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Pyridine Derivatives Stimulate Phosphatidylcholine Secretion in Primary Cultures of Rat Type II Pneumocytes

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Abstract

We have examined the effects of pyridine derivatives on phosphatidylcholine secretion in primary cultures of rat type II pneumocytes.

Of 12 pyridine derivatives, 4-aminopyridine, 4-dimethylaminopyridine and 4-pyrolidinopyridine had a stimulatory effect on phosphatidylcholine secretion, whereas other derivatives had little effect. The stimulatory effect of 4-aminopyridine was concentration- and time-dependent, and was inhibited by the acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N*, *N*, *N'*, *N'*-tetraacetic acid (3 μM), an intracellular Ca^{2+} chelator. In addition, the stimulatory effect of 4-aminopyridine was suppressed by W-7 (*N*-(6-aminohexyl)-5-chloro-1-napthalene-sulphonamide) (10 μM), a calmodulin inhibitor, and sphingosine (10 μM) and staurosporine (0·1 μM), protein kinase C inhibitors.

These results indicate that several pyridine derivatives stimulate phosphatidylcholine secretion in type II pneumocytes.

Pulmonary surfactant, which is produced in type II pneumocytes, lowers the surface tension at the air-liquid interface in the lung and provides for alveolar stability. Insufficient surfactant at birth can lead to respiratory distress syndrome, a leading cause of morbidity among premature infants. Thus, medicines to increase pulmonary surfactant in the lung have been sought. In type II pneumocytes, the secretion of phosphatidylcholine, a major component of pulmonary surfactant, is activated by numerous intracellular signal-transduction systems, such as an increase of intracellular Ca^{2+} or cAMP, and activation of Ca^{2+}-dependent protein kinase C or calmodulin-dependent kinase (Wright & Dobbs 1991). Of the signal-transduction systems, Ca^{2+} plays a key role in the regulation of phosphatidylcholine secretion. Recently, two types of K^+ channel, the one blocked by 4-aminopyridine and the other blocked by tetraethylammonium, were demonstrated in type II pneumocytes (Peers et al 1990). In general, blocking of outward K^+ current in various mammalian cells activates the secretory process through an increase in intracellular Ca^{2+}. In this study, to find new compounds which stimulate phosphatidylcholine secretion in type II pneumocytes, we screened pyridine derivatives, including 4-aminopyridine. These results have been reported in part in abstract form (Kai et al 1991, 1992b).

Materials and Methods

Isolation and culture of type II pneumocytes

Type II pneumocytes were isolated from male 180–200 g pathogen-free Wistar rats (Kyudo Farm, Fukuoka, Japan) under anaesthesia with sodium pentobarbitone (25 mg kg^{-1}, i.p.). The lung lavage, trypsin digestion, mechanical dissociation, and plating of cells on bacteriological plastic dishes coated with immunoglobulin G were according to a modification of the method of Dobbs et al (Dobbs et al 1986; Oda et al 1991; Kai et al 1992a). This method routinely yielded 1×10^7 cells per rat. The cells were suspended at 1×10^6 cells mL^{-1} in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 2 μCi mL^{-1} [methyl-^{3}H] choline (specific activity, 80·0 Ci $mmol^{-1}$), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, and plated at a density of 1×10^6 cells per well on a 24-well tissue-culture plate (Falcon) and then cultured at 37°C in 5% CO_2 in an air water-saturated atmosphere for 18 h. Non-adherent cells were removed from the wells by washing before the assay. The purity of the type II pneumocytes monolayer was 95 ± 3%. For cellular identification, the sample was stained with a tannic acid and polychrome stain (Mason et al 1985), and alkaline phosphatase stain (Edelson et al 1988). The viability of type II pneumocytes was 98 ± 2% as judged by the trypan blue exclusion test.

Phosphatidylcholine secretion

Secretion of phosphatidylcholine by cultured type II pneumocytes was determined according to the method of Rice & Singleton (1987). The cells were rinsed with fresh serum- and antibiotic-free medium to remove [^{3}H]choline and unattached cells. After 30 min, the test agents were added and, unless indicated otherwise, the incubation was continued for 90 min. All inhibitors were added 30 min before the addition of stimulants. At the end of the incubation period, the medium was aspirated, the cells lysed with 2 mL ice-cold 0·05% Triton X-100 solution and lipids

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extracted from both cells and medium with chloroform and methanol according to the method of Folch et al (1957). 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 the addition of 5 mL Aquasol II to each sample. Secretion was expressed as the amount of [^{3}H]phosphatidylcholine in the medium after 90-min incubation, as a percentage of that in the cells plus medium:

$$\text{Secretion (\%)} = \frac{[^3\text{H}]\text{phosphatidylcholine in the medium}}{\text{total } [^3\text{H}]\text{phosphatidylcholine}} \times 100 \quad (1)$$

