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# Influence of the Protein Kinase C Inhibitor 3-{1-[3-(Amidinothio)propyl]-1*H*-indoyl-3-yl}-3-(1-methyl-1*H*-indoyl-3-yl)maleimide Methane Sulfonate (Ro-318220) on Surfactant Secretion inType II Pneumocytes

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**ABSTRACT.** We have investigated the influence of 3-{1-[3-(amidinothio)propyl]-1*H*-indoyl-3-yl}-3-(1-methyl-1*H*-indoyl-3-yl)maleimide methane sulfonate (Ro-318220), a potent and selective inhibitor of protein kinase C, on phosphatidylcholine secretion in response to surfactant secretagogues in rat type II cells. Freshly isolated cells were cultured overnight with [$^3$H]choline to label the phosphatidylcholine pool and were preincubated for 30 min in fresh medium with or without Ro-318220. Secretagogues were then added, and the incubation was continued for 90 min after which [$^3$H]phosphatidylcholine secretion was measured. Ro-318220 (10 μM) almost completely abolished the stimulatory effects of 36 μM terbutaline, 10 μM ATP, and 1 μM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and significantly antagonized the effects of 10 μM 5′-(*N*-ethylcarboxyamido)adenosine (NECA), 100 μM dioctanoylglycerol, and 0.05 μM ionomycin. The effect of Ro-318220 was dependent on concentration. The $IC_{50}$ values for Ro-318220 inhibition of the effects of terbutaline, NECA, TPA, and ionomycin were not significantly different. The $IC_{50}$ value for Ro-318220 inhibition of the effect of TPA in the type II cell (0.05 μM) was similar to that reported for inhibition of protein kinase C *in vitro* (0.08 μM). We conclude that Ro-318220 antagonizes the effects of the different surfactant secretagogues by antagonizing a step common to the different signaling pathways. It remains to be established if it is protein kinase C or another step that is inhibited. BIOCHEM PHARMACOL 53;4:597–601, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** phosphatidylcholine secretion; lung surfactant; type II pneumocyte; protein kinase C inhibitor; signal-transduction; surfactant secretagogues

Lung surfactant is a lipoprotein complex that lines the alveolar surface and is essential for normal pulmonary function. Although it contains a number of unique proteins, surfactant consists largely of phospholipid, of which phosphatidylcholine is by far the most abundant component [1, 2]. Surfactant phosphatidylcholine is synthesized in type II alveolar epithelial cells, stored in lamellar inclusion bodies, and secreted by exocytosis [1–4].

Secretion of surfactant phosphatidylcholine has been studied extensively in primary cultures of type II cells and shown to be influenced by several physiological and pharmacological agents [1, 3, 4]. Surfactant phospholipid secretagogues include β-adrenergic agonists, $A_2$ (adenosine) and $P_2$ (ATP) purinoceptor agonists, direct activators of PKC† and ionophores that promote $Ca^{2+}$ uptake into the cell. These agents act via at least three signal-transduction mechanisms between which there are additive and synergistic interactions [1, 5, 6]. Protein phosphorylation has a major role in signal transduction in many systems [7], and there is evidence that several protein kinases are activated by surfactant secretagogues. They include PKC, PKA, and CaCM-PK [1].

Previous data suggested that sustained activation of PKC may be important in the maintenance of high levels of surfactant secretion [8]. Inhibitors have been employed extensively in order to elucidate the importance and/or involvement of specific protein kinases in numerous biological processes [9–11]. However, the lack of inhibitor specificity has often made interpretation of such data ques-

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† *Abbreviations:* CaCM-PK, $Ca^{2+}$/calmodulin-dependent protein kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IgG, immunoglobulin G; $IP_3$, inositol trisphosphate; NECA, 5′-(*N*-ethylcarboxyamido)adenosine; PKA, cyclic AMP-dependent protein kinase (protein kinase A); PKC, protein kinase C; PPI-PLC, phosphoinositide-specific phospholipase C; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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tionable [12]. The bisindolyl maleimide 3-{1-[3-(amidinothio)propyl]-1*H*-indoyl-3-yl}-3-(1-methyl-1*H*-indoyl-3-yl)maleimide methane sulfonate (Ro-318220) is a potent and selective PKC inhibitor [12, 13]; the $IC_{50}$ values for its inhibitory effects on rat brain PKC, bovine heart PKA and rat brain CaCM-PK were reported to be 0.08, 1.5, and 17 μM, respectively [13]. Ro-318220 is much more selective than other agents such as staurosporine, K252a, H-7, and calphostin that have been employed extensively as PKC inhibitors [12, 13].

