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INHIBITION OF PHOSPHATIDYLCHOLINE SECRETION BY STILBENE DISULFONATES IN ALVEOLAR TYPE II CELLS

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Abstract—Various agents stimulate the secretion of lung surfactant from alveolar type II cells by increasing intracellular Ca^{2+}, cyclic adenosine-3′:5′-monophosphate (cAMP), or diacylglycerol. A few agents, including the purified surfactant protein A, are known to inhibit the secretion by an unknown mechanism. In the present study, we demonstrated that stilbene disulfonic acids, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), are potent but reversible inhibitors of lung surfactant secretion. The inhibition was concentration dependent, and the EC_{50} was 5 μM for DIDS and 50 μM for SITS. The inhibition was not specific to agonists for any one type of receptor, and was also observed for secretion stimulated by 8-bromo-cAMP, or tetradecanoyl phorbol acetate, suggesting that the site of inhibition was distal to the generation of intracellular second messengers. This was also supported by the failure of DIDS to block the stimulus-mediated increase in diacylglycerol content of type II cells. Further, DIDS and SITS were also inhibitory for basal secretion. Based on the reversibility of inhibition and the fact that inhibition was observed with both basal and stimulated secretion, we suggest that stilbene disulfonic acids affect a component of the exocytosis process that occurs at or near the plasma membrane.

The secretion of phosphatidylcholine (PC)†, the major surface active component of lung surfactant, from alveolar type II cells can be modulated by various agents [1]. Agonists for β-adrenergic, purinergic, vasopressin, and histamine receptors increase [2–7], while compound 48/80, lectins, and surfactant protein A inhibit, the secretion of surfactant PC from isolated type II cells [7–9]. The inhibitory effect of lectins and surfactant protein A are reversible and are not specific to any single type of stimulus. These observations suggest that the inhibitory effects of these agents occur at a process common to secretion mediated via different mechanisms, and is most likely at or very close to the plasma membrane.

Nord and associates [10] have demonstrated that in type II cells recovery from intracellular alkalosis depends on Cl^-/HCO_3^- exchange and can be blocked by the exchange inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Using an isolated perfused lung model, we have demonstrated previously that alkalosis due to ventilation with low CO_2 in air increases the secretion of surfactant [11]. In our subsequent studies, we observed that DIDS inhibited the alkalosis-mediated secretion. These results were somewhat unexpected in that inhibition of Cl^-/HCO_3^- exchange (and recovery from alkalosis) may, in fact, augment the alkalosis-stimulated secretion. Using alveolar type II cells in primary culture, we now provide evidence that both the stimulated and basal secretions of PC are strongly inhibited by stilbene disulfonic acids. Our results demonstrate that the site of DIDS action was distal to generation of second messenger, and was likely at the plasma membrane of type II cells. Part of these studies has been reported in abstract form [12].

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† Abbreviations: AMDS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; cAMP, adenosine-3′:5′-cyclic monophosphate; DETAPAC, diethylenetriamine pentaacetic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DPPC, dipalmitoyl PC; MEM, Eagle's Minimum Essential Medium; PC, phosphatidylcholine; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TEA, tetraethylammonium chloride; and TPA, tetradecanoyl phorbol acetate.

MATERIALS AND METHODS

[^{3}H-*methyl*]Choline, [γ-^{32}P]ATP, and [^{14}C]dipalmitoyl phosphatidylcholine (DPPC) were purchased from the Amersham Corp., Arlington Heights, IL. DIDS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), rat serum IgG, quinine sulfate, furosemide, amiloride, vanadate free adenosine-5′-triphosphate (ATP), 8-bromo-cyclic adenosine-3′:5′-monophosphate (8-Br-cAMP), tetradecanoyl phorbol acetate (TPA), and other standard chemicals were obtained from the Sigma Chemical Co., St. Louis, MO. 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMDS) was purchased from Molecular Probes Inc., Eugene, OR. Terbutaline was obtained as a pharmacological formulation. Charybdotoxin was obtained from Receptor Chemicals Inc., Baltimore, MD, and tetraethylammonium chloride (TEA) was purchased from Fluka, Ronkonkoma, NY. Cell culture supplies were obtained from Flow Laboratories, McLean, VA, and bacteriological plates were obtained from Beckton

Dickenson Labware, Lincoln, NJ. Tissue culture plastic was obtained from Costar, Cambridge, MA. Diacylglycerol kinase were purchased from CalBiochem Corp., La Jolla, CA. Cardiolipin was purchased from Avanti Polar Lipids, Pelham, AL. Porcine elastase was purchased from the Worthington Biochemical Corp., Freehold, NJ.

