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Adrenomedullin increases phosphatidylcholine secretion in rat type II pneumocytes

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Abstract

Adrenomedullin, a novel hypotensive peptide, has been reported to be produced in the lung as well as in the adrenal medulla. However, the effect of adrenomedullin on lung function is still poorly understood. In this study, we detected the expression of both adrenomedullin mRNA and putative adrenomedullin receptor mRNA in primary cultures of rat type II pneumocytes. Adrenomedullin increased the secretion of phosphatidylcholine, the predominant component of pulmonary surfactant, by type II pneumocytes. The increase was partly inhibited by pretreatment with the calcitonin gene-related peptide (CGRP) receptor antagonist CGRP-(8-37). Furthermore, the increased phosphatidylcholine secretion was significantly inhibited by several protein kinase C inhibitors, such as sphingosine, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]3-(1*H*-indol-3-yl) maleimide (Gö6983), 3-[1-(3-amidinothio)-propyl-1*H*-indoyl-3-yl]3-(1-methyl-1*H*-indoyl-3-yl) maleimide methane sulfonate (Ro-31-8220), and staurosporine. Our results suggest that adrenomedullin can be considered a candidate autocrine modulator of surfactant secretion in type II pneumocytes.

Keywords: Pneumocyte (type II); Phosphatidylcholine secretion; Adrenomedullin; Surfactant; Protein kinase C

1. Introduction

Adrenomedullin, a novel vasorelaxant peptide, was originally isolated from a human adrenal pheochromocytoma during the process of looking for agents with the ability to increase adenosine 3′,5′-cyclic monophosphate levels (Kitamura et al., 1993a). Adrenomedullin shares homologous structures with calcitonin gene-related peptide (CGRP) and is as potent a vasodilator as CGRP. Under physiological conditions, this peptide is produced in adrenal medulla, heart, kidney, and lung (Kitamura et al., 1993b). However, the functional role of adrenomedullin in the lung is unclear. In type II pneumocytes, the abundant expression of the cloned adrenomedullin receptor has recently been elucidated by in situ hybridization (Martinez et al., 1997). In addition, the plasma adrenomedullin concentration in asthmatic patients is higher than that in normal subjects (Kohno et al., 1996). Further, circulating adrenomedullin is removed when it passes through the pulmonary vascular bed (Yoshibayashi et al., 1994). These findings lead to the speculation that adrenomedullin may have a role in type II pneumocytes.

Type II pneumocytes produce lung surfactant which reduces the surface tension of the alveolar air–liquid interface, thereby providing mechanical stability and preventing alveolar atelectasis (Chander and Fisher, 1990). In addition to this vital role, lung surfactant also has important roles in stimulating macrophage phagocytosis (Mariencheck et al., 1999) and in facilitating and maintaining airway mucociliary clearance (Kai et al., 1989; Rubin et al., 1992). Lung surfactant consists mainly of proteins and lipids, of which phosphatidylcholine is the predominant lipid. Phosphatidylcholine secretion is increased by a variety of intrinsic physiological agents, including histamine (Chen and Brown, 1990), leukotrienes (Gilfillan and Rooney, 1986), glucagon-like peptide-1 (Benito et al., 1998), and eosinophil granule major basic protein (Okumura et al.,

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1999), as well as pharmacological agents (Chander and Fisher, 1990; Kai et al., 1992; Okumura et al., 1995).

Therefore, we examined whether adrenomedullin and its receptor mRNA are expressed in primary cultures of rat type II pneumocytes and, if so, whether adrenomedullin affects phosphatidylcholine secretion from type II pneumocytes.

2. Materials and methods

2.1. Animals and chemicals

Rats were purchased from Kyudo Farm (Fukuoka, Japan), tissue culture medium was from Nissui Pharmaceutical (Tokyo, Japan), and fetal bovine serum was from JRH Bioscience (Lenexa, KS, USA). [Methyl-^{3}H]choline and Aquasol II (*P*-xylene) were obtained from NEN Research Products (Boston, MA, USA). Rat adrenomedullin and CGRP were obtained from Peptide Institute (Osaka, Japan). Rat CGRP-(8-37) was obtained from Peninsula Laboratories (Belmont, CA, USA); while 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo [2,3-*a*]pyrrolo-[3,4-*c*]carbazole (Gö6976), 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]3-(1*H*-indol-3-yl) maleimide (Gö6983), and 3-[1-(3-amidinothio)-propyl-1*H*-indoyl-3-yl]3-(1-methyl-1*H*-indoyl-3-yl) maleimide methane sulfonate (Ro-31-8220) were obtained from Calbiochem Novabiochem (San Diego, CA, USA). Other reagents and biochemicals were purchased from Sigma (St. Louis, MO, USA). The Ethics Review Committees for Animal Research of Miyazaki Medical College and the Faculty of Pharmaceutical Sciences, Kumamoto University, approved the experimental protocol.

