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Effect of adrenomedullin on hepatic pericytes (stellate cells) of the rat

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Abstract When hepatic stellate cells (Ito cells, fat-storing cells) were incubated with adrenomedullin, they underwent relaxation as monitored by the silicone-rubber membrane method; 43%, 65% and 87% of stellate cells relaxed 5, 10 and 20 min, respectively, after addition of 10^{-6} M adrenomedullin. Adrenomedullin also triggered the dissociation of F-actin and induced transformation of stellate cells to dendritic cell-like structure. When incubated with 10^{-6} M of adrenomedullin for 30 min, cellular levels of cAMP increased from the basal value of 10.2 ± 1.4 to 107 ± 2.8 pmol/2×10^5 cells without affecting cGMP levels. The reaction occurred dose-dependently and was inhibited by an antagonist of calcitonin gene-related peptide. Adrenomedullin had negligible effects on DNA and protein synthesis in proliferating stellate cells. Thus, adrenomedullin is a potent relaxing peptide to hepatic stellate cells and may contribute to the regulation of sinusoidal microcirculation.

Key words: Stellate cell; Sinusoidal microcirculation; Adrenomedullin; Calcitonin gene-related peptide

1. Introduction

Recent studies revealed that stellate cells (fat-storing cells, Ito cells) play important roles in the pathogenesis of liver disease. These cells storage retinyl palmitate and deliver retinol to extrahepatic tissues. These cells synthesize extracellular matrix proteins and play a role in fibrotic scar formation in chronic hepatitis [1,2]. Since stellate cells have abundant contractile filaments such as desmin [3] and α-smooth-muscle actin [4] and encompass the contraluminal surface of sinusoidal endothelial cells with their long branching cytoplasmic processes [5–7], the contraction of these cells has been postulated to regulate the sinusoidal microcirculation [8–11]. In fact, when exposed to vasoactive compounds, such as eicosanoids [8,9], endothelin-1 [10], nitric oxide [10] and substance P [11], cultured stellate cells undergo reversible contraction. Recently, contraction of sinusoidal lumen was reported in endothelin-challenged rats particularly at the sites of stellate cells localization [12,13].

Adrenomedullin is a novel peptide isolated from human pheochromocytoma; the peptide has activity to increase cAMP levels in platelets and to induce hypotension [14]. The amino acid sequence of adrenomedullin has little homology to that of calcitonin gene-related peptide (CGRP) which functions as neuropeptide [15]. Adrenomedullin is abundant in adrenal medulla and is widely distributed in human tissues [16]. Since adrenomedullin has recently been shown to occur in endothelial cells, the peptide may be classified as endothelium-derived relaxing factors. Considerably high concentration of adrenomedullin was found to exist in the blood. Thus, adrenomedullin has been postulated to function as a circulating hormone that regulates vascular tonus [17,18,19].

The present study reports for the first time that adrenomedullin induces strongly relaxation of hepatic stellate cells by some cAMP-dependent mechanism.

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Abbreviations: CGRP, calcitonin gene-related peptide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GBSS, Gey's balanced salt solution; TRITC, tetramethylrhoadamine isothiocyanate.

2. Materials and methods

2.1. Chemicals

Pronase E was purchased from Merck (Darmstadt, Germany). DNase was from Boehringer Mannheim (Mannheim, Germany). Collagenase was from Wako Pure Chemical Co. (Osaka). Rat adremomedullin, rat CGRP and human CGRP(8–37) were from Peptide Institute Inc. (Osaka). Kits for the determination of cAMP and cGMP were from Amersham (Bucks., UK). Tetramethylrhodamine isothiocyanate (TRITC)-phalloidin and 3-isobutyl-1-methyxanthine (IBMX) were from Sigma (St. Louise, MO).

