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Adrenomedullin inhibits angiotensin II-induced contraction in human aortic smooth muscle cells

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Abstract

The vasodilating peptide adrenomedullin (AM) has been reported to regulate vascular tone as well as proliferation and differentiation of various cell types in an autocrine/paracrine manner.

Our study was designed to investigate the effect of AM on Ang II-induced contraction on human aortic smooth muscle cells (HASMC) in vitro, evaluating the signal pathways involved.

Our findings indicate that AM was able to inhibit HASMC Ang II-induced contraction (IC_{50} 19 nM). AM stimulated cAMP production in a dose-dependent fashion as well. SQ 22.536, an adenylate cyclase inhibitor, and KT5720, a PKA inhibitor, blunted the AM effect, suggesting that it was mediated by the activation of the cAMP transduction pathway.

Our results suggest that AM plays a role in the regulation of HASMC contraction by antagonizing the Ang II effects and may be involved in conditions of altered regulation of the blood vessels.

Keywords: Vasodilation; cAMP; Paracrine; NO-independent

1. Introduction

The control of blood pressure is regulated by a complex network of various systems that cooperate to maintain the homeostatic balance in which the regulation of vascular tone is a major mechanism [1,2].

Adrenomedullin (AM) is a multifunctional regulatory peptide originally isolated from the extract of human pheochromocytoma which was subsequently shown to be ubiquitously secreted by endothelial cells [3,4]. Furthermore, vascular smooth muscle cells express its receptors [5,6] suggesting a role for AM as an autocrine/paracrine factor in the regulation of vascular tone.

In man, intravenous infusion of adrenomedullin results in a potent and sustained hypotension [7,8]. AM increases forearm blood flow when infused into the human brachial artery and reverses the venous constriction in the dorsal hand vein caused by norepinephrine preinjection [9–11]. Intrapulmonary AM infusion in patients with pulmonary hypertension induces a significant dose-dependent increase in blood flow velocity in a segmental pulmonary artery, suggesting a strong pulmonary vasodilator activity of AM [12]. Accordingly, acute and chronic administration of adrenomedullin provoke blood pressure fall by dilating resistance vessels with a drop in total peripheral resistance.

The mechanisms involved in the vasodilation induced by AM are likely to be multiple, but nitric oxide production by endothelial cells with subsequent vascular smooth muscle cells (VSMC) relaxation is usually held as the more important [13]. However, NO-independent vasodilation has been reported as well [14] and attributed to a direct action on VSMC through a marked increase of cyclic adenosine 3′,5′-monophosphate (cAMP) [15].

Our study was designed to investigate whether AM has a direct effect on the contraction of human aortic smooth muscle cells (HASMC) induced by angiotensin II, and to evaluate the signal pathways involved.

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2. Materials and methods

2.1. Materials

AM and AM-(22-52) were purchased from Peptide Institute (Osaka, Japan). Ang II was obtained from SIGMA (Milan, Italy), and PD 98059, SQ 22536, KT5720 from Calbiochem (Milan,Italy). Losartan was kindly provided by Merck, Sharp & Dohme (Rome, Italy), and human VSMC was obtained from BioWhittaker, Europe.

2.2. HASMC culture

After thawing, according to the method suggested by BioWhittaker, cryopreserved cells were placed at density of 2.5×10^3 cells/cm^2 in 75 ml plastic flack (Falcon) with SmGM-2 medium (BioWhittaker Europe) containing 0.5 μg/ml hEGF (human recombinant Epidermal Growth Factor), 0.5 mg/ml insulin, 1 μg/ml hFGF (human recombinant Fibroblast Growth Factor), 1 mg/ml TGF-β1 (Transforming Growth Factor β1), 50 mg/ml Gentamicin, 50 μg/ml Amphotericin-B and 10% fetal calf serum. They were maintained at 37 °C in 5% CO_2 atmosphere, and the medium was changed every 48 h. When the cells were about at 70–80% confluence they were subcultured.