2.2. Extraction of RNA

RNA was extracted from adrenal, liver and lung tissue, and from type II pneumocytes using the acid–guanidinium–phenol–chloroform protocol (Chomczynski and Sacchi, 1987). The RNA pellets were dissolved in diethylpyrocarbonate-treated water and the yield of RNA was quantified by spectrophotometry at 260 nm. The purity of all samples was more than 1.8 (OD_{260}/OD_{280} ratio). Samples were aliquoted and stored at -20°C until further processing.

2.3. Reverse transcription–polymerase chain reaction (RT–PCR) and the analysis of PCR products

RT was performed with 1 μg of total RNA using random primers and avian myeloblastosis virus transcriptase (Takara Shuzo, Kyoto, Japan). For rat adrenomedullin, amplification was performed with 40 cycles of 30 s at 94°C for denaturation, 30 s at 55°C for annealing, and 1 min at 72°C for extension. The specific primer set used for rat adrenomedullin was 5′-TGG GTT CGC TCG CCG TTC TCG-3′ (sense) and 5′-CGT CCT TGT CTT TGT CTG TAA-3′ (antisense) (So et al., 1996). For rat adrenomedullin receptor, amplification was performed with 40 cycles of 30 s at 94°C for denaturation, 30 s at 60°C for annealing, and 1 min at 72°C for extension. The specific primer set used for rat adrenomedullin receptor was 5′-AGC GCC ACC AGC ACC GAA TAC G-3′ (sense) and 5′-AGA GGA TGG GGT TGG CGA CAC AGT-3′ (antisense) (Montuenga et al., 1997). To compare mRNA levels, glyceraldehyde-3-phosphate dehydrogenase was amplified for reference using previously published primers (Terada et al., 1992). The PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and photographed under ultraviolet transillumination. The expression of adrenomedullin and adrenomedullin receptor mRNA was determined in triplicate experiments to avoid false-positive results due to contamination with template.

2.4. Primary cultures of rat type II pneumocytes

Type II pneumocytes were isolated from the lungs of adult specific-pathogen-free male Wistar rats (body weight, 180–200 g) according to the method of Dobbs et al. (1986a). This method yields approximately 1×10^7 cells per rat. Cells were suspended at 1×10^6 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 74 kBq/ml [methyl-^{3}H]choline (specific activity, 3.0 Tbq/mmol), 100 units/ml penicillin, and 100 μg/ml streptomycin, plated on 24-well tissue culture plates (Falcon 3047), and then cultured at 37°C in 5% CO_2-air for 18 h. Non-adherent cells were removed from the wells by washing before the assay. The number of adherent cells was approximately 1×10^5 cells/well. For cellular identification, the sample was stained with tannic acid–polychrome stain (Mason et al., 1985) and alkaline phosphatase stain (Edelson et al., 1988). The purity of the type II pneumocyte monolayer was $96 \pm 1\%$ (mean $\pm$ S.E.M., $n = 10$), and the viability was $98 \pm 1\%$ ($n = 10$) as confirmed by the Trypan blue exclusion test.

2.5. Metabolic labeling of phosphatidylcholine and treatment of cultures

Secretion of phosphatidylcholine by cultured type II pneumocytes was determined in the following way. The cultured cells were rinsed with fresh serum- and antibiotic-free medium to remove [methyl-^{3}H]choline and unattached cells. Adrenomedullin and CGRP were added 30 min after the rinse, and then, the cells were incubated for 90 min. To investigate the phosphatidylcholine secretion mechanisms, various inhibitors of intracellular pathways and CGRP-(8-37) were added 15 min before the addition of adrenomedullin or CGRP. After the incubation period, the medium was aspirated, the cells were lysed

with 2-ml ice-cold 0.05% Triton X-100 (mono-*p*-isooctylphenyl ether) solution, and the lipids were extracted from both the cells and medium using chloroform and methanol (2:1, v/v). Phosphatidylcholine was separated from other phospholipids by thin-layer chromatography (Miyata et al., 1987), and its radioactivity was measured with a liquid scintillation counter after addition of 5 ml Aquasol II to each sample. Secretion is expressed as the amount of [^{3}H]phosphatidylcholine released into the medium after the 90-min incubation, as a percentage of the total in the cells plus medium.