Male Sprague–Dawley rats (180–200 g) were used for all studies reported here. Secretion of PC from isolated perfused rat lung was followed according to previously published protocol [11]. Briefly, lungs from anesthetized rats were removed for ventilation-perfusion according to Fisher *et al.* [13]. Lung PC was labeled with 5 μM [^{3}H-*methyl*]choline (sp. act. 0.1 mCi/nmol) by perfusion for 30 min. During this period, lungs were ventilated with 5% CO_2 in air. At the end of this labeling period, unlabeled choline was added to provide a final concentration of 200 μM. For some experiments, perfusion was terminated at this stage and lungs were lavaged to determine the release of labeled PC during this period ("zero" time). For others, perfusion was continued for the next 60 min and the lungs were either ventilated with 5% or 0% CO_2 in air. In some experiments where lungs were ventilated with 0% CO_2 in air, we also added 80 μM DIDS to the perfusion medium. At the end of each perfusion period, the lungs were lavaged with buffered saline (5 × 7 mL), and the cell-free lavage fluid and the lavaged lungs were processed for lipid extraction [14]; PC radioactivity was measured in the lipid extracts. Secretion was expressed as dpm in the lipid extract of lavage fluid × 100/[dpm in lipids of lavage fluid plus lung homogenate].

Alveolar type II cells were isolated after elastase digestion of lungs [15]. In brief, animals were anesthetized with sodium pentobarbital (50 mg/kg,i.p.) and tracheostomized. The lungs were cleared of residual blood by perfusion via pulmonary artery while maintaining ventilation. The lungs were removed, treated three times with endotracheally instilled elastase (3 U/mL), and minced with a tissue chopper. The tissue mince was suspended and mixed vigorously in a shaking water bath. The suspension was sequentially filtered through nylon filters of 160, 37, and 15 μm, and centrifuged for 10 min at 300 *g*. The pelleted cells were suspended in Eagle's Minimum Essential Medium (MEM), and incubated for 1 hr at 37° on IgG coated bacteriological culture dishes. Subsequently, the free cells were collected by "panning", centrifuged, suspended in MEM containing 10% fetal bovine serum and plated on 35 mm tissue culture dishes at a cell density of 8×10^5 cells in 1.5 mL MEM–10% fetal bovine serum. The cells were then incubated for 20–22 hr (overnight) at 37° in humidified air containing 5% CO_2. Cells attached to the culture dishes after overnight incubation were >92% type II as evaluated by staining with phosphine 3R and >95% of these excluded vital dye, erythrosin B.

PC secretion was studied after labeling of cellular lipids with [^{3}H-*methyl*]choline (0.3 μCi/mL) which was added to the culture medium (MEM) during overnight incubation. At the end of this incubation period, the cells attached to the culture dishes (approximately 2.5×10^5 cells) were washed five times with serum-free MEM, and then three times with incubation buffer (Krebs–Ringer–bicarbonate buffer) that was equilibrated with 5% CO_2 in air and contained 117.6 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 30 mM Hepes, pH 7.4 and 10 mM glucose. The cells were then equilibrated for 30 min in the incubation buffer and in 5% CO_2 in air. Some incubations were terminated at this stage, and results from these were used as the PC secretion at "zero" time. Other culture dishes were further incubated for indicated periods of time without change of the medium but with or without the addition of indicated agents. In some experiments, some of the culture dishes were transferred to 0% CO_2–air (alkalosis) and further incubated for 2 hr. In preliminary experiments, inhibitors of secretion were added during the 30-min equilibration period. Subsequent experiments showed that DIDS also inhibited PC secretion during the equilibration period and was equally effective if added just before the addition of a secretagogue. Therefore, for all studies reported here, DIDS was added at the end of the equilibration period.

At the end of each incubation period, the medium was collected and centrifuged for 10 min at 300 *g* to remove cells (a few) that came off during the incubation. These cells were pooled with those on the plate. Carrier egg PC (400 μg) and tracer [^{14}C]-DPPC were added to both cell and medium samples, and lipids were extracted [14]. Radioactivity was measured in a liquid scintillation counter in total lipid extract, since >95% of the label in lipid extract was in PC (results not shown) similar to that reported by others [16]. The data were corrected for recoveries based on ^{14}C counts in the samples. The secretion was expressed as dpm in the medium lipids × 100/[dpm in medium plus cell lipids].