2.2. Preparation of stellate cells

Male Wistar rats, 450–500 g, were obtained from SLC (Shizuoka) and fed laboratory chow and water ad libitum. Stellate cells were prepared from the liver as described [8]. Briefly, the liver was perfused with a Ca^{2+}/Mg^{2+}-free Gey's balanced salt solution (GBSS) for 10 min at 37°C (10 ml/min) followed by collagenase digestion for 30 min at 37°C. The digested liver was excised, cut into small pieces and incubated in GBSS containing 0.1% pronase E and 20 μg/ml of DNase for 30 min at pH 7.3. The resulting suspension was filtered through a nylon mesh (150 μm in diameter). After centrifugation of the filtrate on an 8.2% Nycodenz cushion ($1,400 \times g$, 4°C, 20 min), a stellate-cell-enriched fraction was obtained. The cells in the upper-whitish layer were washed by centrifugation ($400 \times g$ at 4°C for 10 min) and cultured in DMEM (Gibco) supplemented with 10% fetal-bovine serum (FBS, Flow) and antibiotics (10^5 U/l penicillin G and 100 mg/l streptomycin), and used for experiments.

2.3. Detection of cell relaxation by silicone rubber membrane method

Silicone-rubber sheets were prepared as described elsewhere [8,10,20]. Isolated stellate cells were plated on silicone rubber sheets in 1.5 ml culture medium and cultured for 5 days. After adding various concentrations of adrenomedullin, wrinkle formation was monitored under phase-contrast microscopy (IMT-2, Olympus) using a video-camera system (FCD-10, Olympus). The extent of relaxation was determined by counting the decrease in the number of wrinkles around the cells observed.

2.4. Observation of cell shape and F-actin organization

Stellate cells in primary culture for 5 days were incubated with various concentrations of adrenomedullin for indicated intervals. Morphological change in stellate cells was continuously observed under a phase-contrast microscope equipped with a video-camera system. In some cases, cells were fixed in 3.7% formaldehyde and observed at high magnification using Zeiss inverted microscope. For the staining of F-actin, formaldehyde-fixed cells were permeabilized by 0.1% Triton X-100, followed by incubation with 0.5 μM TRITC-phalloidin. After washing, the cell-associated F-actin was observed under fluorescent microscopy.

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2.5. Determination of cellular level of cAMP and cGMP

The cultured stellate cells were washed twice with GBSS. After incubation at 37°C for 10 min with 1 ml of GBSS containing 0.1% albumin and 0.5 mM IBMX, the cells were added various concentrations of test agents. The reaction was terminated by adding 5% (m/v) trichroloacetic acid. cAMP and cGMP contents in the extracts were measured by radioimmunoassay using cAMP or cGMP assay kits.

2.6. Determination of cell proliferation and protein synthesis

Four-day-old stellate cells were cultured in DMEM in the absence of serum for 48 h. Then they were incubated with adrenomedullin in the presence of 5 μCi/ml of [^{3}H]thymidine or [^{3}H]leucine in DMEM with or without serum. After 24 h of incubation, the cells were washed 3 times with GBSS and treated with 5% (m/v) trichroloacetic acid. Radioactivity in acid-insoluble fractions was determined by a liquid-scintillation counter.

3. Results and discussion

3.1. Effect of adrenomedullin on the contractility of stellate cell

Previous studies demonstrated that the silicone-rubber membrane method is excellent to detect the contractile response of stellate cells to various vasoactive substances; increase or decrease in the number of wrinkles formed on the membranes indicates development or reduction, respectively, of cellular contraction. When exposed to endothelin-1, a thromboxane A2 analogue, prostaglandin F2α and nucleotides such as ATP and UTP, stellate cells undergo contraction. On the contrary, relaxation of cells was induced by a prostacycline analogue, prostaglandin E2 and donors of nitric oxide [8,11]. By using this method, the present study was performed to elucidate the effect of adrenomedullin on stellate cell contraction. Adrenomedullin hardly induced the cell contraction. However, at concentrations higher than 10^{-8} M, adrenomedullin triggered the relaxation of stellate cells that had been contracted (Fig. 1). Since prostacyclin-dependent relaxation occurred at ligand concentrations higher than 5×10^{-7} M, the relaxing activity of adrenomedullin might be stronger than that of prostacyclin, indicating that adrenomedullin is the most potent relaxant for the cells. Relaxation was detectable as early as 3 min after addition of 10^{-6} M adrenomedullin; 43%, 65% and 86% of precontracted stellate cells underwent relaxation at 5, 10 and 20 min, respectively, after addition of 10^{-6} M adrenomedullin. Interestingly, cell relaxation continued until the removal of adrenomedullin from the incubation medium. Thus, adrenomedullin is a potent and long-acting relaxant for stellate cells in primary culture.