2.3. HASMC contraction in microperfusion chamber system

The microperfusion experiments were performed as already described [16]. In brief, a glass coverslip with plated HASMC was placed into a stainless steel microperfusion chamber (0.5 ml volume). Perfusate was circulated by a peristaltic pump. A multiport valve served to switch between perfusates with different composition. Control of the temperature inside the cell chamber (37 °C) was achieved by external control of perfusate temperature. Measurements were performed upon continuous cell perfusion on an Olympus microscope using a 20 × phase contrast objective. Images were video captured by a Panasonic B/W camera coupled to a Sony video recorder. After perfusion (1 ml/min) with regular buffer for 3 min, time necessary for thermal equilibration, cells were flushed with test compounds. To evaluate cell contraction, the video captured images were acquired on computer (Screen Machine II, FAST Multimedia AG, Munchen, Germany), cell areas were calculated from the digitized images (SigmaScan Pro, Jandel Scientific, Erkrath, Germany), and percent of contraction (C) computed according to the formula:

$$C = [(A - a/A] \times 100$$

where A is the area of a single cell before stimulation and a is the area of the same cell after stimulation. Experiments were performed at least 3 times in triplicate.

2.4. Determination of cAMP

Confluent HASMC (10×10^3 cells/well in 24-well trays) were washed twice with the assay buffer (M199 containing 0.1 % BSA, 20 mM Hepes, and 0.5 mM isobutyl-methylxanthine, pH 7.4) and subsequently incubated for 15 min at 37 °C in the absence or in the presence of AM and antagonists. Cells were lysed in 6% trichloroacetic acid, and the acid was removed by water-saturated diethyl ether extraction. After lyophilization of the aqueous phase, intracellular cAMP was measured by an RIA kit (Amersham, England).

2.5. Statistical analysis

Data are presented as means ± standard error of mean (S.E.M.). Statistical analysis was performed by using the Student's paired t-test. $P < 0.05$ was taken as statistically significant.

3. Results

Angiotensin II, as expected, was able to induce contraction in HASMC in a dose-dependent manner with EC_{50} value of 1.3 nM (1.07–1.73 nM, 95% confidence limits). Losartan inhibited its effect and produced a rightward parallel shift of the curve (Fig. 1). Schild analysis of the data gave a pA_2 value of 7.27 (7.23–7.30 % confidence limits) and a slope of 1.1 (0.8–1.18, 95% confidence limits), showing a receptor–antagonist effect. When cells were pretreated with adrenomedullin (10 nM) 1 h before treatment with increasing dose of Ang II, HASMC contraction induced by angiotensin was blunted, as illustrated in Fig. 2.

To elucidate this inhibitory effect, we performed a set of experiments using increasing doses of adrenomedullin. In this set AM inhibited HASMC contraction induced by Ang II (100