For diacylglycerol studies, about 2.5×10^6 cells were plated on 35 mm tissue culture plastic dishes. After overnight incubation the attached cells (approximately 10^6) were equilibrated for 30 min in the incubation buffer, and in 5% CO_2 in air, and then were further exposed for 30 sec to either 5% CO_2 without or with the addition of 1 mM ATP, or 0% CO_2. In some cases, 100 μM DIDS was added to the culture dishes and cells were incubated for an additional 1 min, before the 30-sec exposure to each of the conditions as given above. At the end of the 30-sec incubation, the medium was aspirated rapidly and 1 mL chloroform:methanol (1:2, v/v) was layered over the cells. In view of the short period of treatment, the incubations were staggered so that only one culture dish was processed through the whole procedure at any one time.

Diacylglycerol in total lipid extract was measured after phosphorylation to phosphatidic acid with [γ-^{32}P]ATP [17]. In brief, an aliquot of lipid extract was dried in a stream of N_2, and then was solubilized by sonication in a solution containing 7.5% octyl-β-D-glucoside, 5 mM cardiolipin, and 1 mM diethylenetriamine pentaacetic acid (DETAPAC). The phosphorylation mixture contained 20 μL of solubilized lipids, 50 μL of reaction buffer [100 mM imidazole–HCl, pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM ethyleneglycolbis(aminoethyl-

ether)tetra-acetate (EGTA)], 2 μL of freshly prepared dithiothreitol, 10 μL of diacylglycerol kinase (20 mU/μL), and water to a total volume of 90 μL. The reaction was started with the addition of 10 μL of 10 μM [γ-^{32}P]ATP (sp. act., 4 μCi/nmol) in 100 mM imidazole plus 1 mM DETAPAC, pH 6.6. The reaction was conducted for 30 min at room temperature, and stopped with 1 mL of chloroform:methanol (1:2, v/v). Lipids were extracted [14], and phosphatidic acid was separated by thin-layer chromatography on silica gel plates in chloroform: methanol: acetic acid (65:15:5, by vol.). The radioactivity co-migrating with authentic phosphatidic acid was quantitated by scintillation counting. In agreement with Preiss *et al.* [17], the diacylglycerol kinase catalyzed reaction demonstrated a stoichiometry of 1 nmol of ATP incorporated per nmol of diacylglycerol (results not shown). Accordingly, the amount of diacylglycerol was calculated from cpm in phosphatidic acid/cpm per nmol of ATP. These results were normalized to total phospholipid calculated from determination of phospholipid phosphorus according to Marinetti [18].

The toxicity of various treatments was evaluated by analyzing the cells on the plate for erythrosin B exclusion. None of the treatments decreased erythrosin B exclusion. Toxicity of DIDS was also evaluated by investigating the reversibility of DIDS inhibition. In these experiments, type II cells were incubated for 30 min in the presence of 10, 50 or 100 μM DIDS. The cells were then washed (×3) with the incubation medium, incubated for 30 min, and then incubated in DIDS-free medium as described for the secretion studies.

All results on secretion from type II cells are expressed as net after subtraction of "zero" time secretion. Similarly, results with the isolated perfused lung model are given as the net secretion over that observed at the end of the 30 min of labeling with choline.

All experiments on secretion from type II cells were conducted in duplicate, and the results were averaged to obtain single data points for each set of observations. These data points were then evaluated for statistical significance by one-way analysis of variance or Student's *t*-test for paired experiments as appropriate, and were considered significantly different at a P value of < 0.05. Secretion experiments with the perfused lung model were conducted as unpaired experiments and were evaluated for statistical significance by one-way analysis of variance.

RESULTS

The secretion of PC in the perfused lung preparation was 0.9 ± 0.04% (mean ± SEM, N = 6) of the total lung PC during 1 hr of perfusion and ventilation with 5% CO_2 in air (control). Ventilation of lungs with 0% CO_2 in air increased the secretion to 2.2 ± 0.2% (N = 5, $P < 0.05$), as previously reported [11]. Addition of 80 μM DIDS to the perfusion medium, before change of the ventilation gas from 5% to 0% CO_2 in air, prevented the increase in PC secretion (0.89 ± 0.06%, N = 4). The perfusion medium pH after ventilation for 1 hr with 0% CO_2 in air was 7.71 ± 0.03 (N = 5) in the absence and 7.65 ± 0.01 (N = 3) in the presence of 80 μM DIDS, while the control pH was 7.40. These results suggest that the inhibitory effect of DIDS was unrelated to changes in the perfusate pH.