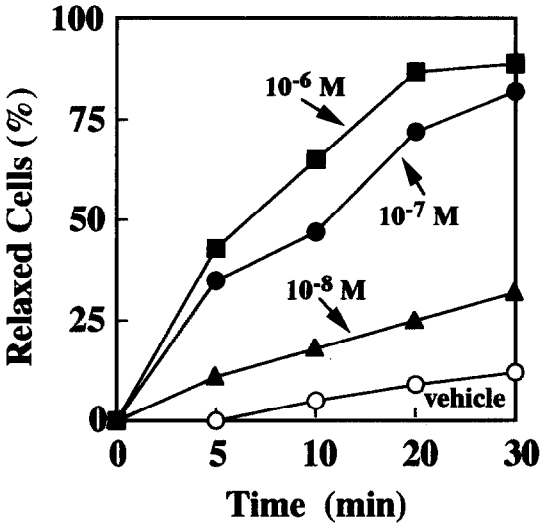

Fig. 1. Effect of adrenomedullin on stellate cell relaxation. Stellate cells were cultured on silicone-rubber films for 4 days. Then the cells were allowed to react with adrenomedullin (10^{-8} to 10^{-6} M) or vehicle (1% GBSS) for 30 min. Increased or decreased wrinklings of spontaneously pre-contracted stellate cells were monitored under phase-contrast microscopy connected to video-camera system. The relaxation of stellate cells was determined by counting the decrease in the number of wrinkles around the cells. Reaction of at least fifty cells was observed for each treatment.

3.2. Changes in cell shape and F-actin fibers

As reported previously, cell relaxation was frequently accompanied by a dendritic transformation of cell shape and structural change in F-actin fibers [8,11]. As shown in Fig. 2A, four-day-cultured stellate cells had numerous lipid droplets and enlarged cell bodies with membranous processes. These cells expressed 'stress fibers' of F-actin as revealed by TRITC-phalloidin staining (Fig. 2E). When exposed to 10^{-6} M of adrenomedullin, the size of cells decreased and their shape transformed to star-like configuration with dendritic processes (Fig. 2B–D). F-Actin in these dendritic cells showed spotty granule- or pliant fiber-like structure instead of stress fibers (Fig. 2F). At concentrations higher than 10^{-7} M, adrenomedullin showed similar effect. Stellate cells exhibited such dendritic cell structure for at least 2 h after adding the agonist and returned to the initial shape with stressed-configuration at about 8 h later.

3.3. Determination of cellular levels of cAMP and cGMP

Our previous studies demonstrated that stellate cell contraction was mediated by the increased metabolism of inositol phosphates and intracellular calcium concentrations while their relaxation was induced by the elevation of cellular levels of cAMP or cGMP [11]. As adrenomedullin induced relaxation of stellate cells, cellular levels of cAMP and cGMP were determined in the presence of 0.5 mM IBMX. cAMP concentration in non-stimulated stellate cells was 10.2 ± 1.4 pmol/2×10^5 cells ($n = 4$). Their levels increased dose- and time-dependently and reached to 107 ± 2.8 pmol/well ($n = 4$) in the presence of 10^{-6} M adrenomedullin for 30 min (Fig. 3). EC_{50} was 8.4×10^{-8} M.

