal polypeptide (VIP) reduced it to 74% of the control. We checked 3 catecholamines, but only adrenaline showed 42% increase of ir-AM concentration. FCS and TPA stimulated AM production, as described below. The other substances listed in Table 1 did not induce any significant effects on AM production under the conditions used in the present study.

We examined effects of Ang II, TPA and FCS on AM production in detail. As shown in Fig. 1, Ang II dose-dependently stimulated AM production with an ED_{50} value of about 0.02 μM and maximal ir-AM level was 156% of the control, indicating that Ang II was the most potent stimulant among the vasoactive peptides examined. TPA and FCS did not dose-dependently stimulate AM production. TPA increased ir-AM concentration to 127% of the control at 10 nM, but decreased it to 75% at 10 μM. 0.1-1% of FCS maximally stimulated AM production, and higher concentrations of FCS induced weaker effects.

Effects of forskolin, thrombin, VIP, INF-γ and 8-Br-cAMP were further examined, as shown in Fig. 2. Forskolin and thrombin suppressed AM production in a dose-dependent manner with ED_{50} values of 0.2–0.5 μM and 0.02–0.05 NIH U/ml and decreased ir-AM concentration in the incubation medium to 42 and 43% of the control after 16 h incubation. These substances also decreased AM mRNA level to 46 and 19% of the control. VIP decreased ir-AM concentration in a dose-dependent manner to 72% of the control with an ED_{50} value of 1 nM. INF-γ and 8-Br-cAMP were found to induce significant inhibitory effects only at 100 U/ml and 1 mM, respectively (data not shown).

We have about 10 different rat VSMC cell lines, which show

Table 1
Effects of various stimulants on AM production in cultured rat vascular smooth muscle cells

Stimulants	Concentration	ir-Am (fmol/10^5 cells/24 h)
control		10.37 ± 0.30
INF-α	100 units/ml	11.09 ± 0.42
INF-β	100 units/ml	10.67 ± 0.87
INF-γ	100 units/ml	7.51 ± 0.23*
Ang II	2 μM	16.20 ± 0.56*
AVP	2 μM	9.87 ± 0.33
ANP	1 μM	8.75 ± 0.34
BNP	1 μM	9.63 ± 0.44
CNP	1 μM	9.05 ± 0.52
bradykinin	2 μM	12.03 ± 0.39*
substance P	2 μM	13.27 ± 0.99*
ET-1	2 μM	12.79 ± 0.25*
CGRP	0.1 μM	10.98 ± 0.98
VIP	0.1 μM	7.76 ± 0.17*
oxytocin	0.1 μM	10.88 ± 0.71
neuropeptide Y	0.5 μM	9.58 ± 0.20
adrenaline	0.1 μM	14.80 ± 0.96*
noradrenaline	0.1 μM	12.26 ± 1.98
dopamine	0.1 μM	9.63 ± 0.73
carbachol	0.1 μM	9.27 ± 0.43
serotonin	10 μM	8.21 ± 0.45
PGI_2	10 μM	9.43 ± 0.37
histamine	10 μM	10.60 ± 0.43
PAF C_{16}	1 μM	9.86 ± 0.88
adenosine	1 μM	10.50 ± 0.28
thrombin#	10 units/ml	3.38 ± 0.12*
FCS	0.1 %	14.53 ± 0.88*
TPA	10 nM	13.19 ± 0.42*
forskolin	2 μM	3.15 ± 0.08*
8-Br-cAMP	1 mM	7.10 ± 0.44*
8-Br-cGMP	1 mM	9.69 ± 0.45

Each value represents mean ± S.E.M. of three or six separate wells.
*Statistically significant difference from control ($P < 0.01$).
#NIH units.

