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Short communication

Down-regulation of adenylate cyclase coupled to adrenomedullin receptor in vascular smooth muscle cells

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Abstract

Adrenomedullin activates receptor-mediated adenylate cyclase to cause vasorelaxation. To elucidate whether desensitization of adenylate cyclase coupled to vascular adrenomedullin receptors occurs, we studied the adenylate cyclase activity after treatment with rat adrenomedullin in cultured rat aortic vascular smooth muscle cells. Cyclic AMP (cAMP) generation induced by adrenomedullin was markedly decreased by pretreatment with adrenomedullin: a maximal reduction (~ 80%) was induced after 2 h and persisted during 24 h. Desensitization was independent of protein kinase A, protein kinase C, protein tyrosine kinase or receptor sequestration, because pretreatment with either isoproterenol, forskolin, tetradecanoylphorbol acetate, cytochalasin D, or colchicine did not affect the adrenomedullin-stimulated cAMP response. Furthermore, preincubation with inhibitors for these protein kinases prior to pretreatment with adrenomedullin failed to affect the adrenomedullin-induced decrease in cAMP response following the second stimulation with adrenomedullin. The present results provide the evidence for the existence of desensitization of adenylate cyclase coupled to vascular adrenomedullin receptors.

Keywords: Adrenomedullin; Smooth muscle cell, vascular; Desensitization; Adenylate cyclase

1. Introduction

Adrenomedullin is a novel vasorelaxant peptide originally isolated from human pheochromocytoma (Kitamura et al., 1993). Subsequent studies have revealed that adrenomedulli transcripts and protein are expressed in various tissues such as cultured rat endothelial cells and vascular smooth muscle cells (Sugo et al., 1994). Immunoreactive adrenomedullin has been shown to circulate in human plasma (Kitamura et al., 1994), suggesting its possible role as a vasoactive hormone.

We have demonstrated the presence of specific receptors for adrenomedullin in cultured vascular smooth muscle cells through which AM induces increase in intracellular cyclic AMP (cAMP) concentrations (Eguchi et al., 1994). Recently, a cDNA clone encoding 395 residues has been identified as a putative adrenomedullin receptor (Kapas et al., 1995), which has seven transmembrane domains belonging to the G protein-coupled receptor super-family. However, no information is yet available as to the sensitivity of adenylate cyclase by prolonged adrenomedullin receptor stimulation. Therefore, the present study was designed to elucidate whether desensitization of adenylate cyclase coupled to adrenomedullin receptors occurs in cultured rat vascular smooth muscle cells.

2. Materials and methods

2.1. Materials

Rat adrenomedullin was purchased from Peptide Institute (Osaka, Japan), Dulbecco's Modified Eagle Medium (DMEM) from GIBCO (Grand Island, NY, USA), fetal calf serum from Hyclone Laboratories (Logan, UT, USA), 3-isobutyl-1-metylxanthine (IBMX), isoproterenol, forskolin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and genistein from Sigma (St. Louis, MO, USA), KT5720 from Kyowa Medix (Tokyo, Japan), GF109203X from Calbiochem-Novabiochem (La Jolla, CA, USA). All other reagents were of analytical grade.

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2.2. Cell culture

Vascular smooth muscle cells were prepared from the thoracic aorta of 8-week old male Wistar rats by the explant method as previously described (Eguchi et al., 1994), and cultured in DMEM containing 10% fetal calf serum at 37°C in a humidified atmosphere of 95% air–5% CO_2. Vascular smooth muscle cells harvested between 10th and 20th passages were used in the experiments.

2.3. Incubation

Confluent vascular smooth muscle cells ($\sim 5 \times 10^5$ cells/well) were incubated with rat adrenomedullin at 37°C for the indicated times in serum-free DMEM. After completion, cells were washed three times with fresh DMEM to remove unbound adrenomedullin.

2.4. Measurement of intracellular cAMP generation

For measurement of intracellular cAMP, cells were incubated with or without adrenomedullin in the presence of 0.5 mM IBMX at 37°C for 10 min. After extraction with 1 M HCl, intracellular cAMP was determined by a radioimmunoassay kit (Yamasa Shoyu, Chiba, Japan).