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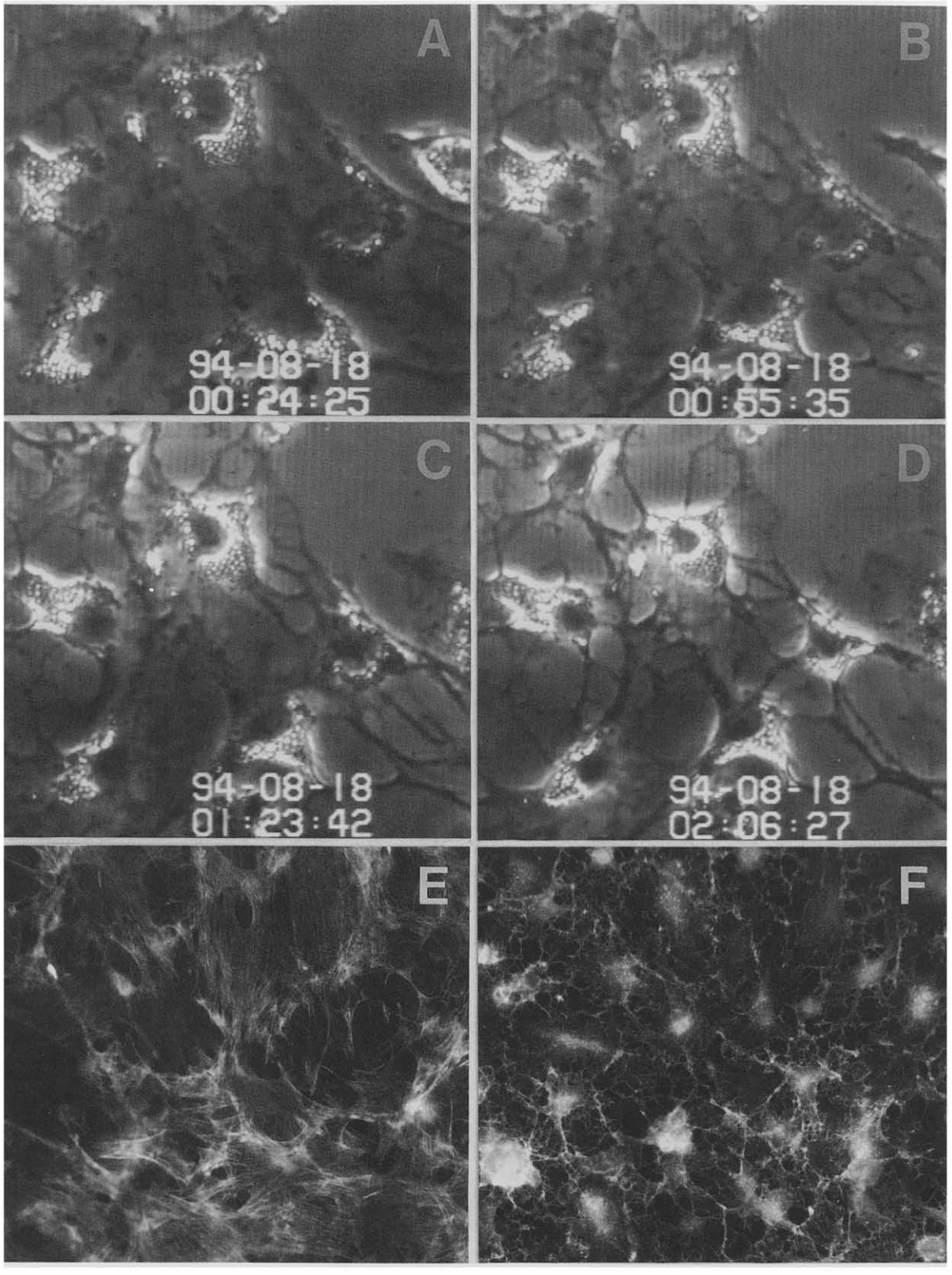
Fig. 2. Effect of adrenomedullin on the transformation of stellate cell shape and F-actin conformation. (A–D) Stellate cells cultured on uncoated plastic dish for 4 days in 10% FBS/DMEM were incubated with 10^{-6} M of adrenomedullin for indicated times. Morphological change in stellate cells was continuously observed under a phase-contrast microscope equipped with video-camera system. (A) Basal tonic state. The cells had numerous lipid droplets and showed membranous processes. (B) At about 30 min after addition of adrenomedullin. (C) At about 60 min. The cells exhibited dendritic shape. (D) At about 100 min. Note that the cells had long and branched cytoplasmic processes. (E and F) Stellate cells before and after treatment with 10^{-6} M adrenomedullin were fixed with formaldehyde and incubated with 0.5 μM TRITC-phalloidin. The cell-associated F-actin was observed under fluorescent microscopy. (E) Stellate cells without treatment. The cells showed stress fibers of F-actin. (F) Stellate cells treated with adrenomedullin for 45 min. F-actin showed spotty granule- or pliant fiber-like structure instead of stress fibers.