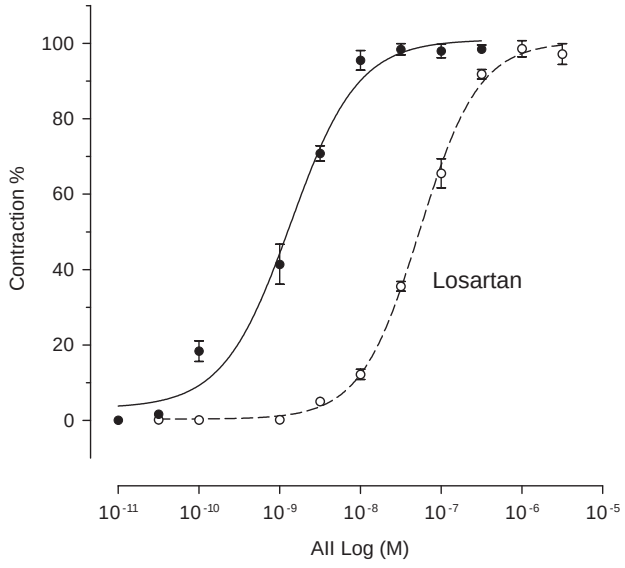

Fig. 1. Dose–response curve of the contraction induced in HASMC by graded concentration of Ang II (closed circle), and competitive inhibition exerted by losartan (open circle). Losartan produced a rightward parallel shift in the dose–response curve. The results are expressed as percentage of contraction computed as the difference in the areas before and after stimulation. Student's paired t-test was used for the statistical analysis of data. Each point is expressed as the mean of 3 separate experiments run in triplicate. Bars show S.E.M.

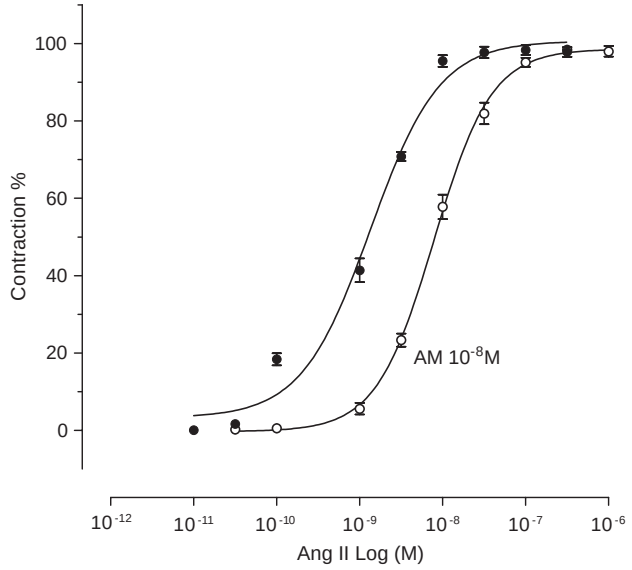

Fig. 2. Dose–response curve of HASMC contraction induced by graded concentration of Ang II in the presence or the absence of AM (10 nM). The results are expressed as percentage of contraction computed as the difference in the areas before and after stimulation. Student's paired *t*-test was used for the statistical analysis of data. Each point is expressed as the mean of 3 separate experiments run in triplicate. Bars show S.E.M.

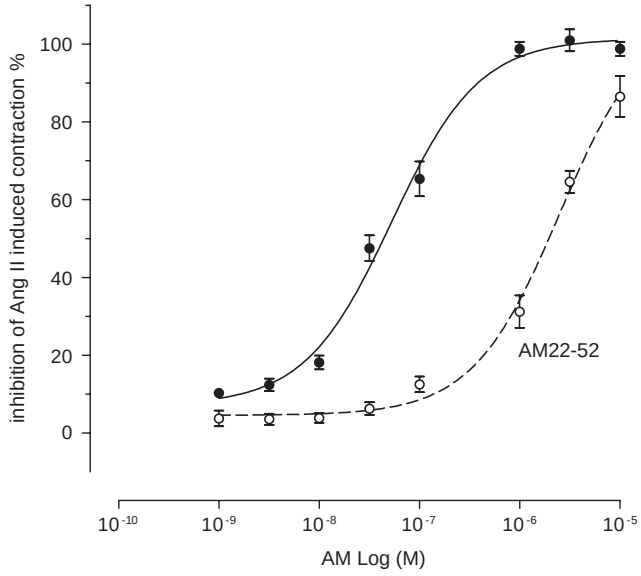

Fig. 4. Adrenomedullin inhibition curve of Ang II-induced HASMC contraction. Pretreatment with the specific antagonist AM-(22-52) (10 nM) produced a rightward parallel shift in the dose–response curve. The results are expressed as percentage of contraction computed as the difference in the areas before and after stimulation. Student's paired *t*-test was used for the statistical analysis of data. Each point is expressed as the mean of 3 separate experiments run in triplicate. Bars show S.E.M.

