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European Journal of Pharmacology 407 (2000) 205–210

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Desensitization and resensitization of adrenomedullin-sensitive receptor in rat mesangial cells

Narayanan Parameswaran [a], Nambi Aiyar [b], Hsia-ling Wu [b], David P. Brooks [b], Ponnal Nambi [b], William S. Spielman [a,*]

[a] *Department of Physiology, Michigan State University, 103 Giltner Hall, East Lansing, MI 48824-1101 USA*
[b] *Department of Cardiovascular and Renal Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Philadelphia, PA, USA*

Received 9 March 2000; received in revised form 16 August 2000; accepted 22 August 2000

Abstract

Adrenomedullin is a potent adenylate cyclase activator and a vasodilatory peptide, that has anti-proliferative and apoptotic effects in rat mesangial cells. The present study was designed to determine the mechanisms of desensitization and resensitization of adrenomedullin-sensitive receptor in mesangial cells. Adrenomedullin caused a rapid desensitization of cAMP response evident within 5 min that was almost complete by 1 h of treatment. Pretreatment of cells with forskolin, that activates protein kinase-A by direct activation of adenylate cyclase, also caused adrenomedullin receptor desensitization. In addition, H89 [{*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], a potent protein kinase-A inhibitor inhibited adrenomedullin-induced desensitization of cAMP response. Adrenomedullin also caused desensitization of isoproterenol- and epinephrine-mediated cAMP accumulation. Furthermore, adrenomedullin induced cross-desensitization of endothelin-stimulated inositol phosphate accumulation. The attenuated cAMP response of adrenomedullin was restored to original levels within 2 h of agonist removal. This resensitization response was blocked by treatment with okadaic acid, a protein phosphatase (protein phosphatase-1/protein phosphatase-2A) inhibitor, during the 2 h resensitization period, indicating that protein phosphatase-1/protein phosphatase-2A may be involved in the resensitization of the adrenomedullin-sensitive receptor. We demonstrate for the first time in rat mesangial cells that the adrenomedullin-sensitive receptor undergoes heterologous desensitization and resensitization, and that it likely involves protein kinase-A and protein phosphatase-1/protein phosphatase-2A, respectively.

Keywords: Potent adenylate cyclase activator; Protein kinase-A; Protein phosphatase-1/protein phosphatase-2A

1. Introduction

Adrenomedullin is a recently discovered potent vasodilatory peptide that is also a potent activator of adenylate cyclase (Kitamura et al., 1993). Adrenomedullin has been shown to have positive chronotropic and inotropic effects in the heart, diuretic and natriuretic effects in the kidney, inhibitory effects on angiotensin II-stimulated aldosterone secretion, thirst and salt appetite, and vasopressin secretion, etc. (Samson, 1998). Plasma levels of adrenomedullin have been shown to be elevated in a number of disease states including chronic cardio-renal disorders (Ishimitsu et al., 1994; Cheung and Leung, 1997). It is not known, however, whether this elevation has any pathophysiological significance in terms of adrenomedullin-receptor signaling. Chronic elevation in the plasma and tissue levels of drugs or hormones has been shown to result in a decrease in response to these agents to subsequent challenges. This phenomenon, termed desensitization, is classified into two types, homologous and heterologous desensitization. Homologous desensitization occurs as a result of agonist-dependent activation of the same receptor, while heterologous desensitization results from activation of a different receptor (Hausdorff et al., 1990; Bohm et al., 1997). Resensitization of a receptor occurs after it has been desensitized, depending on the receptor and the desensitization mechanism. At least, in cases where a phosphorylation mechanism is involved in desensitization, the receptor undergoes endocytosis through complex

* Corresponding author. Tel.: +1-517-355-6475 ext. 1210; fax: +1-517-355-5125.
E-mail address: spielman@psl.msu.edu (W.S. Spielman).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00656-7

mechanisms to be dephosphorylated by phosphatases and then recycled back to the plasma membrane (Ferguson and Caron, 1998; Ferguson et al., 1998). We hypothesized that if the adrenomedullin-sensitive receptor desensitization involves a phosphorylation mechanism, then the resensitization might involve dephosphorylation. For that, we have used okadaic acid, a protein phosphatase-1/2A inhibitor to evaluate the role of protein phosphatase-1/protein phosphatase-2A in the resensitization process. Accordingly, the present study was undertaken (1) to identify the type of desensitization that the adrenomedullin-sensitive receptor in mesangial cells undergoes, and (2) to understand the possible mechanisms involved in the desensitization and resensitization processes.