To assess the importance of PKC in the overall regulation of surfactant secretion, we examined the influence of Ro-318220 on increased phosphatidylcholine secretion in type II cells in response to selected agonists. The agonists include terbutaline, a β-adrenergic agonist; NECA, an adenosine $A_2$ receptor agonist; ATP, the prototypical $P_2$ purinoceptor agonist; TPA and dioctanoylglycerol, direct activators of PKC; and ionomycin, an ionophore that promotes $Ca^{2+}$ uptake into the cell [1].

## MATERIALS AND METHODS

Type II cells were isolated from adult male Sprague-Dawley rats by the method of Dobbs *et al.* [14] as described previously [15]. This method involves digestion of the blood-free lungs with elastase and separation of type II pneumocytes from contaminating cells by panning on bacteriological dishes coated with IgG. The freshly isolated cells were plated at a density of 3–5 × $10^6$ cells per dish on 35-mm diameter plastic tissue culture dishes (Falcon; Becton Dickinson, Lincoln Park, NJ) and cultured in 1.5 mL DMEM containing 10% FBS, streptomycin (10 μg/mL), and penicillin (100 U/mL) for 18–20 hr at 37° in a humidified atmosphere of 90% air–10% $CO_2$. We previously reported that more than 95% of the cultured cells were identifiable as type II pneumocytes [16].

The cells were cultured overnight with [$^3$H]choline (2 μCi/mL) to label the phosphatidylcholine pool after which the medium was removed and the cells were washed three times with fresh DMEM without FBS, antibiotics, or radioactivity. Fresh DMEM with or without Ro-318220 was then added, and the cells were returned to the incubator. After a 30-min preincubation in the fresh medium, solvent vehicle or agonists were added, and the incubation was continued for 90 min. The medium was then aspirated and the cells were lysed with ice-cold water. The medium was centrifuged at 200 *g* for 10 min to remove any floating cells; lipids were extracted from both cells and medium with a mixture of chloroform and methanol by the method of Bligh and Dyer [17] and quantitated by liquid scintillation counting [16]. As phosphatidylcholine accounts for over 95% of the lipid radioactivity in both cells and medium [16], lipids were not fractionated into individual components. Phosphatidylcholine secretion is expressed as the amount of total lipid radioactivity in the medium after 2 hr as a percentage of the total in cells and medium combined. Stimulation in response to agonists, either alone or combined with Ro-318220, was calculated as a percentage increase over the rate of secretion in the corresponding control group with negative values being considered 0% stimulation. The inhibitory effect of Ro-318220 was calculated from the percent stimulation values.

In each experiment, type II cells were isolated from the pooled lungs of 2–4 rats and distributed among the various treatment groups, three dishes per group. The dishes were processed separately and the values were averaged to give a single data point per group per experiment. Data are presented as means ± SEM from the number of experiments indicated. Statistical analysis was done with either Student's two-tailed *t*-test or ANOVA followed by the Newman–Keuls test using Instat 2.04 (GraphPad Software, San Diego, CA). The $IC_{50}$ values were calculated as described [18].

Terbutaline sulfate (Brethine®) was obtained from Geigy Pharmaceuticals, West Caldwell, NJ. ATP (disodium salt), NECA, TPA and dioctanoylglycerol were from Sigma, St. Louis, MO; and IgG and ionomycin from Calbiochem, San Diego, CA. Porcine pancreatic elastase (EL357) was obtained from Elastin Products, Owensville, MO; FBS from Hyclone, Logan, UT; DMEM from Gibco-BRL, Grand Island, NY; and [$^3$H]choline chloride from DuPont NEN, Boston, MA. Ro-318220 was a gift from Dr. Geoffrey Lawton, Roche Products, Welwyn Garden City, Hertfordshire, U.K. TPA, ionomycin, and Ro-318220 were dissolved in dimethyl sulfoxide and other agents in DMEM. The final concentration of dimethyl sulfoxide in the culture medium was 1%, and this amount was also added to the corresponding control dishes. Dioctanoylglycerol was dispersed by sonication in DMEM.