3. Results

To determine whether pretreatment with adrenomedullin leads to desensitization of adenylate cyclase activity, adrenomedullin-stimulated cAMP generation was examined (Fig. 1A). Pretreatment with 100 nM of adrenomedullin decreased adrenomedullin (100 nM)-induced cAMP generation by 23% as early as 15 min; the maximal loss of cAMP response (~ 80%) occurred after 2 h and persisted during 24 h. The adrenomedullin-stimulated cAMP response was examined after pretreatment with various doses of adrenomedullin for 24 h (Fig. 1B). Pretreatment with adrenomedullin resulted in a dose-dependent (1 nM–1 μM) decrease in adrenomedullin-induced cAMP generation; a half maximal dose to induce desensitization of cAMP response was 3 nM.

To determine whether protein kinase A or protein kinase C is responsible for the adrenomedullin-induced reduction of adenylate cyclase response, we examined the effects of compounds that stimulate protein kinase A or protein kinase C on adrenomedullin-induced cAMP generation (Fig. 2A). In contrast to a marked decrease in cAMP response after rechallenge with adrenomedullinin in adrenomedullin-pretreated cells, adrenomedullin increased cAMP production to the same extent as in control cells after pretreatment with either isoproterenol (1 μM), a β-adrenoceptor agonist, forskolin (1 μM), an adenylate cyclase activator, or TPA (1 μM), a protein kinase C activator. To determine whether protein kinase(s) is responsible for the adrenomedullin-induced reduction of cAMP response, we examined the effects of relatively selective inhibitors for several protein kinases (Fig. 2B). Neither protein kinase A inhibitor (KT5720: 500 nM), a protein kinase C inhibitor (GF109203X: 2 μM) nor a tyrosine kinase inhibitor (genistein: 100 μM) preincubated prior to adrenomedullin pretreatment affected the adrenomedullin-induced decrease in cAMP response. The adrenomedullin-induced reduction in cAMP response (21.3

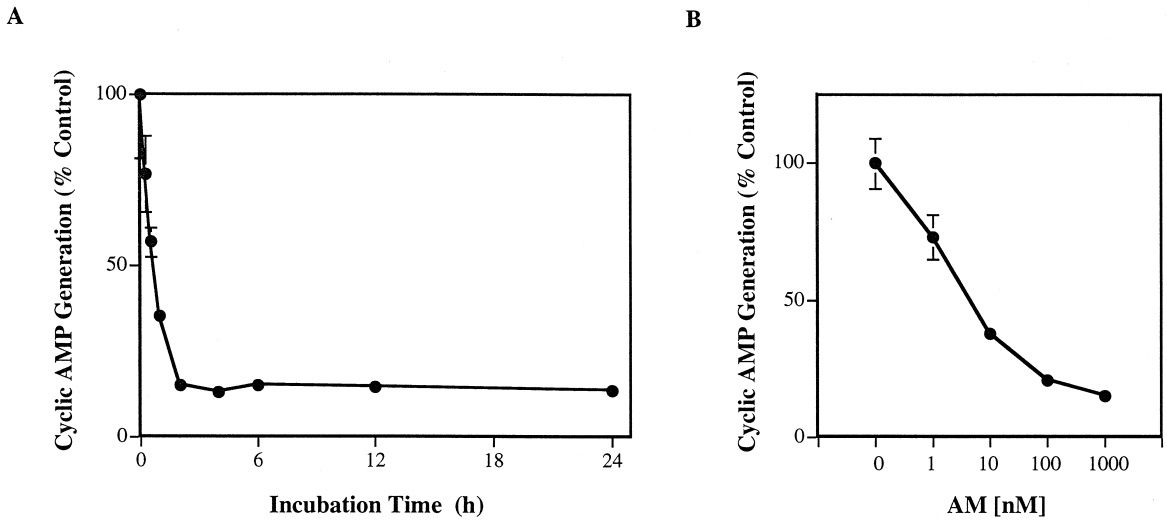

Fig. 1. Desensitization of adrenomedullin-stimulated cAMP generation after pretreatment with adrenomedullin in rat vascular smooth muscle cells. (A) Cells were exposed to adrenomedullin (100 nM) for the indicated times, washed and rechallenged with adrenomedullin (100 nM) for 10 min; concentrations of intracellular cAMP were determined. (B) Dose-dependent decrease of adrenomedullin (100 nM)-stimulated cAMP generation after pretreatment with various doses of adrenomedullin (AM) for 24 h. Each point represents the percentage to cAMP generation without adrenomedullin pretreatment; bars show S.E.M. ($n = 4$).