nM) in a dose–response fashion with an IC_{50} value of 19 nM (14.2–25.2 nM, 95% confidence limits) (Fig. 3). Under these conditions we evaluated the action of the specific receptor antagonist AM-(22-52). Pretreatment with 10 nM AM-(22-52) produced a rightward parallel shift in the dose–response curve of AM effect on Ang II-induced contraction. Schild analysis of the data gave a pA_2 value of 5.56 (5.46–5.73, 95% confidence limits) and a slope of 0.93 (0.55–1.31, 95% confidence limits), showing a typical receptor–antagonism fashion (Fig. 4).

To analyze the signaling pathways involved in the AM effect, cAMP production was measured. AM was able to induce cAMP production in a dose-dependent fashion (Fig. 5). In addition, pharmacological inhibitors were used to study the cAMP pathway. Two compounds were used, 9-(tetrahydro-2-

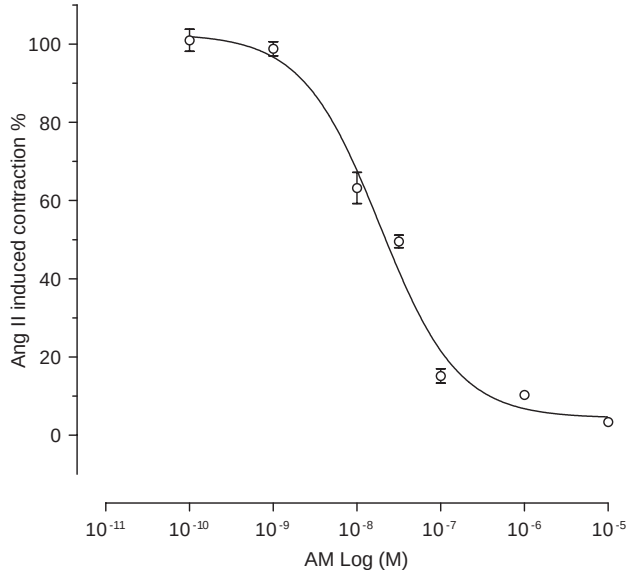

Fig. 3. Effect of graded concentration of AM on HASMC contraction induced by Ang II (10 nM). Adrenomedullin inhibited contraction with an IC_{50} of 19 nM (14.2–25.2 nM, 95% confidence limits). The results are expressed as percentage of contraction computed as the difference in the areas before and after stimulation. Student's paired *t*-test was used for the statistical analysis of data. Each point is expressed as the mean of 3 separate experiments run in triplicate. Bars show S.E.M.

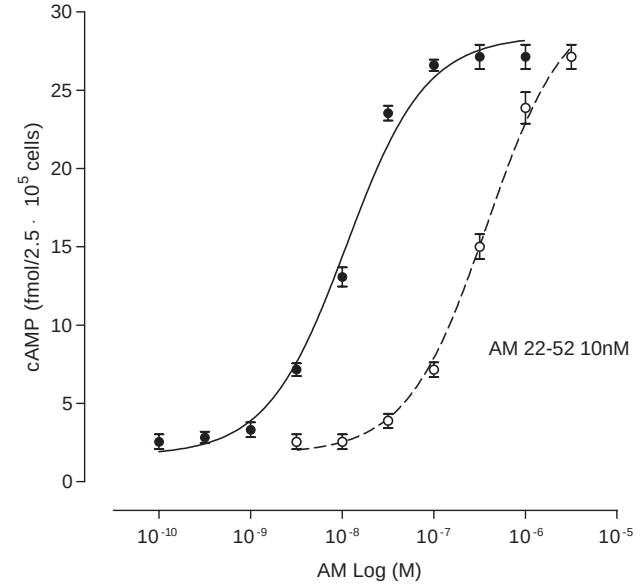

Fig. 5. Adrenomedullin effect on intracellular cAMP. AM elicited an increase of intracellular cAMP in HASMC in a dose-dependent manner with an EC_{50} of about 10 nM (10–13 nM, 95% confidence limits). AM(22-52) (10 nM) inhibited AM effect, inducing a right parallel shift of the curve with an IC_{50} of 290 nM (240–350 nM, 95% confidence limits). Each point is expressed as the mean ± S.E.M. of 3 separate experiments run in triplicate. Bars show S.E.M.