## RESULTS

The effect of Ro-318220 on phosphatidylcholine secretion in type II cells in response to surfactant secretagogues is shown in Table 1. Ro-318220 almost completely abolished the stimulatory effects of terbutaline, ATP, and TPA and significantly antagonized the effects of NECA, dioctanoylglycerol, and ionomycin. Ro-318220 alone had little effect on surfactant secretion. Although it tended to decrease the basal rate of secretion by 22% from 1.72 ± 0.16 to 1.35 ± 0.16% phosphatidylcholine secreted in 2 hr, that effect was not significant ($P = 0.14$, paired *t*-test; $N = 3$).

We used 10 μM Ro-318220 in initial experiments because at that concentration it maximally inhibits phospholipase D activation in response to TPA in type II cells [8]. We reported previously that 10 μM Ro-318220 was not cytotoxic as it did not increase significantly the rate of lactate dehydrogenase release [8]. However, 10 μM is a high concentration when compared to the $IC_{50}$ values reported for Ro-318220 inhibition of protein kinases *in vitro* [13]. We therefore considered the possibility that Ro-318220 antagonism of the effects of the different secreta-

**TABLE 1. Effect of Ro-318220 on phosphatidylcholine secretion in response to surfactant secretagogues in rat type II cells in primary culture***

| Agonist | N | Control | Agonist alone (% secretion) | Agonist + Ro-318220 | % Inhibition |
|---|---|---|---|---|---|
| Terbutaline ($3.6 \cdot 10^{-5}$ M) | 8 | 1.71 ± 0.22 | 2.55 ± 0.24† | 1.48 ± 0.17 | 97 ± 1.9 |
| NECA ($10^{-5}$ M) | 7 | 1.56 ± 0.15 | 3.06 ± 0.32† | 1.69 ± 0.09‡ | 83 ± 9.2 |
| ATP ($10^{-5}$ M) | 3 | 1.72 ± 0.16 | 5.96 ± 0.49† | 1.82 ± 0.15‡ | 96 ± 2.4 |
| TPA ($10^{-6}$ M) | 6 | 1.91 ± 0.22 | 6.64 ± 0.58† | 2.17 ± 0.21‡ | 93 ± 2.3 |
| Dioctanoylglycerol ($10^{-4}$ M) | 4 | 1.91 ± 0.42 | 4.65 ± 0.55§ | 2.79 ± 0.25‡ | 65 ± 20.8 |
| Ionomycin ($5 \cdot 10^{-8}$ M) | 6 | 1.51 ± 0.18 | 3.02 ± 0.25† | 1.75 ± 0.26‡ | 76 ± 10.5 |

* Type II cells prelabeled with [$^3$H]choline were incubated with or without $10^{-5}$ M Ro-318220 for 30 min. Secretagogues were then added, and the incubation was continued for 90 min after which lipids were extracted from the cells and medium. Secretion is expressed as the amount of [$^3$H]phosphatidylcholine in the medium as a percentage of the total in cells + medium after the 2-hr incubation. Percent inhibition was calculated from the percentage increase over the rate of secretion in the control group as described in Materials and Methods. The data are means ± SEM from N experiments and were analyzed statistically by repeated measures ANOVA.

† $P < 0.001$ vs control.

‡ Not significantly different from control.

§ $P < 0.01$ vs control.

gogues was due to inhibition of a number of different protein kinases. To test that hypothesis, we examined the relationship between the concentration of Ro-318220 and its antagonism of the effects of terbutaline, NECA, TPA, and ionomycin. We did not include ATP as it is known to act via multiple signaling mechanisms [1] and, therefore, might exhibit a complex Ro-318220 concentration–response relationship. Ro-318220 concentration–response curves are shown in Fig. 1 and $IC_{50}$ values in Table 2. Concentration–response curves were similar for all four secretagogues, and there were no significant differences between the $IC_{50}$ values. These data suggest that Ro-318220 antagonizes the effects of the different surfactant secretagogues by inhibiting a signal-transduction step that is common to all four secretagogues.