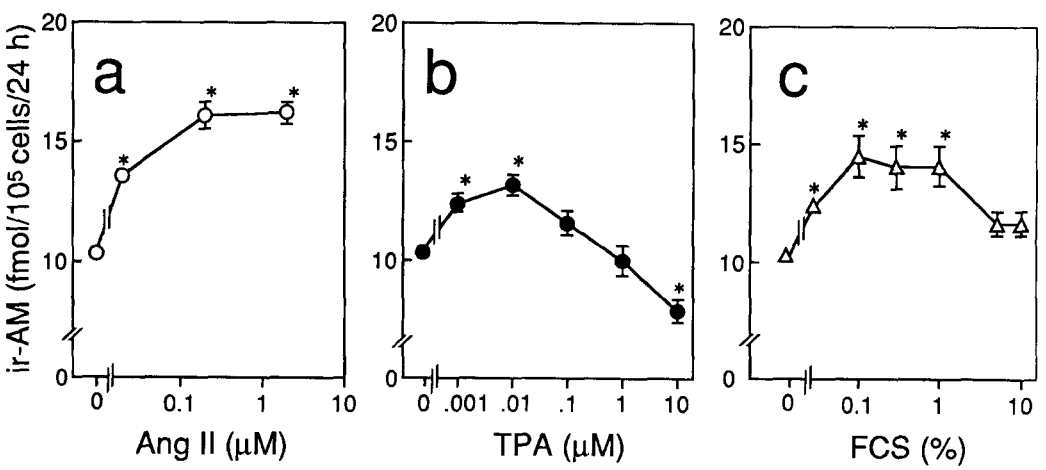

Fig. 1. Dose-dependent effect of Ang II, TPA and FCS on AM production in cultured rat VSMC (VSMC-1). Ir-AM concentration in culture medium of rat VSMC was measured after 24 h incubation with (a) Ang II, (b) TPA and (c) FCS. Each point represents mean ± S.E.M. of six separate wells. *$P < 0.01$.