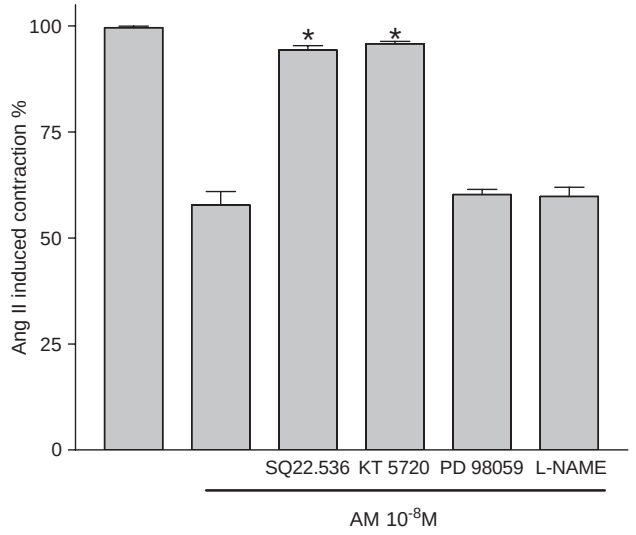

Fig. 6. Effects of the inhibition of cAMP and MAPK pathways and of nitric oxide production on AM inhibition of Ang II (10 nM)-induced HASMC contraction. SQ22.536 and KT5720 inhibited the AM effect, whereas MAPK inhibitor PD 98059 (5 μM) and nitric oxide synthase inhibitor L-NAME (100 μM) were ineffective. The results are expressed as percentage of contraction. Data are normalized assuming a 100% response evoked by Ang II (100 nM). Each histogram represents the mean±S.E.M. of 3 separate experiments run in triplicate. *Indicates significant difference ($P<0.005$) vs. samples treated with AM.

furanyl) adenine (SQ 22.536), an adenylate cyclase inhibitor, and KT5720, a PKA inhibitor. Both compounds blunted the inhibitory effect of AM on Ang II, further indicating that the cAMP pathway is involved in the effect.

To evaluate whether nitric oxide production was involved in the inhibition of Ang II-induced contraction, the cells were pretreated with the nitric oxide synthase L-NAME (100 μM) which was unable to reverse the AM effect (Fig. 6).

Because the extracellular signal-regulated kinase (ERK) subfamily of MAPKs are activated by AM [17] and these kinases are able to modulate Ang II-induced contraction in VSMC of spontaneously hypertensive rats [18], the relevance of the MAPK pathway was also analyzed using the MAPK inhibitor PD 98059 (5 μM). No change of the AM effect was induced by PD 98059 (Fig. 6).

4. Discussion

Our investigation documents that AM is able to inhibit Ang II-induced contraction of human aorta smooth muscle cells (HASMC). Ang II is well known to elicit a rapid, reversible, contractile response in VSMC, inhibited by pretreatment with the AT1 competitive antagonist [19], via the tyrosine kinase pathway [20]. The present documentation of the AM inhibitory effect on Ang II-induced contraction is in line with previous investigations in our laboratory which demonstrated that AM is able to inhibit Ang II and ET1-induced contraction in testicular peritubular myoid cells [16,21].