2.6. Detection of cell membrane damage

The presence or absence of cytoplasmic leakage due to cell membrane damage following adrenomedullin and CGRP treatment was determined by measuring lactate dehydrogenase (LDH) activity in the culture medium with a commercial LDH assay kit (Nippon Shoji). The activity of LDH released into the medium did not exceed 1% of the total cell content in all experiments (data not shown).

2.7. Statistical analysis

Phosphatidylcholine secretion is expressed as the mean ± S.E.M. Differences among groups were assessed using Dunnett's test after the analysis of variance (ANOVA), and statistically significant differences were indicated by $P < 0.05$ and 0.01.

3. Results

3.1. Expression of adrenomedullin mRNA and adrenomedullin receptor mRNA

RT–PCR was used to evaluate the expression of adrenomedullin and adrenomedullin receptor mRNA, because the level of expression was predicted to be low. The expression in rat adrenal, liver, and lung tissue was investigated as a control. All the PCR reactions amplified single products of the predicted sizes for adrenomedullin (360 bp) and adrenomedullin receptor (470 bp). Adrenomedullin mRNA and adrenomedullin receptor mRNA were observed in type II pneumocytes as well as adrenal, liver, and lung tissue (Fig. 1). Further, triplicate experiments in this study were similar to each other, and the negative control without RNA or reverse transcriptase did not form the specific bands indicative of adrenomedullin mRNA and adrenomedullin receptor mRNA.

3.2. Effects of adrenomedullin on phosphatidylcholine secretion

Adrenomedullin and CGRP increased phosphatidylcholine secretion in primary cultures of rat type II pneumocytes to approximately 2.2- and 1.8-times control levels, respectively, without increasing LDH activity in the culture medium (Fig. 2: top). The increased phosphatidylcholine secretion induced by adrenomedullin occurred in a concentration-dependent manner (1×10^{-10}–1×10^{-7} M). The kinetics of phosphatidylcholine secretion showed that secretion commenced within 5 min of the addition of adrenomedullin (1×10^{-7} M). Secretion increased sharply for the first 30 min, and there was a small increase over the remaining 60 min (Fig. 2: bottom). In addition, the secretion profile differed from that of the control, which increased progressively throughout the observation period. The profile of the control is characteristic of basal secretion.

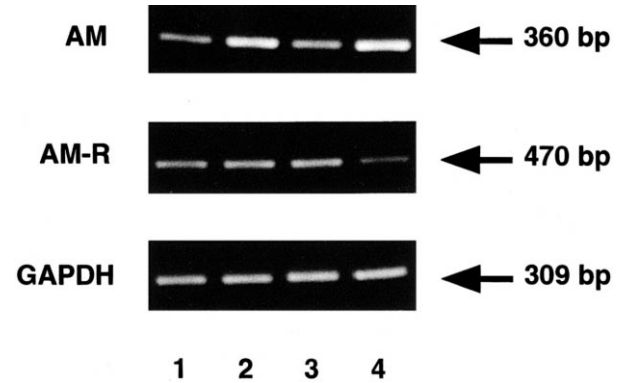

Fig. 1. Expression of adrenomedullin- and adrenomedullin receptor-mRNA in rat type II pneumocytes. RNA was also extracted from aliquots of rat adrenal medulla, liver, and lung as negative and positive controls. RT–PCR was performed three times with 1 μg of total RNA. The size of the adrenomedullin and adrenomedullin receptor product was 360 and 470 bp, respectively. Lane 1: liver; Lane 2: adrenal medulla; Lane 3: lung; Lane 4: type II pneumocytes.

3.3. Effects of CGRP receptor antagonist (CGRP-(8-37)) on the increased phosphatidylcholine secretion induced by adrenomedullin and CGRP

Pretreatment with CGRP-(8-37) dose dependently inhibited the increased phosphatidylcholine secretion from type II pneumocytes induced by adrenomedullin and CGRP (Fig. 3). The percentage inhibition for CGRP-induced secretion was 88.1 ± 2.5% at a concentration of 1×10^{-7} M. In contrast, the percentage inhibition for adrenomedullin-induced secretion was 58.5 ± 4.1%.