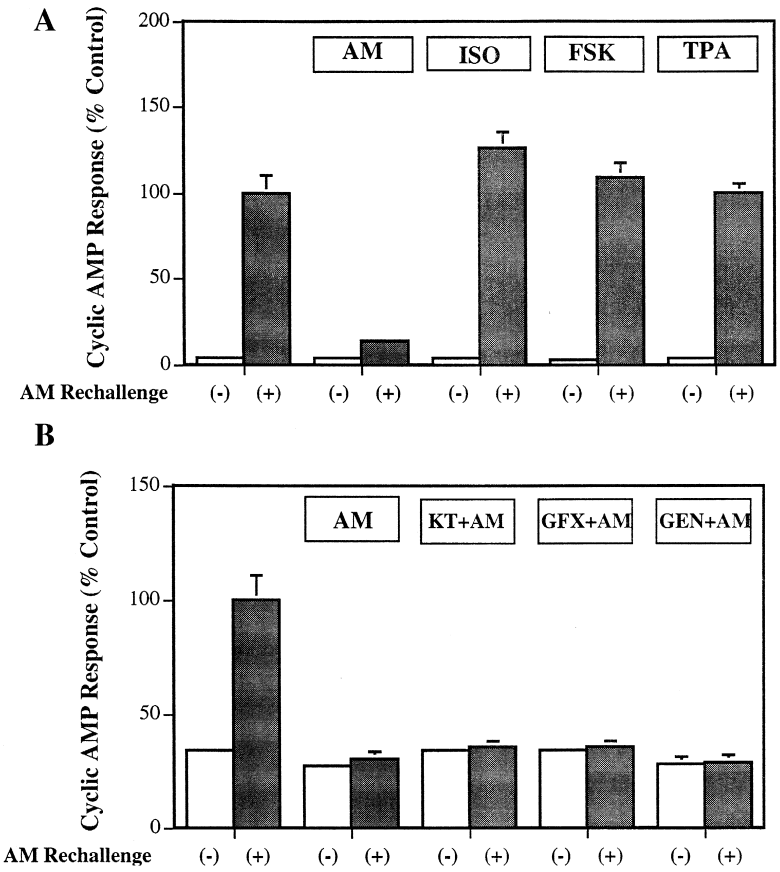

Fig. 2. (A) Effects of adrenomedullin, isoproterenol, forskolin and phorbol ester pretreatment on basal and adrenomedullin-stimulated cAMP generation in rat vascular smooth muscle cells. Cells were pretreated with or without adrenomedullin (AM: 100 nM), isoproterenol (ISO: 1 μM), forskolin (FSK: 1 μM) and tetradecanoylphorbol acetate (TPA: 1 μM) for 5 h, washed, and rechallenged with (■) or without (□) adrenomedullin (100 nM) for 10 min; intracellular cAMP levels were determined. Each column represents the percentage to the adrenomedullin-stimulated cAMP generation in control cells; bars show S.E.M. ($n = 4$). (B) Effects of protein kinase A inhibitor (KT5720), protein kinase C inhibitor (GF109203X) and tyrosine kinase inhibitor (genistein) pretreatment on adrenomedullin-induced reduction of cAMP response in rat vascular smooth muscle cells. Cells were pretreated with or without KT5720 (KT: 500 nM), GF109203X (GFX: 2 μM) and genistein (GEN: 100 μM) 30 min prior to pretreatment with or without adrenomedullin (100 nM) for 5 h, washed, and rechallenged with (■) or without (□) adrenomedullin (AM: 100 nM) for 10 min; intracellular cAMP levels were determined. Each column represents the percentage to the adrenomedullin-stimulated cAMP generation in control cells; bars show S.E.M. ($n = 4$).