We demonstrate for the first time that the adrenomedullin-sensitive receptor in mesangial cells undergoes heterologous desensitization, and that it is dependent at least in part on the activation of protein kinase-A. Moreover, exposure of mesangial cells to adrenomedullin also causes desensitization of isoproterenol- and epinephrine-induced cAMP responses, and endothelin-stimulated inositol phosphates. Adrenomedullin-stimulated cAMP response is resensitized and restored to original levels within 2 h after the removal of agonist. Resensitization is inhibited by okadaic acid, a serine/threonine phosphatase inhibitor. Our results raise the issue of whether chronic elevation of plasma adrenomedullin levels could lead to desensitization of adrenomedullin-sensitive receptors in the peripheral organs in diseased states and also suggests some important role for some serine/threonine kinases and phosphatases in the adrenomedullin-sensitive receptor desensitization and resensitization processes.

2. Materials and methods

2.1. Materials

Adrenomedullin was purchased from Phoenix Pharmaceuticals (Belmont, CA), RPMI-1640, fetal bovine serum, penicillin and streptomycin were from Gibco (Grand Island, NY). All other reagents were of high quality available.

2.2. Cell culture

Rat mesangial cells were obtained from the glomeruli of kidney cortex isolated from Sprague–Dawley rats as described before (Albrightson et al., 1992), and were grown in RPMI-1640 with 15% fetal bovine serum. Passages between 15 and 30 were used for the experiments.

2.3. Measurement of cAMP accumulation

CyclicAMP measurements were performed as described before, with slight modifications (Haneda et al., 1996). Cells were plated in 24 well plates (50,000 cells/well) and grown for 2 days prior to serum starvation for overnight. Cells were preincubated with 0.5 mM isobutyl methyl xanthine for 10 min and then agonist solutions (prepared in phosphate buffered saline containing 0.2% bovine serum albumin, 0.2% magnesium chloride and 0.1% glucose)

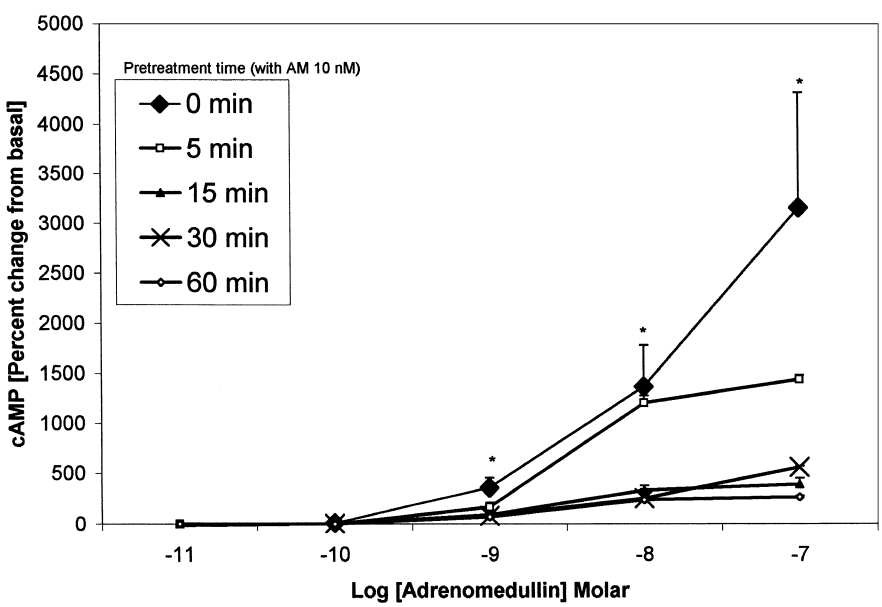

Fig. 1. Time-dependent desensitization of adrenomedullin-sensitive receptor in rat mesangial cells: rat mesangial cells were pretreated with 10-nM adrenomedullin for the different time periods indicated. At the end of the time periods, cells were washed thoroughly and treated again with different concentrations of adrenomedullin for 5 min in triplicate wells. Measurement of cAMP accumulation was done as described in Sec. 2.3. Adrenomedullin-sensitive receptor desensitization was evident within 5 min of pretreatment and was almost complete in 60 min ($n = 3$). $^{*}P < 0.01$ compared to the 15, 30, and 60 min pretreatment periods.