## DISCUSSION

There is evidence that activation of at least three protein kinases, PKC, PKA and CaCM-PK, results in increased surfactant phospholipid secretion [1]. Surfactant secretagogues include agents that directly activate those kinases and agents that act via signal-transduction mechanisms coupled to their activation. TPA and dioctanoylglycerol are known to directly activate PKC [19, 20]. PKA is activated by agents such as terbutaline and NECA that act at β-adrenergic and adenosine $A_2$ receptors, respectively, that are coupled to adenylate cyclase. Thus, terbutaline has been reported to increase cyclic AMP formation [21] and to activate PKA [22] in type II cells. NECA also increases cyclic AMP formation in type II cells [21, 23] and, as its stimulatory effect on phosphatidylcholine secretion is not additive to that of terbutaline [21], it is likely that it also activates PKA. Ionomycin is an ionophore that increases uptake of $Ca^{2+}$, and its effect on phosphatidylcholine secretion in the type II cell is antagonized by calmodulin antagonists [6], suggesting that it may activate $Ca^{2+}$/calmodulin-dependent protein kinase.

There is evidence that ATP may activate all three protein kinases in the type II cell [1]. ATP acts at a $P_{2u}$ purinoceptor that is coupled to activation of PPI-PLC [24, 25] as well as at another receptor coupled to adenylate cyclase activation [23, 26]. Activation of adenylate cyclase can be expected to result in subsequent activation of PKA, whereas activation of PPI-PLC results in the formation of the two second messengers, diacylglycerol and $IP_3$ [23, 27, 28]. Diacylglycerol is known to activate PKC [19, 20], and ATP has been reported to activate PKC in type II cells [28]. $IP_3$ promotes mobilization of $Ca^{2+}$ from intracellular stores [29], and ATP also has been reported to increase $Ca^{2+}$ levels in type II cells [28]. $Ca^{2+}$ may then activate CaCM-PK as well as act synergistically with diacylglycerol to activate PKC [20]. That ATP activates CaCM-PK is suggested by the finding that calmodulin antagonists decrease its effect on phosphatidylcholine secretion in type II cells [6, 30].

The current data show that Ro-318220 antagonizes the stimulatory effects of NECA, terbutaline, ATP, TPA, dioctanoylglycerol, and ionomycin on phosphatidylcholine secretion. The $IC_{50}$ value for Ro-318220 inhibition of the effect of TPA in the type II cell, 0.05 μM, is similar to that reported for its inhibition of partially purified rat brain PKC *in vitro*, 0.08 μM [13]. However, its $IC_{50}$ values for inhibition of the effects of terbutaline, NECA, and ionomycin in the type II cells are in the same range, 0.11 to 0.32 μM, and are not significantly different from that for TPA. The fact that its concentration–response curves and $IC_{50}$ values are virtually the same for terbutaline, NECA, TPA, and iono-