3.4. Effects of various inhibitors on the adrenomedullin-induced increase in phosphatidylcholine secretion

To determine which intracellular pathways are involved in the adrenomedullin-induced increase in phosphatidylcholine secretion, we examined the effects of several intracellular pathway inhibitors. In preliminary experiments, H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]5-isoquinolinesulfonamide; 6×10^{-6} M), a protein kinase A in-

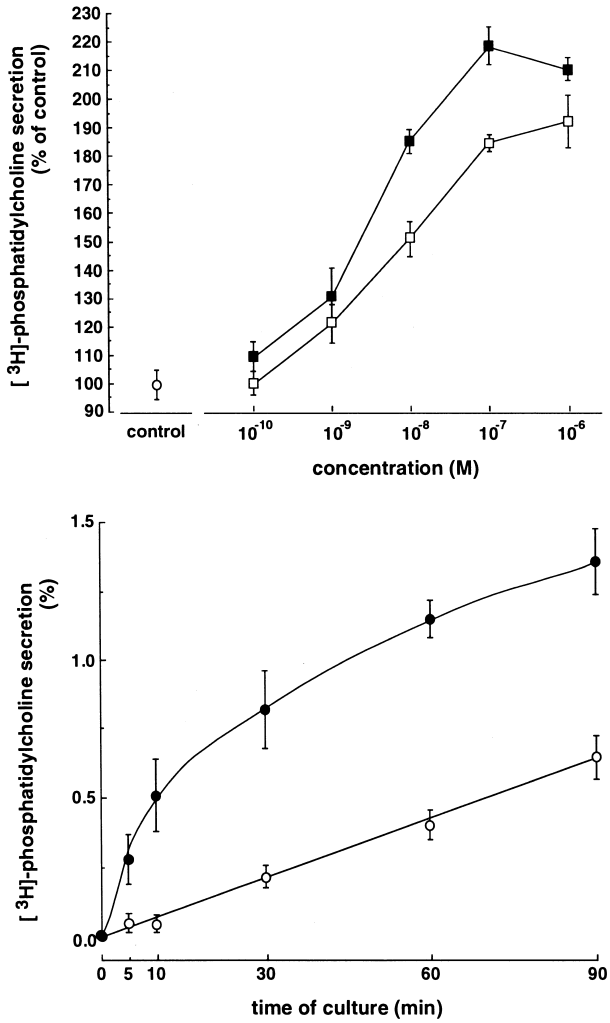

Fig. 2. Top: effects of adrenomedullin and CGRP on the secretion of phosphatidylcholine from rat type II pneumocytes. Isolated pneumocytes were incubated with the indicated concentrations of adrenomedullin or CGRP for 90 min. Secretion is expressed as the amount of [^{3}H]phosphatidylcholine released into the medium after a 90-min incubation, as a percentage of that in the cells plus medium. [^{3}H]phosphatidylcholine secretion over 90 min was $0.66 \pm 0.08\%$ (mean $\pm$ S.E.M., $n = 5$) in control cultures (not incubated with adrenomedullin or CGRP). Open circle: control; open square: CGRP (1×10^{-7} M); solid square: adrenomedullin (1×10^{-7} M). Bottom: kinetics of adrenomedullin-stimulated phosphatidylcholine secretion in rat type II pneumocytes. Phosphatidylcholine secretion is expressed as a percentage of [^{3}H]phosphatidylcholine secretion in the medium relative to that in cells plus medium at the end of the incubation period. [^{3}H]phosphatidylcholine secretion over 90 min was $0.63 \pm 0.08\%$ (mean $\pm$ S.E.M., $n = 5$) in control cultures. Open circle: control; solid circle: adrenomedullin (1×10^{-7} M). Data represent the mean ($\pm$ S.E.M.) of five experiments.

hibitor, did not influence the adrenomedullin-induced increase in phosphatidylcholine secretion (data not shown). As shown in Fig. 4, the effects of several protein kinase inhibitors were examined on the adrenomedullin-induced increase in phosphatidylcholine secretion. Sphingosine (5×10^{-6} M), a general protein kinase C inhibitor, strongly inhibited the increased phosphatidylcholine secretion. Gö6983 (1×10^{-7} M), which inhibits protein kinases