were added to the wells and incubation continued for an additional 5 min at 37°C. Reactions were stopped by adding 50 μl of 100% trichloroacetic acid. The cells in trichloroacetic acid were collected in separate tubes and centrifuged. The supernatants were collected after brief centrifugation and ether extracted three times with water-saturated ether. After overnight evaporation of the ether, a portion of the sample was used for measurement of cAMP levels, using a radio-immunoassay kit purchased from PerSeptive Biosystems (Framingham, MA).

2.4. Measurement of inositol phosphate accumulation

The assay for inositol phosphate accumulation was done as described before (Oppermann et al., 1996). Briefly, cells were plated in 24 well plates (50,000 cells/well) and grown for 2 days prior to serum starvation and treatment with [^{3}H] *myo*-inositol for 16–18 h. On the day of the experiment, cells were washed and preincubated with 10 mM LiCl for 10 min at 37°C. Agonists were added at the indicated concentrations and the reaction was stopped by adding 50 μl of 100% trichloroacetic acid. The cells were centrifuged, the supernatants collected and ether extracted and neutralized with 1 M Tris base. Total [^{3}H] inositol phosphates were separated from the free [^{3}H] *myo*-inositol using ion exchange column chromatography following the procedure of Cotecchia et al. (1992) and counted in Beckman LS counter.

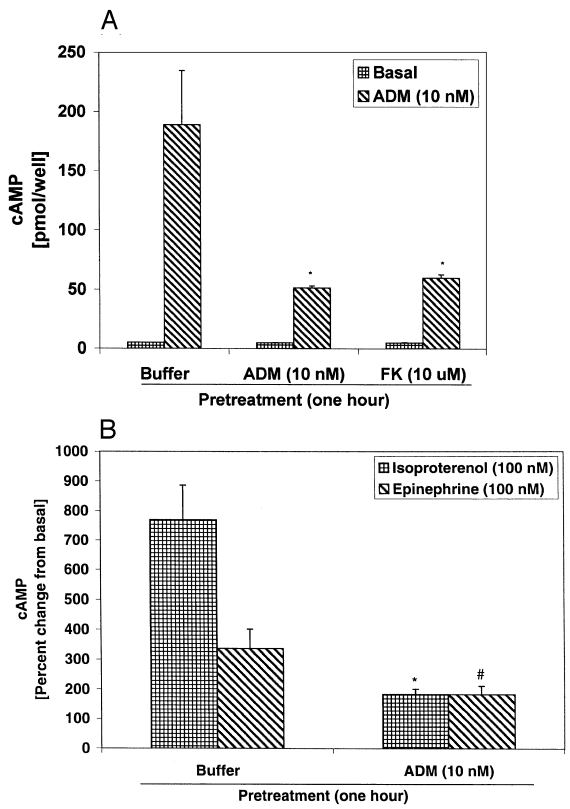

Fig. 2. (A) Rat mesangial cells were pretreated with buffer, adrenomedullin (10 nM) or forskolin (10 μM) for 1 h. At the end of the pretreatment period, cells were washed thoroughly and treated without (basal) or with adrenomedullin (10 nM) for 5 min. Measurement of cAMP accumulation was done as described in Sec. 2.3 ($n = 3$). Forskolin induced adrenomedullin receptor desensitization suggesting PKA-mediated event. $^{*}P <$ 0.01 compared to pretreatment with buffer alone. (B) Rat mesangial cells were pretreated with buffer alone or with adrenomedullin (10 nM) for 1 h. At the end of the pretreatment period, cells were washed thoroughly and treated without (basal) or with isoproterenol (100 nM) or epinephrine (100 nM) for 5 min. Measurement of cAMP accumulation was done as described in Sec. 2.3 ($n = 3$). Isoproterenol and epinephrine significantly induced cAMP response in rat mesangial cells. This effect was attenuated in cells pretreated with adrenomedullin ($n = 3$). $^{*}P < 0.01$ compared to pretreatment with buffer alone for isoproterenol; $\#P < 0.05$ compared to pretreatment with buffer alone for epinephrine.