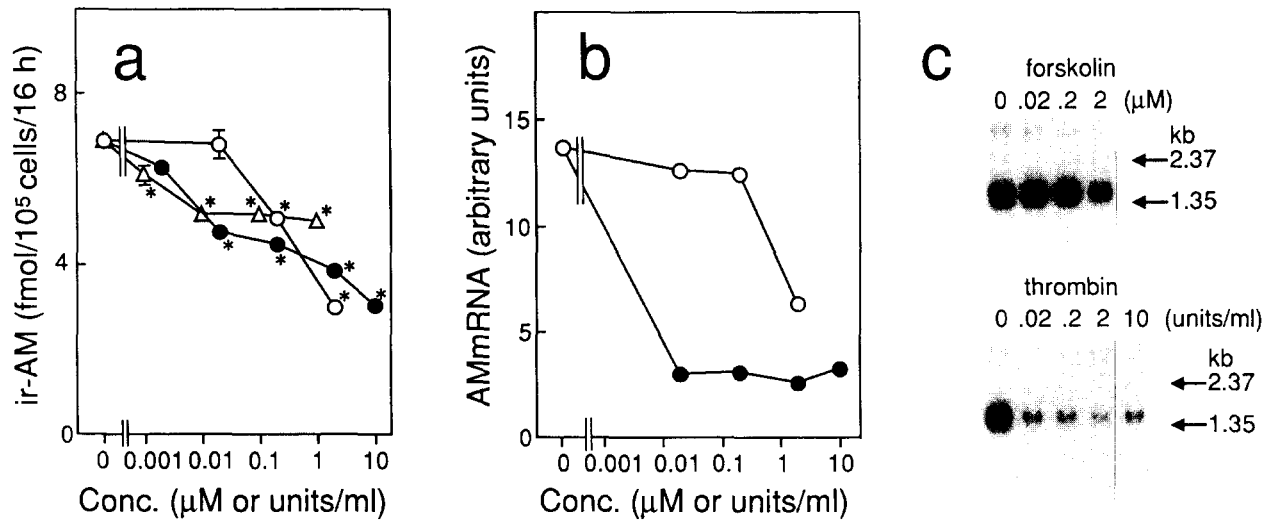

Fig. 2. Dose-dependent inhibition of AM production and gene expression in cultured rat VSMC (VSMC-1) by forskolin, thrombin and VIP stimulations. (a) Ir-AM concentration in culture medium after 16 h incubation with forskolin (○, μM), thrombin (●, NIH U/ml) and VIP (△, μM). Each point represents mean ± S.E.M. of six separate wells. *$P < 0.01$. (b) AM mRNA level in rat VSMC incubated with forskolin (○) and thrombin (●) for 16 h. (c) RNA blot analysis of AM transcripts in rat VSMC incubated with forskolin (upper panel) and thrombin (lower panel) for 16 h. Each lane contains 25 μg of total RNA.

different morphological features and biochemical properties such as cAMP generation. Three of these cell lines were characterized by stimulating with typical substances (Fig. 3). After 24 h stimulation, ir-AM concentrations in the medium of VSMC-1, VSMC-2 and VSMC-3 were increased to 410, 149 and 552% of the control by IL-1β (10 ng/ml) and to 471, 358 and 694% by TNF-α (20 ng/ml), while forskolin (2 μM) decreased ir-AM concentration to 30, 31 and 68% of the control. Thrombin (10 NIH U/ml) decreased ir-AM concentration to 33 and 26% of the control in VSMC-1 and VSMC-2, but increased it to 284% in VSMC-3.

4. Discussion

Among the substances examined, forskolin potently inhibited AM production and gene transcription in cultured rat VSMC (Fig. 2). 8-Br-cAMP also suppressed AM production to a lesser extent. These results suggest that production and gene transcription of AM are down-regulated by an intracellular cAMP concentration. AM receptors, coupled with the adenylate cyclase system, have been shown to be present on VSMC [9,10]. AM is produced and secreted from VSMC and the secreted AM then binds AM receptors on VSMC, resulting in elevation of intracellular cAMP level. If AM concentration is high enough, AM production might be suppressed by a feedback mechanism through cAMP. VIP, which increases intracellular cAMP concentration, also inhibits AM production. These results support the presence of a cAMP-mediated inhibitory pathway of AM production in VSMC. Although cAMP-regulated enhancers are present in the 5′-upstream region and in-