Interestingly, CGRP mimicked the effect of adrenomedullin. On the other hand, cellular cGMP levels in the same samples remained unchanged after adding adrenomedullin and CGRP (data not shown).

3.4. Effect of CGRP[8–37] on cell relaxation and cAMP accumulation in adrenomedullin-treated stellate cells

The effect of CGRP(8–37), a CGRP antagonist, on stellate cell relaxation was studied by using silicone-rubber-membrane



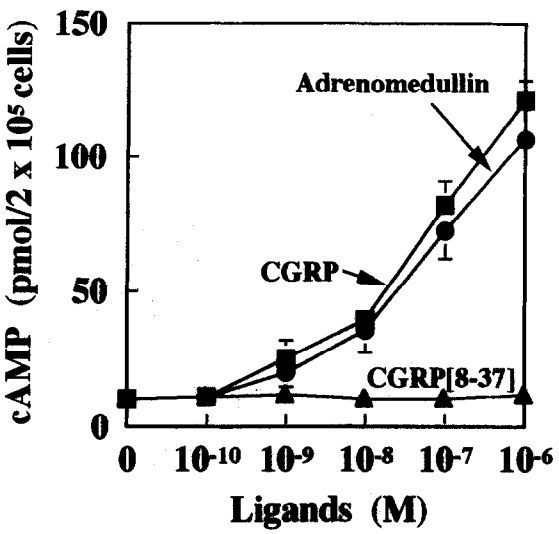


Fig. 3. Effect of adrenomedullin on cellular level of cAMP. The cultured stellate cells were incubated with various concentrations of adrenomedullin, CGRP or CGRP(8–37) for 30 min in the presence of 0.1% albumin and 0.5 mM IBMX. The reaction was terminated by addition of 5% trichloroacetic acid. Level of cAMP in the extracts was determined by using radioimmunoassay. Mean ± S.E.M. of 4 different experiments was expressed.

method. As shown in Fig. 4, while CGRP(8–37) itself had negligible effects on cell motility, 1×10^{-5} M of CGRP(8–37) remarkably attenuated the effect of adrenomedullin. In addition, CGRP(8–37) dose-dependently inhibited the increase of cAMP level induced by adrenomedullin with IC_{50} value of 3.3×10^{-7} M (Fig. 5). CGRP(8–37) also inhibited the increase in cAMP level induced by CGRP (data not shown) with IC_{50}

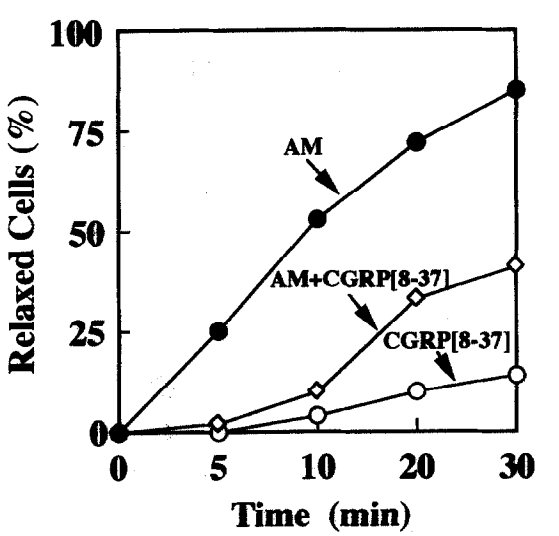


Fig. 4. Effect of CGRP(8–37) on adrenomedullin-induced stellate cell relaxation. Stellate cells were cultured on silicone-rubber films for 4 days. Then the cells were allowed to react with 10^{-7} M of adrenomedullin in the presence or absence of 10^{-5} M of CGRP(8–37) for 30 min. Increased or decreased wrinklings of spontaneously pre-contracted stellate cells were monitored under phase-contrast microscopy connected to video-camera system. The relaxation of stellate cells was determined by counting the decrease in the number of wrinkles around the cells. Reaction of at least fifty cells was observed for each treatment. AM, adrenomedullin.

value of 2.4×10^{-6} M. These results indicate that the relaxing effect of adrenomedullin may be mediated by cAMP.