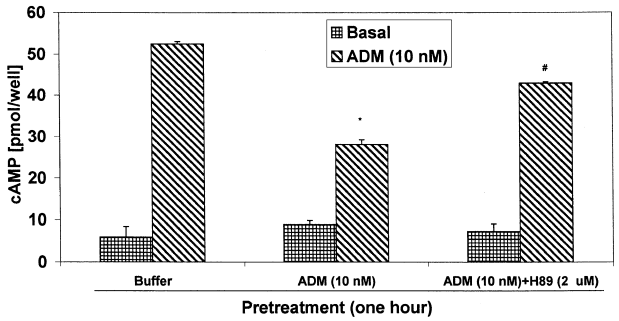

Fig. 3. Rat mesangial cells were pretreated with buffer alone or with adrenomedullin (10 nM) or with adrenomedullin (10 nM) + protein kinase A inhibitor [H89 (2 μM)]. At the end of the pretreatment period, cells were washed thoroughly and treated without (basal) or with adrenomedullin (10 nM) for 5 min. Measurement of cAMP accumulation was done as described in Sec. 2.3 ($n = 3$). Inhibition of protein kinase-A with H89 reversed the desensitization effect of adrenomedullin pretreatment. $^{*}P <$ 0.01 compared to pretreatment with buffer alone. $\#P < 0.01$ compared to pretreatment with adrenomedullin.

2.5. Desensitization protocols

The cells were plated as described for cAMP and inositol phosphate measurements. For desensitization, the cells were washed first with 1 ml Dulbecco's phosphate buffered saline containing 0.1% glucose, 0.2% bovine serum albumin, and 10 mM $MgCl_2$ (DPBS + +). These cells were then treated with adrenomedullin or forskolin or other compounds as indicated at 37°C for the indicated time points. At the end of treatment, the cells were washed four times with DPBS + +. Then, cAMP or inositol phosphate measurements in response to different agents were done as explained before.

2.6. Resensitization protocols

The resensitization protocols were similar to desensitization protocols up to washing the cells four times with DPBS + +. That is, after the 1-h treatment period (see Desensitization protocols) with different agents, cells were washed four times with DPBS + +. The cells were then

incubated with DPBS + + with or without adrenomedullin or okadaic acid for a period of 2 h at 37°C. cAMP measurements were done as described above after the incubation period.

3. Results

3.1. Desensitization of adrenomedullin-sensitive receptor in rat mesangial cells

Adrenomedullin-sensitive receptor in rat mesangial cells underwent rapid desensitization, which was evident as early as 5 min of adrenomedullin pretreatment. The desensitization was almost complete by 30–60 min after treatment with adrenomedullin (Fig. 1). The cAMP accumulation in response to 100 nM adrenomedullin in control cells without any pretreatment was 3162 ± 1149% increase over basal, while after 1 h, it was reduced to 270 ± 14.9% increase. In addition, forskolin pretreatment, which increases protein kinase-A activity by directly activating adenylate cyclase, also caused desensitization of adrenomedullin-stimulated adenylate cyclase activity (Fig. 2A). Adrenomedullin pretreatment also caused heterologous desensitization of isoproterenol- and epinephrine-induced adenylate cyclase activity (Fig. 2B). Furthermore, pretreatment of mesangial cells with H89 [{*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, hydrochloride}], an inhibitor of cAMP-dependent protein kinase significantly inhibited adrenomedullin-mediated adrenomedullin-sensitive receptor desensitization suggesting that adrenomedullin-mediated desensitization involved at least in part, activation of cAMP-dependent protein kinase (Fig. 3). Surprisingly, adrenomedullin pretreatment of mesangial cells also resulted in desensitization of endothelin-stimulated inositol phosphate accumulation (Fig. 4).