The investigation of the transduction pathway showed that cAMP inhibitor SQ22.536 and PKA inhibitor KT5720 blunted the AM inhibitory effect on Ang II-induced contraction, suggesting that the cAMP pathway, a known intracellular messenger of AM, is involved. The contraction inhibition was not mediated by NO, as pretreatment with L-NAME was not able to reverse the AM effect. This is at variance with what has been reported on resistance vessels and coronary arteries in man, in which the nitric oxide synthase inhibition is capable to blunt the AM-induced vasodilation [7,22]. In addition, in contrast with what reported in other species [17], the MAPK pathway does not seem to be involved as demonstrated by the lack of effect of PD 98059.

In conclusion, our findings indicate that AM antagonizes the HASMC contraction induced by Ang II, by acting directly on HASMC via the cAMP pathway. The contractile activity in smooth muscle cell is regulated by the extent of Ca^{+} myosin light chain phosphorylation [23], catalyzed by a myosin light chain kinase regulated through the calcium binding protein calmodulin [24]. Smooth muscle cell myosin can be phosphorylated by the catalytic subunit of PKA [25], and this phosphorylation results in a high increase in the amount of calmodulin required for half-maximal activity [26]. Thus AM, increasing intracellular cAMP, can induce Ca^{+} desensitization of aortic smooth muscle cell, and inhibit their contraction. On the other hand AM did not change the Ca^{+} transient provoked by Ang II (data not shown).

On this grounds, in man, AM is likely to regulate aorta tone by directly affecting HASMC contraction, even if our experimental model cannot exclude that in vivo AM may also stimulate NO production by endothelial cells with additional HASMC relaxation and, as the VSMC phenotype changes in cultured cells, different data could be gathered with the denuded vessel model. The cAMP-dependent relaxation in the human aorta is in keeping with porcine coronaries and cat hind-limb [13,14] regulation but is in contrast with what has been reported for rat aorta and resistance vessels and coronary arteries in man. Taken all together, this indicates that, beyond species difference, different vascular districts may be regulated by AM via different transduction pathways.

The evidence shown in our study suggests that AM plays a direct role in the regulation of HASMC, and may be involved in conditions of altered regulation of the vascular tone as an autocrine or paracrine regulatory peptide. However, further investigation is required to elucidate the complex role of AM in the intricate regulatory mechanisms of cell contraction in which a large number of agonists are involved.

Acknowledgments

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References

[1] Lundgren O. Role of splanchnic resistance vessels in overall cardiovascular homeostasis. Fed Proc 1983;42:1673–7.
[2] Mulvany MJ, Aalkjær C. Structure and function of small arteries. Physiol Rev 1990;70:921–61.
[3] Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. Adrenomedullin: a novel hypotensive peptide isolated from human phaeochromocytoma. Biochem Biophys Res Commun 1993;192:553–60.