The basal secretion of PC from type II cells incubated in 5% CO_2 in air (control) increased as a function of time for up to 2 hr (results not shown). Cells that were equilibrated in 5% CO_2 in air for 30 min and then incubated for 2 hr in 0% CO_2 in air (alkalosis) showed an approximately 100% increase in PC secretion over that released by cells maintained in 5% CO_2–air (Table 1). This increase in PC secretion was blocked by 100 μM DIDS. In control cells also, the basal secretion was blocked by 100 μM DIDS.

Next, we evaluated the effect of DIDS on PC secretion elicited by a β-adrenergic agonist, terbutaline, and a P_{2y}-purinergic agonist, ATP. Terbutaline (0.1 mM) and ATP (1 mM) increased PC secretion by about 160 and 300%, respectively (Table 1). However, in each case, 100 μM DIDS blocked the secretion by about 80–90%. These results show that DIDS inhibition of secretion was not specific to any one type of stimulus.

DIDS inhibits Cl^-/HCO_3^- exchange in a number of cell types including type II cells [10]. Another structurally similar compound, SITS, is also an anion-exchange inhibitor and blocks the Cl^-/Cl^- exchange. We found that SITS was also inhibitory for the terbutaline- and ATP-stimulated secretion, although the degree of inhibition was less (Table 1). The inhibition of ATP-dependent secretion was further evaluated for the concentration dependence of DIDS and SITS (Fig. 1). The apparent 50% inhibition was observed at approximately 5 μM DIDS and 50 μM SITS. The fact that one of the isothiocyanato groups on the stilbene moiety of DIDS is substituted with an acetamido group in SITS suggests that the isothiocyanato groups contribute to the secretion inhibitory property of stilbene disulfonic acids. This was also supported by the observation that in one experiment AMDS (100 μM), another structural analog where both isothiocyanato groups of DIDS are substituted, did not inhibit ATP-stimulated secretion (results not shown).

We also investigated if the secretion of PC could be inhibited with DIDS when added after stimulation of cells with ATP. Cells were incubated in the presence of 1 mM ATP for various periods of time for up to 120 min. In some of the culture dishes, DIDS was added 30 and 60 min after incubation with ATP and the incubation was continued for a total of 2 hr. ATP-stimulated PC secretion increased with the time of incubation and, in this particular experiment, reached a plateau by 2 hr (Fig. 2). Addition of DIDS after 30 and 60 min of stimulation with ATP prevented any further increase in PC secretion. These observations show that the effect of DIDS was quite rapid.

DIDS and SITS were also inhibitory to surfactant secretion stimulated by 8-Br-cAMP (a cell permeable analog of cAMP) or TPA. In the absence of stilbene disulfonates, the secretion was increased by about

Table 1. DIDS and SITS inhibition of PC secretion from isolated type II cells

Addition	% [^{3}H]PC secreted in 2 hr		
	None	100 μM DIDS	100 μM SITS
None	0.91 ± 0.02 (13)	0.09 ± 0.06* (4)	0.02 ± 0.02 (2)
Alkalosis	2.50 ± 0.2 (3)	0.2 ± 0.2 (3)	
ATP, 1 mM	3.62 ± 0.63† (8)	0.36 ± 0.21* (4)	2.06 ± 0.47* (4)
Terbutaline, 0.1 mM	2.49 ± 0.29† (6)	0.50 ± 0.09* (3)	1.95 ± 0.22* (3)

Results are means ± SEM or range (where N = 2) of experiments conducted on the number of cell preparations indicated in parentheses. Cells were incubated for 30 min in Krebs–Ringer–bicarbonate buffer containing 30 mM Hepes, pH 7.4, and 10 mM glucose, in 5% CO_2–air. For alkalosis, cells were then transferred to 0% CO_2–air for the next 2 hr with or without the addition of DIDS or SITS. All other incubations were carried out in 5% CO_2–air for 2 hr in the absence or presence of the indicated agents.

* $P < 0.05$ vs in the absence of inhibitor.

† $P < 0.05$ vs no addition.