The degree of the stimulatory effects was expressed as the percentage of the stimulated secretion:

$$\text{Stimulation (\%)} = \frac{\text{stimulated secretion}}{\text{unstimulated secretion}} - 100 \quad (2)$$

Lactate dehydrogenase assay. The rate of lactate dehydrogenase release into the medium was determined to assess cellular integrity. After the secretion experiments, lactate dehydrogenase activity in the cells and medium was assayed using LDH kits (Nippon Shoji Co. Ltd.). The lactate dehydrogenase activity released into the medium did not exceed 1% of the total cellular content in all experiments.

Chemicals and drugs. DMEM was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan), trypsin from Difco Laboratories (Detroit, MI, USA) and foetal bovine serum from Sera-Lab Ltd. (Sussex, UK). [Methyl-^{3}H]choline, and Aquasol II were obtained from NEN Research Products (Boston, MA, USA). Acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N*, *N*, *N'*, *N'*-tetraacetic acid (BAPTA/AM) was from Dojin Laboratories (Kumamoto, Japan) and *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide (W-7) from Seikagaku Co. (Tokyo, Japan) and other reagents and biochemicals from Sigma Chemicals Co. (St Louis, MO, USA).

Sphingosine was dissolved in ethanol, and phorbol 12-myristate-13-acetate (PMA) and BAPTA/AM in dimethyl sulphoxide (DMSO) and stored at −20°C. The final concentration of ethanol and DMSO in the experiments never exceeded 1%, and this amount was also added to the media of the corresponding control groups.

Statistical analysis

All data were mean values ± s.e. Statistical analysis was performed by means of Duncan's multiple-range test or two-tailed unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Results

We screened pyridine derivatives for phosphatidylcholine secretion in type II pneumocytes. Pyridine derivatives were used at 3 mM, in general, a concentration sufficient to induce biological effects in several electrophysiological studies. Of 12 pyridine derivatives, 4-aminopyridine, 4-dimethylaminopyridine and 4-pyrolidinopyridine stimulated phosphatidylcholine secretion, whereas other derivatives had little effect (Fig. 1). 4-Aminopyridine, 4-dimethylaminopyridine and 4-pyrolidinopyridine at 0·3 mM had no effect (data not shown). These derivatives did not cause cell damage at the concentrations used in the experiments, as determined by lactate dehydrogenase release. We focused on

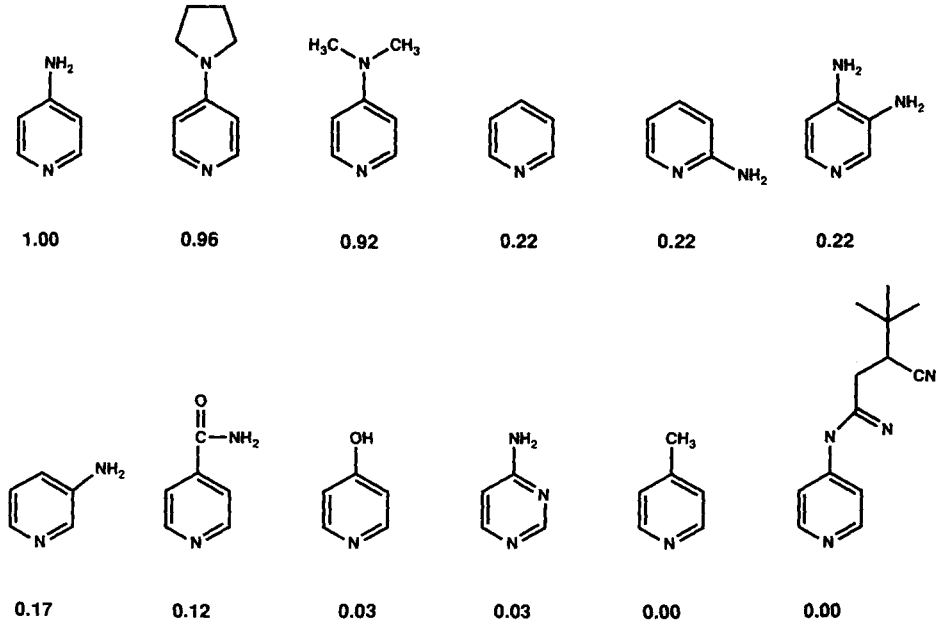

FIG. 1. Structure–activity relationship of pyridine derivatives on phosphatidylcholine secretion in type II pneumocytes. The cells were incubated in the presence or absence of the indicated compounds at 3 mM for 90 min, after which [^{3}H]phosphatidylcholine in the cells and medium was measured. The values indicate the ratio in comparison with the mean value of the stimulatory effect of 3 mM 4-aminopyridine from 6 wells of cells from eight rats.