3.5. *Effect of adrenomedullin on proliferation and protein synthesis of stellate cells*

When cultured in the presence of serum, stellate cells undergo transformation to myofibroblast-like cells. Myofibroblast-like cells start to proliferate and produce large amount of extracellular matrix proteins [21]. Some growth factors in the serum, such as platelet-derived growth factor and epidermal growth factor, have been postulated to trigger such transformation. In the present study, effect of adrenomedullin on the proliferation and the protein synthesis in stellate cells was analyzed. Serum-starved stellate cells that were cultured in the absence of FCS for 48 h incorporated 3114 ± 217 cpm/2 × 10^5 cells/24 h ($n = 4$) of [^{3}H]thymidine. This value did not change significantly when they were incubated with 10^{-6} to 10^{-10} M of adrenomedullin. When stellate cells were cultured in DMEM supplemented with 10% FCS, the amount of incorporated [^{3}H]tymidine increased to 12,327 ± 478 cpm/2 × 10^5 cells/24 h ($n = 4$). Adrenomedullin again failed to affect [^{3}H]thymidine uptake by these serum-stimulating stellate cells. Thus, adrenomedullin seemed to have negligible effects on stellate cell proliferation. In addition, adrenomedullin also failed to affect [^{3}H]leucine uptake by stellate cells.

3.6. *Concluding remarks*

The present work revealed that adrenomedullin strongly induced relaxation of stellate cells by some cAMP-dependent mechanism without affecting proliferation and protein synthesis. CGRP mimicked the effect of adrenomedullin. Since the CGRP antagonist inhibited cAMP accumulation induced by adrenomedullin, the two peptides might share the same receptors on stellate cells. Since considerably high concentration of adrenomedullin is present in the blood [16,17], it might function as a circulating hormone that regulates contraction of stellate

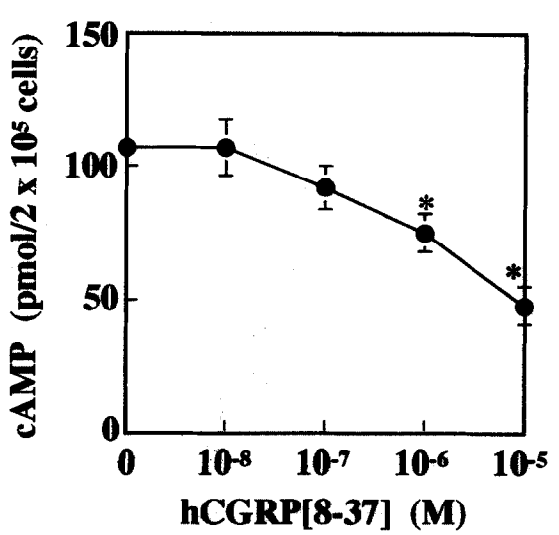


Fig. 5. Effect of CGRP receptor antagonist on adrenomedullin-induced cAMP formation in stellate cells. Stellate cells were incubated with 10^{-7} M adrenomedullin in the presence or absence of CGRP(8–37) in concentrations as indicated for 30 min. The reaction was terminated by addition of 5% trichloroacetic acid. Level of cAMP in the extracts was determined by using radioimmunoassay. Mean ± S.E.M. of four different experiments was expressed. *$P < 0.05$ vs. control.

cells, thereby controlling sinusoidal microcirculation. Pathophysiological roles of adrenomedullin and its receptors on stellate cells are under our current investigation. The extension of the studies now adds adrenomedullin as a novel regulator of sinusoidal microcirculation and stellate cell function.

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