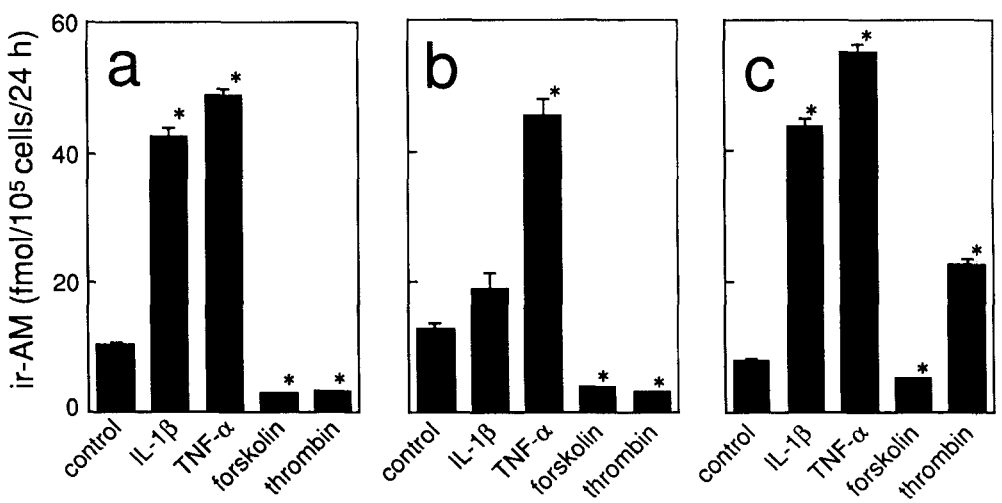

Fig. 3. Differential responses of AM production to IL-1β, TNF-α, forskolin and thrombin stimulations in three rat VSMC cell lines. Ir-AM concentration in culture medium of (a) VSMC-1, (b) VSMC-2 and (c) VSMC-3 was measured after 24 h incubation. IL-1β, TNF-α, forskolin and thrombin were administered at the final concentrations of 10 ng/ml, 20 ng/ml, 2 μM and 10 NIH U/ml. Each value represents mean ± S.E.M. of three or six separate wells. *$P < 0.01$.

trons of human AM gene [16], the present study indicates that these enhancers do not directly function in AM production of VSMC.

Thrombin markedly suppressed AM production and gene transcription in two of three VSMC cell lines, but stimulated it in one cell line (Figs. 2,3). Thrombin is reported to activate protein kinase C (PKC) and cAMP-mediated pathway has not been recognized as a major intracellular signaling pathway [17,18]. Calcitonin gene-related peptide (CGRP), which utilizes cAMP as an intracellular signal [19], induced no effect on AM production. These data suggest that regulation of AM production and gene transcription may not be simply attributed to the cAMP-mediated pathway.

TPA, an activator of PKC, elicited weak stimulatory effects on AM production in a lower concentration and inhibitory effects in a higher concentration (Fig. 1). Ang II and ET-1 elicit potent vasoconstriction mainly through a PKC-mediated pathway. Although these peptides stimulated AM production, increments of AM production were at most 56% that of the control. Several AP-1 and AP-2 sites are found in the 5′-upstream region of the human AM gene [16]. However, these data indicate that the PKC-mediated pathway is not enough to induce a potent stimulatory effect. Natriuretic peptides, which activate receptor guanylate cyclases on VSMC, and 8-Br-cGMP showed no effect, indicating that cGMP does not regulate AM production in VSMC.

In other substances examined, FCS, adrenaline, substance P and bradykinin stimulated AM production (Table 1). Stimulatory effect of FCS is not potent but is altered in each FCS concentration (Fig. 1). The effects of FCS may fluctuate in different lots or producers of FCS and may alter effects of stimulants. Thus, we employed the serum-free system for the experiments. Since only adrenaline induced significant effects in the catecholamines, detailed experiments are required for analysing the mechanism of catecholamines. AM induction mechanism of substance P and bradykinin, which stimulate production of nitric oxide in EC, remains unsolved.

Among the three INFs, only INF-γ inhibited AM production in VSMC. Although 13 INF responsive elements are present in the 5′-upstream region and introns of AM gene [16], AM production was not stimulated by INFs, in contrast to inducible NO synthase (iNOS) which has the many elements and has its gene expression augmented by INF-γ [20,21]. In this study, cAMP analogs are also shown to induce quite different effects on AM and iNOS production in VSMC [22], although both AM and iNOS relax VSMC and their gene expressions are commonly augmented by IL-1, TNF and LPS [23]. Regulation mechanism of AM gene transcription and its relationship to the gene structure is the next problem to be elucidated.

Three representative cell lines of VSMC uniformly responded to IL-1β, TNF-α and forskolin stimulation (Fig. 3), i.e. IL-1β and TNF-α stimulated and forskolin suppressed AM production regardless of cell shapes and properties. These data suggest that IL-1, TNF and intracellular cAMP regulate AM production even in the in vivo system.

In the present study, we verified that various substances influencing VSMC functions also regulate AM production in VSMC. These substances probably act simultaneously and in the same regions of the vascular wall. Therefore, the AM production level may be determined after integration of these effects, suggesting a complex regulation system of AM production in VSMC.