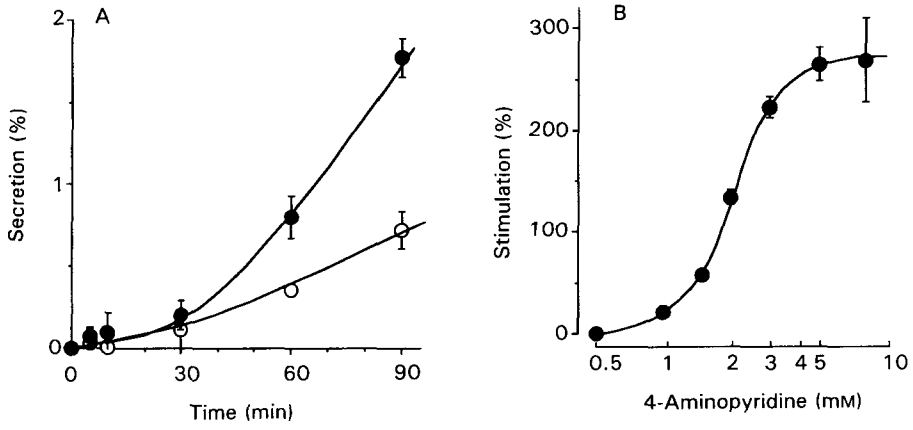

FIG. 2. A. Time course of the effects of 4-aminopyridine (2 mM) on phosphatidylcholine secretion in type II pneumocytes. After a 30-min pre-incubation, the cells were incubated with no agonist (○) and 2 mM 4-aminopyridine (●) for the times indicated, after which [^{3}H]phosphatidylcholine in the cells and medium was measured. Secretion is expressed as the percent of increase over the rate in 30-min preincubation. The data are the mean ± s.e. from 8 wells of cells from nine rats. The secretion of [^{3}H]phosphatidylcholine by type II pneumocytes in primary culture was linear with time with a basal rate secretion of 0·77 ± 0·06% (n = 24) of total cellular phosphatidylcholine over 90 min. B. Concentration-response curves for the effect of 4-aminopyridine on phosphatidylcholine secretion in type II pneumocytes. The cells were incubated in the presence of the indicated concentrations of agonist for 90 min, after which [^{3}H]phosphatidylcholine in the cells and medium was measured. Secretion is expressed as percent stimulation over the rate in control cells cultured without 4-aminopyridine. The data are the mean ± s.e. from 8 wells of cells from six rats.

the effect of 4-aminopyridine in the following experiments. The stimulatory effect of 4-aminopyridine (2 mM) was seen 60 min after its addition and then the secretion was time-dependent (Fig. 2A). Further, 4-aminopyridine stimulated phosphatidylcholine secretion in a concentration-dependent manner in the range 0·5–5 mM (Fig. 2B); the EC50 was found to be 1·7 mM.

To examine whether intracellular Ca^{2+} is involved in the stimulatory effect of 4-aminopyridine, we used BAPTA-AM as an intracellular Ca^{2+} chelator. BAPTA-AM, an acetoxymethyl ester of a double aromatic analogue of EGTA, can cross plasma membranes and is hydrolysed by cellular esterases to BAPTA, which has high selectivity for Ca^{2+} over Mg^{2+} (Tsien 1980). Type II pneumocytes were incubated with 3 μM BAPTA-AM for 30 min before the addition of 4-aminopyridine, after which the medium containing BAPTA-AM was removed and then 4-aminopyridine was added. BAPTA suppressed the stimulatory effect of 3 mM 4-aminopyridine and 10 nM PMA (Table 1). These findings suggest that the effect of 4-aminopyridine may require intracellular Ca^{2+}.

To determine whether the stimulatory effect of 4-aminopyridine is through Ca^{2+}-dependent calmodulin kinase or Ca^{2+}-dependent protein kinase C, we used W-7 as a calmodulin inhibitor, and sphingosine and staurosporine as protein kinase C inhibitors. W-7 (10 μM) significantly inhibited the phosphatidylcholine secretion induced by 3 mM 4-aminopyridine or 10 nM PMA (Table 2). Sphingosine (10 μM) and staurosporine (0·1 μM) significantly inhibited the stimulatory effect of 4-aminopyridine and PMA (Table 2).

Table 1. 4-Aminopyridine- and PMA-stimulated phosphatidylcholine secretion in the BAPTA-AM loaded type II pneumocytes.