[4] Marutsuka K, Hatakeyama K, Sato Y, Yamashita A, Sumiyoshi A, Asada Y. Immunohistological localization and possible functions of adrenomedullin. Hypertens Res 2003;26:S33–40.
[5] Fukai N, Shichiri M, Ozawa N, Matsushita M, Hirata Y. Coexpression of calcitonin receptor-like receptor and receptor activity-modifying protein 2 or 3 mediates the antimigratory effect of adrenomedullin. Endocrinology 2003;144:447–53.
[6] Kamitani S, Asakawa M, Shimekake Y, Kuwasako K, Nakahara K, Sakata T. The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells. FEBS Lett 1999; 448:111–4.
[7] Nakamura M, Yoshida H, Makita S, Arakawa N, Niinuma H, Hiramori K. Potent and long-lasting vasodilatory effects of adrenomedullin in humans. Comparisons between normal subjects and patients with chronic heart failure. Circulation 1997;95:1214–21.
[8] Lainchbury JG, Cooper GJ, Coy DH, Jiang NY, Lewis LK, Yandle TG, Richards AM, Nicholls MG. Adrenomedullin: a hypotensive hormone in man. Clin Sci 1997;92:467–72.
[9] Cockcroft J, Noon J, Gardner-Medwin J, Bennett T. Haemodynamic effects of adrenomedullin in human resistance and capacitance vessels. Br J Clin Pharmacol 1997;44:57–60.
[10] Rademaker M, Charles C, Espiner E, Nicholls M, Richards A. Long-term adrenomedullin administration in experimental heart failure. Hypertension 2002;40:667–72.
[11] Nagaya N, Satoh T, Nishikimi T, Uematsu M, Furuichi S, Sakamaki F, Oya H, Kyotani S, Nakanishi N, Goto Y, Masuda Y, Miyatake K, Kangawa K. Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. Circulation 2000; 101:498–503.
[12] Nagaya N, Miyatake K, Kyotani S, Nishikimi T, Nakanishi N, Kangawa K. Pulmonary vasodilator response to adrenomedullin in patients with pulmonary hypertension. Hypertens Res 2003;26:S141–6.
[13] Yoshimoto R, Mitsui-Saito M, Ozaki H, Karaki H. Effects of adrenomedullin and calcitonin gene-related peptide on contractions of the rat aorta and porcine coronary artery. Br J Pharmacol 1998;123:1645–54.
[14] Champion HC. Comparison of responses to rat and human adrenomedullin in the hindlimb vascular bed of the cat. Regul Pept 1997;70:161–5.
[15] Ishizaka Y, Ishizaka Y, Tanaka M, et al. Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. Biochem Biophys Res Commun 1994;200:642–6.
[16] Santiemma V, Rossi F, Guerrini L, Markouizou A, Pasimeni G, Palleschi A, Fabbrini A. Adrenomedullin inhibits the contraction of cultured rat testicular peritubular myoid cells induced by endothelin-1. Biol Reprod 2001;64:619–24.
[17] Iwasaki H, Shichiri M, Marumo F, Hirata Y. Adrenomedullin stimulates proline-rich tyrosine kinase 2 in vascular smooth muscle cells. Endocrinology 2001;142:564–72.
[18] Touyz RM, El Mabrouk M, He G, Wu XH, Schiffrin EL. Mitogen-activated protein/extracellular signal-regulated kinase inhibition attenuates angiotensin II-mediated signaling and contraction in spontaneously hypertensive rat vascular smooth muscle cells. Circ Res 1999;84:505–15.
[19] Gunther S, Alexander RW, Atkinson WJ, Gimbrone MA. Functional angiotensin II receptors in cultured vascular smooth muscle cells. J Cell Biol 1982;92:289–98.
[20] Torrecillas G, Boyano-Adanez MC, Medina J, Parra T, Griera M, Lopez-Ongil S, Arilla E, Rodriguez-Puyol M, Rodriguez-Puyol D. The role of hydrogen peroxide in the contractile response to angiotensin II. Mol Pharmacol 2001;59:104–12.
[21] Rossi F, Ferraresi A, Romagni P, Silvestroni L, Santiemma V. Angiotensin II stimulates contraction and growth of testicular peritubular myoid cells in vitro. Endocrinology 2002;143:3096–104.
[22] Terata K, Miura H, Liu Y, Loberiza F, Gutterman DD. Human coronary arteriolar dilation to adrenomedullin: role of nitric oxide and $K(^{+})$ channels. Am J Physiol Heart Circ Physiol 2000;279:H2620–6.
[23] Adelstein RS, Eisenberg E. Regulation and kinetics of the actin–myosin–ATP interaction. Annu Rev Biochem 1980;49:921–56.
[24] Blumenthal DK, Stull JT. Activation of skeletal muscle myosin light chain kinase by calcium(2^{+}) and calmodulin. Biochemistry 1980;19:5608–14.
[25] Conti MA, Adelstein RS. Phosphorylation by cyclic adenosine 3′5′-monophosphate-dependent protein kinase regulates myosin light chain kinase. Fed Proc 1980;39:1569–73.
[26] Conti MA, Adelstein RS. The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3′5′cAMP-dependent protein kinase. J Biol Chem 1981;256:3178–81.