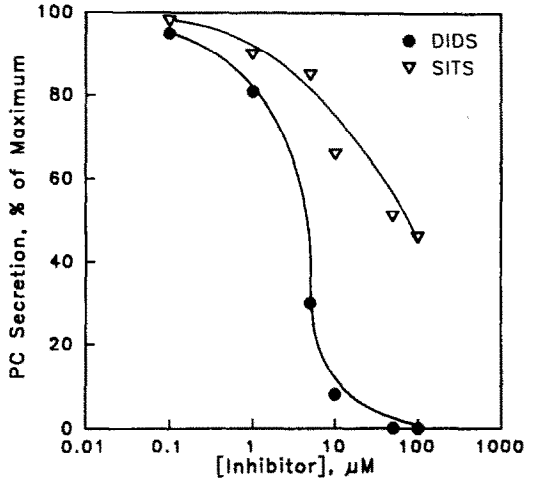

Fig. 1. Concentration dependence of DIDS and SITS inhibition of ATP-stimulated secretion of lung surfactant. DIDS and SITS were added 1 min before the addition of 1 mM ATP, and PC secretion during the 2-hr incubation was measured. Results are from one experiment representative of three with similar results. PC secretion in the presence of 1 mM ATP was 2.6% in the experiment with DIDS and 3.3% in the experiment with SITS. Control secretion was 0.5 and 0.7%, respectively.

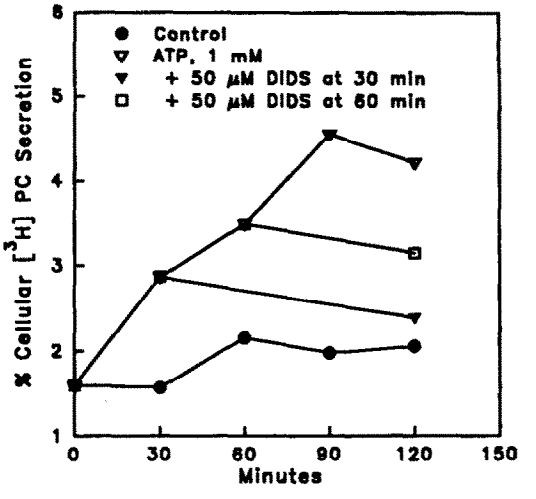

Fig. 2. DIDS inhibition of surfactant secretion from alveolar type II cells. ATP was added to cells at time 0, and the incubation continued for the next 2 hr. In some cases, 50 μM DIDS was added 30 or 60 min after the addition of ATP and the incubation continued for a total of 2 hr. Results are from one experiment in duplicate.

40% with 100 μM 8-Br-cAMP and 200% with 50 ng/mL TPA (Table 2). In the presence of 100 μM DIDS, the secretion with 8-Br-cAMP and TPA was inhibited by 93 and 98%, respectively. In parallel experiments, 100 μM SITS inhibited the secretion with each of these secretagogues by only 75 and 60%. Thus, the inhibitory potency (DIDS > SITS) was similar to that observed with ATP- and terbutaline-stimulated secretion. These results suggest that the site of action of DIDS and SITS is distal to the generation of second messenger in response to stimulation of β-adrenergic or purinergic receptors.

ATP stimulation of cells prelabeled with [^{3}H]-arachidonic acid increases the labeling of the diacylglycerol pool, suggesting elevated levels of diacyglycerol [5, 19]. We measured the diacylglycerol pool in type II cells in the presence and absence of 100 μM DIDS, with and without stimulation with a secretagogue. Exposure of cells to 0% CO_2 (alkalosis), or 1 mM ATP, for 30 sec increased the diacylglycerol content of cells by about 58 and 48%,

Table 2. Effect of stilbene derivatives on cAMP- and TPA stimulated PC secretion

Addition	%[^{3}H]PC secretion in 2 hr		
	None	100 μM DIDS	100 μM SITS
None	0.91 ± 0.20 (13)	0.09 ± 0.06* (4)	0.02 ± 0.02 (2)
8-Br-cAMP, 100 μM	1.26 ± 0.19† (3)	0.08 ± 0.05* (3)	0.32 ± 0.20* (3)
TPA, 50 ng/mL	2.78 ± 0.51† (3)	0.03 ± 0.03* (3)	1.11 ± 0.64* (3)

Results are means ± SEM, or range (where N = 2) of paired experiments conducted on the number of cell preparations indicated in parentheses. Stilbene derivatives were added 1 min before the addition of secretagogue.

* $P < 0.05$ vs control in the absence of inhibitor.

† $P < 0.05$ vs without agonist.