	No inhibitor	BAPTA-AM
	Stimulation (%)	
4-Aminopyridine	175·8 ● 22·3	38·4 ± 11·0*
PMA	225·4 ± 27·5	35·1 ± 15·4*

Cells were incubated with BAPTA-AM for 30 min, and cultured in the presence and absence of 4-aminopyridine (3 mM) and PMA (10 nM) for 90 min, after which [^{3}H]phosphatidylcholine in the cells and medium was measured. The data, expressed as percent stimulation, are the mean ± s.e. from 6 wells of cells from three rats and were analysed statistically with an unpaired Student's *t*-test. *$P < 0·05$ compared with BAPTA-AM loaded groups.

Table 2. Effects of W-7, sphingosine, and staurosporine on 4-aminopyridine- and PMA-stimulated phosphatidylcholine secretion in type II pneumocytes.

	No inhibitor	W-7	Sphingosine	Staurosporine
		Stimulation (%)		
4-Aminopyridine	176·3 ± 20·1	24·3 ± 16·2*	41·3 ± 10·0*	38·5 ± 21·4*
PMA	212·1 ± 30·5	77·4 ± 30·1*	75·1 ± 14·4*	99·5 ± 17·6*

Cells were cultured in the presence and absence of the indicated agonists for 90 min after the 30-min pre-incubation with no drug or protein kinase inhibitors, after which [^{3}H]phosphatidylcholine in the cells and medium was measured. The data, expressed as percent stimulation over the control (cells incubated without any test agent), are the mean ± s.e. from 5 wells of cells from 5 rats and were analysed statistically with Duncan's multiple-range test. *$P < 0·05$ compared with groups not treated with inhibitor.

Discussion

In the present study, several pyridine derivatives stimulated phosphatidylcholine secretion from cultured type II pneumocytes in a concentration-dependent manner without producing cytotoxicity. Judging from this structure-activity relationship, a pyridine nucleus with 4-amino substituents without steric hindrance may be important in the stimulatory effect. In terms of its structure-activity relationship, interestingly, the effect on phosphatidylcholine secretion differed from that on neurotransmitter release reported by Johns et al (1976). In particular 3,4-diaminopyridine had little effect on phosphatidylcholine secretion, although the compound is undoubtedly active as a blocker of K^+ current in excitable tissues.

The stimulatory effect of 4-aminopyridine was inhibited by the treatment of the cells with BAPTA, suggesting that activation of phosphatidylcholine secretion by 4-aminopyridine occurred through calcium-dependent transduction systems. In addition, pretreatment with the calmodulin antagonist W-7 suppressed the phosphatidylcholine secretion induced by 4-aminopyridine or PMA. This finding may suggest the involvement of calmodulin kinases in phosphatidylcholine secretion activated by 4-aminopyridine or PMA, although it remains unclear whether W-7 specifically inhibits calmodulin kinases in type II pneumocytes. In support of its involvement, calmodulin is found adjacent to lamellar bodies in type II pneumocytes (Hill et al 1984). Further, Voyno-Yasenetskaya et al (1991) have shown that W-7 inhibited the stimulatory effect of PMA on phosphatidylcholine secretion, and hypothesized that the phosphorylation of many high-affinity calmodulin-binding proteins by protein kinase C may cause displacement of bound calmodulin, perhaps leading to activation of Ca^{2+}-calmodulin-dependent processes (Graff et al 1989), and surfactant secretion in type II pneumocytes. This hypothesis may help to explain the effect of 4-aminopyridine. In addition, our present results have shown that the phosphatidylcholine secretion induced by 4-aminopyridine was inhibited by protein kinase C inhibitors. The degree of inhibition was similar to that produced by PMA. These findings suggest that 4-aminopyridine, like PMA, may stimulate phosphatidylcholine secretion through a Ca^{2+}- and phospholipid-dependent protein kinase directly or indirectly in type II pneumocytes.

It has been reported that most type II pneumocytes have time- and voltage-dependent outward currents carried by K^+ (DeCoursey et al 1988; Peers et al 1990), and that 4-aminopyridine and TEA reduce K^+ current (Peers et al 1990). The effective concentrations of 4-aminopyridine on the reduction of the current was similar to that observed in the present results on phosphatidylcholine secretion. In general, all the known K^+ channels are blocked by TEA at high concentrations (Hille 1984). However, 50 mM TEA had no effect on phosphatidylcholine secretion (data not shown). It seems to us that the stimulatory effect of several pyridine derivatives is not involved in K^+-current blocking, although we need further investigation to examine the contribution of the K^+ channel to regulation of phosphatidylcholine secretion.

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