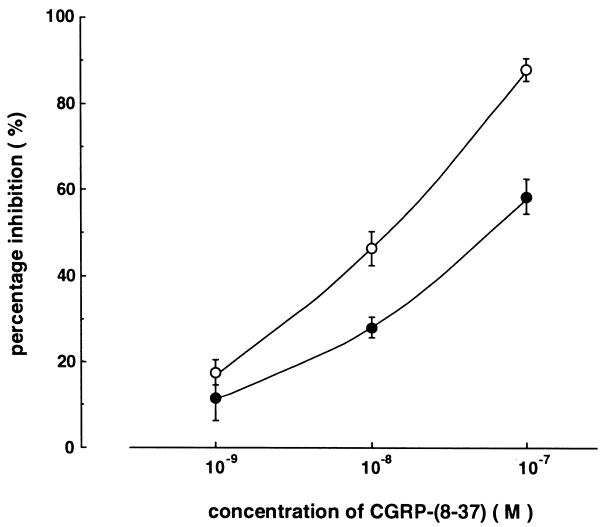

Fig. 3. Effects of CGRP receptor antagonist (CGRP-(8-37)) on phosphatidylcholine secretion stimulated by adrenomedullin and CGRP in rat type II pneumocytes. CGRP-(8-37) was added at the indicated concentration 15 min before the addition of adrenomedullin or CGRP. The percentage inhibition is expressed as the percentage of [^{3}H]phosphatidylcholine secretion inhibited by CGRP-(8-37) treatment, relative to the [^{3}H]phosphatidylcholine secretion after a 90-min incubation with adrenomedullin (1×10^{-7} M) or CGRP (1×10^{-7} M) in the absence of CGRP-(8-37). Data represent the means ($\pm$ S.E.M.) of eight experiments. Open circle: CGRP; solid circle: adrenomedullin.

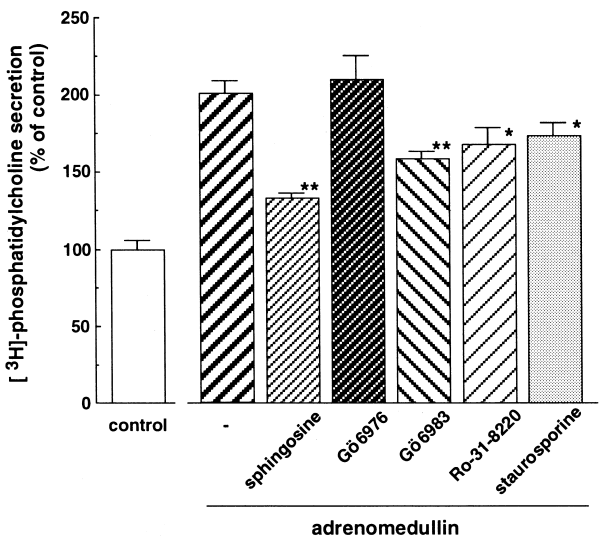

Fig. 4. Effects of protein kinase C inhibitors on adrenomedullin-induced phosphatidylcholine secretion in rat type II pneumocytes. Inhibitors were added 15 min prior to the addition of adrenomedullin (1×10^{-7} M), and the mixture was incubated for a further 90 min. The protein kinase C inhibitors tested were sphingosine (5×10^{-6} M), Gö6976 (1×10^{-7} M), Gö6983 (1×10^{-7} M), Ro-31-8220 (1×10^{-7} M) and staurosporine (1×10^{-6} M). Secretion is expressed as the amount of [^{3}H]phosphatidylcholine released into the medium after a 90-min incubation, as a percentage of that in the cells plus medium. [^{3}H]phosphatidylcholine secretion over 90 min was $0.76 \pm 0.1\%$ (mean $\pm$ S.E.M., $n = 5$) in control cultures. Data represent the means ($\pm$ S.E.M.) of five experiments. * and **: Significant at $P < 0.05$ and 0.01 compared with adrenomedullin alone using Dunnett's test after the ANOVA, respectively.

C-α, -β, -γ, -δ, and -ξ, Ro-31-8220 (1×10^{-7} M), which inhibits protein kinases C-α, -βI, -βII, -γ, and -ε, and staurosporine (1×10^{-6} M), which inhibits protein kinases C-α, -βI, -βII, -γ, -δ, and -ε, all significantly suppressed the adrenomedullin-induced increase of phosphatidylcholine secretion. However, Gö6976 (1×10^{-7} M), which inhibits protein kinases C-α, -βI, and -μ, did not inhibit the adrenomedullin-induced increase in phosphatidylcholine secretion.