Table 3. Diacylglycerol content of alveolar type II cells

Addition	Diacylglycerol (nmol/mg phospholipid)	
	− DIDS	+ 100 μM DIDS
None	48.1 ± 2.7*	44.5 ± 3.7*
ATP, 1 mM	69.6 ± 5.8	57.5 ± 6.7
Alkalosis	76.4 ± 6.2	59.7 ± 4.6

Results are means ± SEM for paired experiments with 5 cell preparations. Cells were equilibrated in 5% CO_2–air for 30 min before the addition of 1 mM ATP or transfer of cells to 0% CO_2–air (alkalosis), and the incubation continued for the next 30 sec. In some cases, cells were incubated with 100 μM DIDS for 1 min before the addition of ATP or exposure to alkalosis.

* $P < 0.05$ vs other groups in the column by one-way analysis of variance.

respectively (Table 3). To investigate the effect of DIDS, cells were incubated with 100 μM DIDS for 1 min before stimulation with 1 mM ATP or 0% CO_2. In either case, the increases in diacylglycerol content in the presence or absence of DIDS were not statistically different. These results suggest that the site of action of DIDS was unlikely prior to phospholipase C mediated increase in diacylglycerol, and are in agreement with our earlier conclusion that the site of action of DIDS was distal to the generation of second messenger (Table 2).

The reversibility of the inhibitory effect was evaluated at 10, 50, or 100 μM DIDS as described in Materials and Methods. In four experiments, PC secretion during a 2-hr incubation in the absence or presence of 1 mM ATP was 0.9 ± 0.2% (mean ± SEM, N = 4) and 3.1 ± 0.2% ($P < 0.05$). In two of these experiments, the secretion was 0.6 ± 0.4% (mean ± range) with 1 mM ATP plus 10 μM DIDS, and 1.3 ± 0.3% in the absence and 3.0 ± 0.2% in the presence of 1 mM ATP in cells preincubated with 10 μM DIDS. Thus, the inhibitory effect of 10 μM DIDS was completely reversible. In one experiment, PC secretion during a 2-hr incubation was 0% with 1 mM ATP plus 50 μM DIDS, and in cells preincubated with 50 μM DIDS, it was 0.4% in the absence and 1.1% in the presence of 1 mM ATP, respectively. In another experiment, the secretion was 0.6% with 1 mM ATP plus 100 μM DIDS, and in cells preincubated with 100 μM DIDS, it was 0% in the absence or presence of ATP. This demonstrates that the inhibition became irreversible as DIDS concentration was increased. These results suggest that with increasing concentrations, DIDS binding to the cellular component is stronger and cannot be dissociated with our experimental protocol.

Several investigators have suggested that stilbene disulfonic acids inhibit secretion by blocking the ion-transport/exchange process [20–24]. Calcium ionophore-stimulated mucin secretion from T84 cells, a human colonic adenocarcinoma cell line, could be blocked with both DIDS and SITS, as well as K^+ channel antagonists, quinine sulfate and barium chloride [22]. In our hands, relatively non-specific inhibitors of Ca^{2+}-activated K^+ channel, quinine sulfate, barium chloride, or triethylamine [25], modestly inhibited (30–40%) ATP-stimulated secretion (Table 4). However, charybdotoxin, a specific antagonist of Ca^{2+}-activated K^+ channel [26], did not inhibit the ATP-stimulated secretion of PC in type II cells. These studies argue against a role for K^+ channel in the secretion of lung surfactant phospholipids.

Both DIDS and SITS anatagonize Cl^- entry into cells which can occur via two antiports: Cl^-/HCO_3^- and Na^+/H^+ exchange, or by Na^+K^+/Cl^- co-transport [21]. Of these, Na^+/H^+ exchange is blocked by amiloride [27], while co-transport is blocked by furosemide and bumetinide. We, therefore, evaluated PC secretion in the presence of amiloride, furoseimide, or bumetinide (Table 5). Of these, only amiloride slightly inhibited ATP-stimulated secretion (30%). The lack of a major inhibition of secretion with any of these compounds suggests that Cl^- transport is not involved in surfactant secretion.