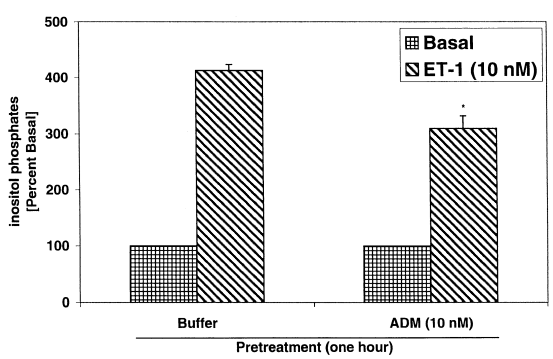

Fig. 4. Rat mesangial cells were pretreated with buffer or with adrenomedullin (10 nM) for 1 h. At the end of the treatment period, cells were washed thoroughly and treated without (basal) or with endothelin-1 (10 nM) for 10 min. Measurement of inositol phosphate accumulation was done as described in Sec. 2.4 ($n = 3$). Adrenomedullin pretreatment caused desensitization of endothelin-1-induced inositol phosphate accumulation. $^{*}P < 0.05$ compared to buffer pretreatment.

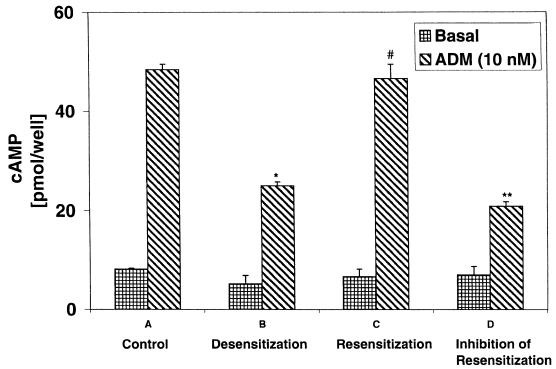

Fig. 5. Rat mesangial cells were treated with buffer alone (A) or with adrenomedullin (10 nM) (B, C, D) for 1 h. The cells were then thoroughly washed and then treated with the following for 2 h: buffer alone (A and C), buffer with adrenomedullin (10 nM) (B), buffer with okadaic acid (125 nM) (D). At the end of the second treatment period, the cells were thoroughly washed and were treated with buffer alone (basal) or adrenomedullin (10 nM) for 5 min. Measurement of cAMP accumulation was done as described in Sec. 2.3. As shown in the figure, adrenomedullin pretreatment induced desensitization of cAMP response to adrenomedullin (B). Treatment with buffer (without adrenomedullin) for 2 h, (after the 1-h treatment with adrenomedullin), resensitized the cAMP response back to original levels (C). However, treatment with okadaic acid for 2 h (after the 1-h treatment with adrenomedullin) completely abolished the resensitization ($n = 3$). Note that the basal cAMP levels are not significantly different from one another. $^{*}P < 0.01$ compared to (A). $\#P < 0.01$ compared to (B). $^{**}P < 0.01$ compared to treatment with (A) and (C).

3.2. Resensitization of adrenomedullin-sensitive receptor in rat mesangial cells

We hypothesized that if the desensitization of adrenomedullin-sensitive receptor is mediated by the activation of cAMP-dependent protein kinase and serine/threonine phosphorylation, then dephosphorylation will result in resensitization. In fact, after 1 h pretreatment with adrenomedullin, the cells were washed thoroughly and incubated with buffer alone, for an additional 2 h. This resulted in the reversal of the attenuated cAMP response due to adrenomedullin-sensitive receptor desensitization (Fig. 5). This process of recovery, also called resensitization, was totally abolished by exposing the cells to okadaic acid, a serine/threonine phosphatase inhibitor, indicating that the activation of serine/threonine phosphatases was critical for the resensitization process.

4. Discussion

Cell surface receptors exhibit a phenomenon of desensitization where in the exposure to agonist ligand leads to a diminished response for a subsequent challenge of the agonist. This property is well known for heterotrimeric G protein-coupled receptors and has been extensively charac-

terized for β_2-adrenoceptors (Hausdorff et al., 1990; Bohm et al., 1997). Attenuation of signals can occur at the level of the agonist (wherein the agonist is made inactive), the receptor, the G protein and at numerous downstream signaling pathways. In the case of β_2-adrenoceptors, after the first few minutes of agonist treatment, uncoupling of the receptor from G_s occurs by either protein kinase-A- or β-adrenergic receptor kinase (βARK)-mediated phosphorylation of receptors (Hausdorff et al., 1990; Bohm et al., 1997). The present study demonstrates that the adrenomedullin-sensitive receptor in mesangial cells, is similarly rapidly desensitized. The desensitization is dependent on the activation of protein kinase-A because (a) forskolin pretreatment causes desensitization of adrenomedullin-induced cAMP response and; (b) H89, a potent protein kinase-A inhibitor, inhibits adrenomedullin-mediated desensitization of cAMP response. These data strongly suggest that the adrenomedullin-sensitive receptor desensitization is mediated through protein kinase-A.