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References

[1] Imura, H., Matsuo, H. and Masaki, T. (1991) Peptide Regulation of Cardiovascular Function, Academic Press Japan Inc., Tokyo.
[2] Furchgott, R.F. and Vanhoutte, P.M. (1989) FASEB J. 3, 2007–2017.
[3] Kawasaki, H., Takasaki, K., Saito, A. and Goto, K. (1988) Nature 335, 164–167.
[4] Loppnow, H. and Libby, P. (1992) Exp. Cell Res. 198, 283–290.
[5] Resink, T.J., Hahn, A.W.A., Scott-Burden, T., Powell, J., Weber, E. and Buhler, F.R. (1990) Biochem. Biophys. Res. Commun. 168, 1303–1310.
[6] Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. 192, 553–560.
[7] Ishiyama, Y., Kitamura, K., Ichiki, Y., Nakamura, S., Kida, O., Kangawa, K. and Eto, T. (1993) Eur. J. Pharmacol. 241, 271–273.
[8] Sugo, S., Minamino, N., Shoji, H., Kangawa, K., Kitamura, K., Eto, T. and Matsuo, H. (1994) Biochem. Biophys. Res. Commun. 203, 719–726.
[9] Ishizaka, Y., Ishizaka, Y., Tanaka, M., Kitamura, K., Kangawa, K., Minamino, N., Matsuo, H. and Eto, T. (1994) Biochem. Biophys. Res. Commun. 200, 642–646.
[10] Eguchi, S., Hirata, Y., Kano, H., Sato, K., Watanabe, Y., Watanabe, T.X., Nakajima, K., Sakakibara, S. and Marumo, F. (1994) FEBS Lett. 340, 226–230.
[11] Sugo, S., Minamino, N., Shoji, H., Kangawa, K., Kitamura, K., Eto, T. and Matsuo, H. (1995) Biochem. Biophys. Res. Commun. 207, 25–32.
[12] Furuya, M., Yoshida, M., Hayashi, Y., Ohnuma, N., Minamino, N., Kangawa, K. and Matsuo, H. (1991) Biochem. Biophys. Res. Commun. 177, 927–931.
[13] Sakata, J., Shimokubo, T., Kitamura, K., Nishizono, M., Ichiki, Y., Kangawa, K., Matsuo, H. and Eto, T. (1994) FEBS Lett. 352, 105–108.
[14] Sugo, S., Minamino, N., Kangawa, K., Miyamoto, K., Kitamura, K., Sakata, J., Eto, T. and Matsuo, H. (1994) Biochem. Biophys. Res. Commun. 201, 1160–1166.
[15] Sakata, J., Shimokubo, T., Kitamura, K., Nakamura, S., Kangawa, K., Matsuo, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. 195, 921–927.
[16] Ishimitsu, T., Kojima, M., Kangawa, K., Hino, J., Matsuoka, H., Kitamura, K., Eto, T. and Matsuo H. (1994) Biochem. Biophys. Res. Commun. 203, 631–639.
[17] Berk, B.C., Taubman, M.B., Cragoe, E.J., Fenton II, J.W. and Griendling, K.K. (1990) J. Biol Chem. 265, 17334–17340.
[18] Huang, C.-L., Cogan, M.G., Cragoe, E.J. and Ives, H.E. (1987) J. Biol. Chem. 262, 14134–14140.
[19] Kubota, M., Moseley, J.M., Butera, L., Dusting, G.J., MacDonald, P.S. and Martin, T.J. (1985) Biochem. Biophys. Res. Commun. 132, 88–94.
[20] Nunokawa, Y., Ishida, N. and Tanaka, S. (1993) Biochem. Biophys. Res. Commun. 191, 89–94.
[21] Nunokawa, Y., Ishida, N. and Tanaka, S. (1994) Biochem. Biophys. Res. Commun. 200, 802–807.
[22] Koide, M., Kawahara, Y., Nakayama, I., Tsuda, T. and Yokoyama, M. (1993) J. Biol Chem. 268, 24959–24966.
[23] Beasley, D. and Eldridge, M. (1994) Am. J. Physiol. 266, R1197–R1203.