$\pm$ 1.5% compared to control) was not affected by co-incubation with either cytochalasin D (1 μM; 19.5 $\pm$ 1.1%) or colchicine (1 μM; 22.8 $\pm$ 3.6%).

4. Discussion

The present study has shown that prior exposure to adrenomedullin led to a rapid (within 15 min) and a marked decrease (80%) after 2 h in adenylate cyclase activity in response to adrenomedullin, which persisted during 24 h. These data are consistent with desensitization of adenylate cyclase system, coupled vascular adrenomedullin receptors.

Receptor desensitization occurs in a variety of G-protein coupled receptors (Wang et al., 1990). In β-adrenoceptor, for example, phosphorylation of the receptor by protein kinase A and by a G protein-coupled receptor kinase mediates heterologous and homologous desensitizaton of the adenylate cyclase in response to β-adrenoceptor agonists, respectively (Bonovic et al., 1985; Hausdorff et al., 1989). The phosphorylation of β-adrenoceptor by protein kinase A occurs at consensus sequences rich in serine and threonine, located in its third intracellular loop or cytoplasmic tail (Hausdorff et al., 1989). Putative adrenomedullin receptor also contains a serine and threonine-rich residues at its carboxyl terminus (Kapas et al., 1995). However, in the present study preincubation with isoproterenol, a β-adrenoceptor agonist, or forskolin, a direct adenylate cyclase activator, did not affect the adrenomedullin-induced cAMP response. Furthermore, preincubation with KT5720, a relatively selective protein kinase A inhibitor, had no effect on decreased cAMP response after adrenomedullin pretreatment. These results indicate that adrenomedullin-induced adenylate cyclase desensitization is not mediated by protein kinase A. The

possible involvement of G protein-coupled receptor kinase in mediation of adrenomedullin-induced desensitization remains to be determined.

Phorbol esters which activate protein kinase C have also shown to induce both desensitization and phosphorylation of β-adrenoceptor (Bouvier et al., 1987). In bovine aortic endothelial cells, AM has been reported to activate not only adenylate cyclase, but also phospholipase C (Shimekake et al., 1995), leading to inositol trisphosphate (IP_3) and diacylglycerol formation, suggesting that adrenomedullin receptor is also coupled with Gq protein/protein kinase C. Although the putative adrenomedullin receptor contains several potential phosphorylation sites by protein kinase C in its cytoplasmic loop and C-terminal tail (Kapas et al., 1995), it seems unlikely that protein kinase C is involved in the process of desensitization because pretreatment with TPA failed to affect adrenomedullin-induced cAMP response and preincubation with GF109203X, a selective protein kinase C inhibitor, had no effect on decreased cAMP response after adrenomedullin pretreatment. We also confirmed that neither intracellular calcium nor IP_3 concentration was increased by adrenomedullin in our rat vascular smooth muscle cells (Iwasaki et al., 1998).

Recently, we have demonstrated that adrenomedullin rapidly induced tyrosine phosphorylation of several proteins and increased association of a tyrosine-phosphorylated protein with adaptor proteins, such as Shc and Grb2 (Iwasaki et al., 1998). However, it seems unlikely that the receptor phosphorylation by tyrosine kinase, if any, involves adrenomedullin-mediated adenylate cyclase desensitization because preincubation with genistein, a potent tyrosine kinase inhibitor, had no effect on decreased cAMP response after adrenomedullin pretreatment.

Receptor sequestration plays an important role for the rapid desensitization of angiotensin II-mediated phospholipase C responses in vascular smooth muscle cells (Ullian and Linas, 1989, 1990). In the present study, adrenomedullin-induced cAMP response was not affected by either cholchicine which interferes with microtubule assembly, or cytochalasin D which disrupts a microfilament. Both agents have been reported to block receptor sequestration (Yahara et al., 1982; Rinder et al., 1987). Thus, the process of desensitization does not appear to involve receptor sequestration.

5. Conclusion

We have shown the first evidence for desensitization of adenylate cyclase system coupled to vascular adrenomedullin receptors. It remains to be determined whether desensitization of vascular adrenomedullin receptor occurs in vivo to dampen its potent vasodilation under physiological and pathological conditions.

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