4. Discussion

In this study, we found that adrenomedullin mRNA as well as adrenomedullin receptor mRNA was expressed in primary cultures of rat type II pneumocytes. The data seem to be consistent with the results of in situ hybridization in the normal human lung (Martinez et al., 1997). Further, adrenomedullin increased phosphatidylcholine secretion in rat type II pneumocytes at low concentrations. This is the first finding to indicate the possible physiological function of adrenomedullin in the lung. Adrenomedullin had a stronger effect on phosphatidylcholine secretion than CGRP. Furthermore, we showed that the phosphatidylcholine secretion increased by adrenomedullin was dose dependently inhibited by pretreatment with CGRP-(8-37), a CGRP receptor antagonist. However, the inhibition was not complete at a concentration of 1×10^{-7} M, which was the concentration that inhibited the CGRP-induced increase in phosphatidylcholine secretion by nearly 90%, and the inhibition was considerably weaker than the inhibition of the CGRP-induced increase in phosphatidylcholine secretion. In addition, the kinetics of adrenomedullin-induced phosphatidylcholine secretion illustrated the characteristics of a receptor-mediated event, such as the terbutaline-stimulated increase in phosphatidylcholine secretion (Okumura et al., 1995). These results suggest that adrenomedullin increases phosphatidylcholine secretion partly through adrenomedullin-specific receptors, and that adrenomedullin may be an autocrine/paracrine modulator of phosphatidylcholine secretion in rat type II pneumocytes.

Adrenomedullin-induced biological effects related to the increased cAMP level have been reported in several cell types, including vascular smooth muscle cells (Ishizaka et al., 1994; Eguchi et al., 1994), vascular endothelial cells (Kato et al., 1995), and glomerular mesangial cells (Chini et al., 1995). In addition, adrenomedullin is also reported to increase intracellular Ca^{2+} in endothelial cells (Shimekake et al., 1995; Yoshimoto et al., 1998). Phosphatidylcholine secretion from rat type II pneumocytes is also partly regulated by cAMP and intracellular Ca^{2+} (Dobbs et al., 1986b; Wright and Clements, 1987; Kai et al., 1992). Hence, in preliminary experiments, we measured the changes in the intracellular cAMP and Ca^{2+} contents of rat type II pneumocytes after addition of adrenomedullin. However, adrenomedullin had no effect on the intracellular cAMP content and intracellular Ca^{2+} mobilization in rat type II pneumocytes (data not shown).

Protein kinase C is activated by 1,2-diacylglycerol, which is produced by the hydrolysis of phosphatidylinositol 4, 5-bisphosphate by phospholipase C and participates in signal transduction cascades and cell activation in a number of cell types. In addition, in rat type II pneumocytes, protein kinase C activation is involved in pulmonary surfactant secretion (Sano et al., 1985). Both phorbol 12-myristate 13-acetate and ATP increase phosphatidylcholine secretion through the activation of protein kinase C (Sen and Chander, 1994). Thus, activation of protein kinase C is an important pathway for increasing phosphatidylcholine secretion. We found here that sphingosine, a selective protein kinase C inhibitor, suppressed the adrenomedullin-induced increase in phosphatidylcholine secretion. So far, there are at least 11 protein kinase C isozymes in mammalian cells. These isozymes are classified into three groups, according to their activation requirements and structural features (Hug and Sarre, 1993; Selbie et al., 1993; Akimoto et al., 1994; Gobran and Rooney, 1999). The first group includes protein kinases C-α, -β, -βI, -βII, and -γ, which are activated by phorbol esters in a Ca^{2+}-dependent manner. The second group includes protein kinases C-δ, -ε, -η, and -θ, which are activated by phorbol esters in a Ca^{2+}-independent manner. The third group includes protein kinases C-ξ, -ι/λ and -μ, which are Ca^{2+}-independent and irresponsive to phorbol esters. Our findings using protein kinase C isozyme inhibitors suggest that activation of protein kinase C-α, -βI and -μ isozymes may not be involved in the adrenomedullin-induced increase in phosphatidylcholine secretion. Linke et al. (1997) identified protein kinases C-α, -β, -δ, -ε, -η, and -ξ in rat type II pneumocytes by immunoblotting and RT–PCR of rat type II pneumocyte cDNA. However, protein kinase C-γ was not detected by either method. Therefore, it seemed that some protein kinase C isozymes, such as protein kinases C-β and -βII, might be related to the adrenomedullin-induced increase in phosphatidylcholine secretion.

In conclusion, this study demonstrated that both adrenomedullin and adrenomedullin receptor were expressed and that adrenomedullin increased phosphatidylcholine secretion via protein kinase C in rat type II pneumocytes. Our findings suggest that adrenomedullin can be considered a candidate autocrine modulator of surfactant secretion in type II pneumocytes.

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