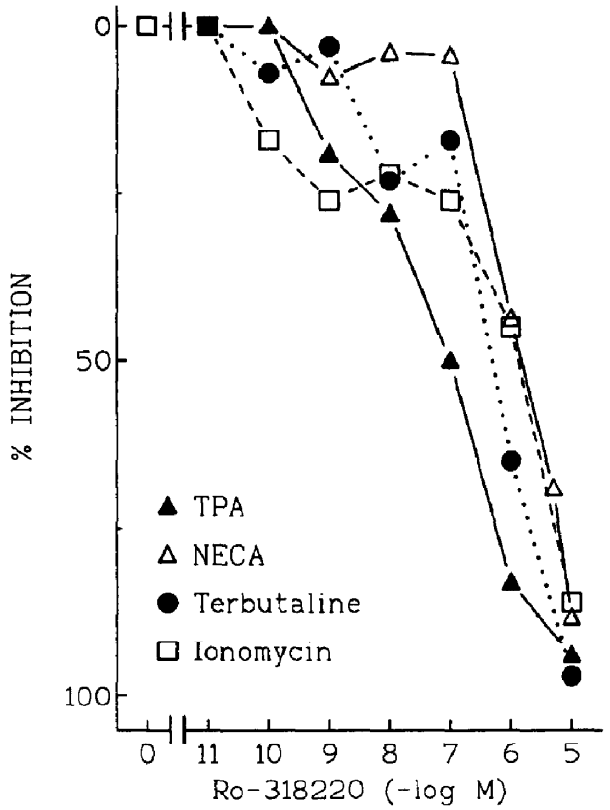


**FIG. 1. Ro-318220 concentration–response relationships. Type II cells were preincubated with the indicated concentrations of Ro-318220 for 30 min before the addition of $10^{-6}$ M TPA, $10^{-5}$ M NECA, $3.6 \cdot 10^{-5}$ M terbutaline, or $5 \cdot 10^{-8}$ M ionomycin. The incubation was then continued for 90 min after which phosphatidylcholine secretion was measured. Percent inhibition was calculated as in Table 1. The data are means from 3–5 experiments. The SE range was 1–3, 0–10, 3–10 and 8–16 in the TPA, NECA, terbutaline, and ionomycin experiments, respectively. SE bars are omitted for clarity. Rates of phosphatidylcholine secretion (% released into the medium in 2 hr) were increased from 2.10 ± 0.42 to 7.63 ± 0.59 by TPA (N = 3; $P < 0.002$, paired *t*-test), 1.44 ± 0.23 to 3.02 ± 0.57 by NECA (N = 4, $P < 0.02$), 1.71 ± 0.35 to 2.59 ± 0.39 by terbutaline (N = 5, $P = 0.0005$), and 1.31 ± 0.31 to 2.84 ± 0.44 by ionomycin (N = 3, $P < 0.02$).**

mycin suggests that Ro-318220 acts at a step that is common to all of those surfactant secretagogues.

As its $IC_{50}$ values for inhibition of the effects of terbutaline, NECA, and ionomycin are considerably less than those reported for inhibition of PKA, 1.5 μM, and CaCM-PK, 17 μM, *in vitro* [13], it is unlikely that the antagonistic effects of Ro-318220 are due to inhibition of the latter protein kinases. Indeed, considering that higher inhibitor concentrations should be required in intact cells than in isolated enzyme preparations [11], it is very unlikely that the effects of Ro-318220 in the type II cell are due to inhibition of either PKA or CaCM-PK. It is possible that Ro-318220 inhibits a PKC isoform acting distal to PKA and CaCM-PK in the adenylate cyclase and $Ca^{2+}$-dependent signal-transduction pathways. That notion is supported by the finding that the effects of NECA and terbutaline on

**TABLE 2. Ro-318220 $IC_{50}$ values for antagonism of surfactant secretagogues***

| Agonist | N | Ro-318220 $IC_{50}$ (μM) |
|---|---|---|
| Terbutaline | 5 | 0.32 ± 0.08 |
| NECA | 3 | 0.27 ± 0.12 |
| TPA | 3 | 0.05 ± 0.01 |
| Ionomycin | 2 | 0.11 ± 0.04 |

* The $IC_{50}$ values were calculated from the concentration-response curves in Fig. 1. Secretagogue concentrations and experimental details are as described in Table 1. The data are means ± SEM from 3–5 experiments or means ± range from 2 experiments. There were no significant differences between the groups ($P > 0.05$; one-way ANOVA).

phosphatidylcholine secretion were virtually abolished by two other PKC inhibitors, staurosporine and H-7 [6], although it is also possible that staurosporine and H-7 inhibit PKA in the type II cell as both agents are known to inhibit PKA and other protein kinases in addition to PKC [9, 10, 12]. On the other hand, the finding that the effect of ionomycin on phosphatidylcholine secretion was only partially blocked by H-7 and not at all by staurosporine [6] argue against PKC being a common component of the different signal transduction pathways. However, multiple isoforms of PKC are known to exist [20], and the sensitivity of individual isomers to various inhibitors is not known [11].

Functional and second messenger parameters have shown that there are synergistic interactions between the PKC, PKA, and CaCM-PK signaling pathways of surfactant secretion [5, 6]. The likelihood of synergism between the PKC and the other two signaling mechanisms is increased by that fact that adenylate cyclase, PKA, and CaCM-PK have each been reported to be substrates for PKC [31]. Although, to our knowledge, there are no data to suggest that the action of PKC is required for activation of those protein kinases, such a requirement would readily explain the inhibitory effect of Ro-318220 in the current study. An alternative, although unlikely, possibility is that Ro-318220 is equally potent in inhibiting PKC, PKA, and CaCM-PK in type II cells. It is also possible that Ro-318220 acts at another distal step common to all three signaling pathways. Further studies will be required to elucidate the precise signaling step or steps in surfactant secretion inhibited by Ro-318220 in the type II cell.

In summary, this paper shows that Ro-318220 antagonizes the effects of surfactant secretagogues acting via at least three different signal-transduction mechanisms. The Ro-318220 $IC_{50}$ values are similar for the different secretagogues, suggesting that it inhibits a step common to the different signaling pathways. Although Ro-318220 is a known PKC inhibitor, it remains to be established if it is PKC or another step that is inhibited.

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