Next, we investigated if DIDS and SITS blocked secretion by inhibition of OH^- transport, which is suggested to facilitate granule swelling prior to

Table 4. Effect of K^+ channel antagonists on ATP-stimulated secretion

	N	% [^{3}H]PC secretion in 2 hr	% Inhibition
Control	12	1.17 ± 0.22	
ATP, 1 mM	12	3.79 ± 0.30*	0
+ TEA, 1 mM	5	2.64 ± 0.41†	30
+ TEA, 10 mM	7	2.73 ± 0.37†	28
+ Quinine sulfate, 0.5 mM	4	2.50 ± 0.37†	34
+ $BaCl_2$, 5 mM	5	2.31 ± 0.43†	39
+ Charybdotoxin, 10 nM	5	3.65 ± 0.70*	4

Results are means ± SEM of experiments with N cell preparations. TEA = tetraethylammonium chloride.
* $P < 0.05$ vs control (paired t-test).
† $P < 0.05$ vs ATP-stimulated secretion (paired t-test).

Table 5. Effect of ion transport inhibitors on [^{3}H]PC secretion from type II cells

	% [^{3}H]PC secretion in 2 hr	% of Control
Control	0.7 ± 0.08* (4)	100
ATP, 1 mM	3.66 ± 0.43 (4)	523 ± 60
+ Amiloride, 0.1 mM	2.62 ± 0.25 (3)	374 ± 35
+ Furosemide, 1 mM	2.96 ± 0.45 (3)	423 ± 62
+ Bumetinide, 0.1 mM†	4.21 ± 0.44 (2)	601 ± 62

Results are means ± SEM or range (where N = 2) of experiments conducted on the number of cell preparations indicated in parentheses.
* $P < 0.05$ from all other groups by one-way analysis of variance.
† The combination of ATP + bumetinide was not evaluated for statistical significance.

exocytosis [24]. We evaluated surfactant secretion in the presence of probenecid and of pyridoxal phosphate. These two are structurally different from DIDS and SITS, inhibit anion transport [28], and block serotonin secretion from platelets [24]. Neither of these agents inhibited ATP-stimulated surfactant secretion. In three paired experiments, the secretion during 2 hr was 0.5 ± 0.1% in the absence and 2.3 ± 0.4% ($P < 0.05$) in the presence of 1 mM ATP. The secretion in the presence of ATP and 1 mM pyridoxal phosphate or probenecid was 2.1 ± 0.5 and 2.0 ± 0.4%, respectively.

Since both Cl^- and OH^- have been implicated for secretory granule swelling during exocytosis, we postulated that either of the two anions (Cl^- or OH^-) was able to provide the driving force for granule swelling. We, therefore, evaluated the effect of probenecid in Cl^- free medium (Cl^- salts of Na^+, Ca^{2+}, and K^+ were replaced by 59 mM Na_2SO_4, 1.3 mM $CaSO_4$, and 4.7 mM K^+-gluconate). Under these conditions 1 mM ATP increased the secretion from 1.6 ± 0.3 to 3.2 ± 0.3% ($N = 4$; $P < 0.05$), an increase of 100%. However, secretion in the presence of ATP and 1 mM probenecid was only 2.2 ± 0.3% ($P > 0.05$ vs controls). Collectively, these observations suggest that anion (Cl^- or OH^-) transport likely contributes to the secretion of lung surfactant.

DISCUSSION

In this report, we have demonstrated that DIDS and SITS, stilbene disulfonic acids, are potent inhibitors of surfactant secretion. The cellular site of action of these compounds is not known. Our experiments on the reversibility of the effect of DIDS suggest multiple sites for the action of DIDS. A partial reversibility of the inhibitory effect at 50 and 100 μM DIDS suggests that the site of action is intracellular. However, a complete reversibility at 10 μM DIDS, a concentration that caused almost 100% inhibition (Fig. 1), suggests the cell surface as another possible site of DIDS action.

One possible site in the vicinity of the plasma membrane is the site of agonist–receptor interaction. However, our results did not support this to be the process affected. First, DIDS inhibited surfactant secretion even when added after incubation of cells with ATP, i.e. after the association of ATP with its putative receptor(s). Second, DIDS inhibited the secretagogue effect of both terbutaline and ATP, each of which acts via a different receptor. Other investigators have also shown that SITS inhibits adenocorticotropin and serotonin secretion in response to agonists for various receptors [21, 24]. It is unlikely that all of these receptors share common

domains for DIDS binding which, in turn, interferes with the agonist–receptor interaction.

The signal from agonist for β-adrenergic or purinoceptor is transduced via activation of adenyl cyclase or phospholipase C to increase cAMP or diacylglycerol, respectively. Our results rule out any of these as the sites for DIDS action, since DIDS did not block the ATP- or alkalosis-stimulated increase in diacylglycerol content of type II cells. Further, DIDS inhibition of secretion stimulated by 8-Br-cAMP or TPA also indicates that the site of DIDS action is distal to the generation of second messengers, similar to that proposed for SITS inhibition of TPA-, forskolin-, or calcium ionophore-stimulated adenocorticotropin secretion from anterior pituitary cells [21].