A recent study in vascular smooth muscle cells by Iwasaki et al. (1998b) indicated that desensitization of adrenomedullin-induced cAMP response was independent of protein kinase-A, protein kinase-C, tyrosine kinase and receptor sequestration suggesting that the desensitization was homologous. Different mechanisms of desensitization are known to occur in different cell systems even for the same receptor. For example, recombinant β_2-adrenoceptors expressed in Chinese hamster ovarian cells and the muscle β-adrenoceptor required β-adrenergic receptor kinase-1 for their desensitization, while β-adrenoceptor in osteosarcoma cells mediated their desensitization through the activation of protein kinase-A. In human epidermoid carcinoma cells, both protein kinase-A- and β-adrenergic receptor kinase-1 were involved (Shih and Malbon, 1994). The difference in both the type and mechanism of adrenomedullin receptor desensitization between rat mesangial cells and vascular smooth muscle cells could be due to the differences in the cell types. Also, the signaling mechanisms of adrenomedullin appears to be different in the preparation of smooth muscle cells as reported by Iwasaki et al. (1998a). Iwasaki et al. (1998b) recently showed that in vascular smooth muscle cells, adrenomedullin actually increased mitogen-activated protein (MAP) kinase activity through a protein kinase-A-independent mechanism, whereas in mesangial cells, adrenomedullin actually decreased MAP kinase activity through a protein kinase-A-dependent mechanism (Parameswaran et al., 1999). These results suggest that a different subtype of adrenomedullin-sensitive receptor and/or different signaling components are involved in these two cell types.

Receptor endocytosis depletes the membrane of high affinity receptors, which is important for both desensitization and resensitization. Also well established for β_2-adrenoceptors is the process of both desensitization and resensitization, which involves phosphorylation and dephosphorylation mechanisms, respectively (Ferguson and Caron, 1998; Ferguson et al., 1998). The process of resensitization depends on the type of receptor because protease-activated receptor resensitization is dependent on the mobilization of golgi stores and synthesis of new receptors and their transport to the plasma membrane (Bohm et al., 1996). In case of β_2-adrenoceptors, resensitization is brought about by dephosphorylation by plasma and vesicular membrane-associated form of protein phosphatase-2A (Krueger et al., 1997). In rat mesangial cells, adrenomedullin receptor resensitization appears to depend on okadaic acid-sensitive phosphatase activity (probably protein phosphatase-2A). We have found that adrenomedullin can increase protein phosphatase-2A activity in rat mesangial cells (Parameswaran et al., 2000). These results suggest that serine/threonine phosphorylation (by protein kinase-A) is important for desensitization of cAMP response by adrenomedullin while the dephosphorylation of these residues leads to the resensitization response.

Adrenomedullin also caused desensitization of endothelin-1-stimulated inositol phosphate accumulation suggesting that adrenomedullin may cause cross-desensitization of phospholipase-c activity. Activation of protein kinase-A has been shown to cause both heterologous desensitization of endothelin receptor and cross desensitization of G protein-regulated phospholipase-c activity (Galas and Harden, 1996; Takemoto et al., 1995). Further experiments will be necessary to establish the mechanism of desensitization elicited by adrenomedullin on endothelin-stimulated inositol phosphate accumulation. These results raise an interesting possibility that the pathophysiological role played by endothelin in diseases like proliferative glomerulonephritis could be attenuated by adrenomedullin by causing desensitization of endothelin-mediated cellular responses.

In summary, we have demonstrated for the first time in rat mesangial cells that adrenomedullin-stimulated cAMP response undergoes rapid desensitization through a protein kinase-A-dependent mechanism and also that the desensitization is heterologous in nature. We have also shown here for the first time that the desensitized adrenomedullin-sensitive receptor undergoes resensitization on removal of the agonist (adrenomedullin), by a putative dephosphorylation mechanism involving serine/threonine phosphatases. These results raise a number of issues regarding the pathophysiological significance of elevated plasma levels of adrenomedullin in a variety of diseases including cardiorenal disorders.

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