The mechanism of stilbene disulfonate inhibition of secretion is not clear. Various investigators have suggested that secretion may be dependent on the transport of ions across the membranes [20–24]. Similar to our results, DIDS inhibited mucin secretion from T84 cells, and the inhibition was reversible at low concentrations of DIDS [22]. Since triethylamine, quinine sulfate, and $BaCl_2$ were also somewhat inhibitory, these investigators suggested a role for K^+ channel in mucin secretion. Marcon *et al.* [22] suggested that an inhibition of K^+ and Cl^- fluxes at the secretory granule level may prevent membrane-fusion events. Although like some other secretory granules [29], lamellar bodies may transport K^+ and Cl^- [30], we observed only a modest inhibition of surfactant secretion with less specific antagonists of K^+ channel (Table 4). Further, charybdotoxin, a specific antagonist of Ca^{2+}-activated K^+ channels [26], failed to block surfactant secretion. Therefore, we propose that DIDS does not act as an antagonist for K^+ channels to inhibit surfactant secretion.

Exocytosis of lung surfactant takes place after fusion of lamellar bodies with the plasma membrane, and is common to both the basal and stimulated secretion of lung surfactant. In other systems, the sequence of events during exocytosis is postulated as a complex process that includes (1) membrane fusion, (2) granule swelling, and (3) extrusion of contents. Granule swelling occurs due to ion transport associated water influx into the granule [23]. The ion species required to be transported appears to be dependent on cell type, since both Cl^- and OH^- have been implicated [20, 21, 23, 24, 31]. However, unlike inhibition of parathyroid hormone [20], we did not observe any inhibition of surfactant secretion in Cl^--free medium (Cl^- substituted with gluconate and sulfate). Instead, the secretion was increased due to possible alkalinization, since transfer of type II cells to Cl^--free medium inhibits the HCO_3^- efflux and raises the pH [10]. Lack of inhibition of surfactant secretion with furosemide or bumetinide also suggests that a specific Cl^- transport is not required for surfactant secretion. Similarly, a specific influx of OH^- to induce granule (lamellar body) swelling [20, 24] is also not required since probenecid and pyridoxal phosphate were ineffective as blockers of surfactant secretion. Nonetheless, it is possible that granule swelling may not be specific to one particular anion, and that either Cl^- or OH^- can sustain exocytosis of surfactant. Some support for this is derived from the fact that probenecid was inhibitory for ATP-stimulated secretion of surfactant in Cl^--free medium.

Finally, DIDS could also be inhibitory to the fusion between lamellar bodies and plasma membrane that occurs prior to suggested granule swelling. DIDS is known to bind to a variety of proteins like Band 3 (Cl^-/HCO_3^- exchange protein) of red cells [32], and ($Ca^{2+} + Mg^{2+}$)ATPase of plasma membrane [33]. Recently, Cardin and associates [34] demonstrated that DIDS binds to Lys^{90} residue of CD4, a glycoprotein expressed on the cell surface of T helper lymphocytes and macrophages [35], and antagonizes CD4 binding to gp120 to block cell–cell fusion. The inhibitory potency (DIDS > SITS) for such cell–cell fusion [34] was similar to that observed for lung surfactant secretion (Fig. 1). Our unpublished results show that DIDS also bound to synexin and blocked synexin-mediated liposome fusion. Such binding of DIDS to synexin could occur in type II cells and conceivably inhibit synexin-augmented fusion between the lamellar bodies and the plasma membrane [36]. Although erythrocytes are impermeable to DIDS [37], cellular differences in permeability to this compound cannot be ruled out. Based on this reasoning, we speculate that DIDS binds to synexin in close proximity to the plasma membrane, and the resulting DIDS–synexin complex is rendered non-functional for membrane fusion.

In summary, we have shown that stilbene disulfonic acids inhibit both the basal and stimulated secretion of lung surfactant. Since the site of this inhibition is distal to the generation of second messengers, we propose that the most likely site of action is the process of exocytosis at the plasma membrane. However, further experiments are needed to demonstrate if this site is the protein-mediated membrane-fusion event and/or subsequent anion-influx-induced granule swelling, that may occur before the extrusion of lamellar body contents.

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