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Reactive blue 2 selectively inhibits P_{2y}-purinoceptor-stimulated surfactant phospholipid secretion from rat isolated alveolar Type II cells

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1 The effect of reactive blue 2 on adenosine 5′-triphosphate (ATP), 12-O-tetradecanoylphorbol 13-acetate (TPA) and terbutaline-induced surfactant phospholipid secretion from rat isolated alveolar Type II cells was studied.

2 Reactive blue 2 significantly inhibited ATP-induced surfactant phospholipid secretion, but was without effect on C-kinase agonist (TPA) or β-adrenoceptor agonist (terbutaline)-stimulated surfactant phospholipid secretion. The IC_{50} for inhibition of ATP-induced surfactant secretion was 1.5×10^{-4} M.

3 These data are consistent with a P_{2y}-purinoceptor regulating surfactant phospholipid secretion from isolated Type II cells and support previous work suggesting reactive blue 2 is a specific inhibitor at P_{2y}-purinoceptors.

Introduction

Type II cells synthesize and secrete surfactant phospholipid in response to a variety of secretagogues (Hollingsworth & Gilfillan, 1984). Adenosine 5′-triphosphate (ATP) remains one of the most potent secretagogues for surfactant phospholipid secretion identified to date (Rice & Singleton, 1986; Gilfillan & Rooney, 1987). While the precise mechanism of ATP-induced surfactant phospholipid secretion is not known, previous work demonstrated calcium mobilization in association with binding of exogenous ATP to a receptor, and based on the rank order of agonist potency suggested a P_{2y}-subtype purinoceptor was responsible for mediating ATP-induced surfactant phospholipid secretion (Rice & Singleton, 1987). Burnstock & Kennedy (1985) had previously suggested the division of P_2- purinoceptors into P_{2x} and P_{2y} subtypes, based on rank order of agonist potency, and further work provided data suggesting reactive blue 2 was a specific antagonist of P_{2y}-purinoceptor-mediated responses (Burnstock & Warland, 1987; Houston *et al.*, 1987). Reactive blue 2, which does not affect responses mediated by P_{2x}-purinoceptors, has previously been proposed as an ATP antagonist (Kerr & Krantis, 1979; Choo, 1981). In the present work, to support further the hypothesis that ATP-induced surfactant phospholipid secretion is mediated via a P_{2y}-purinoceptor, we tested the effect of reactive blue 2 on ATP-induced surfactant phospholipid secretion compared to the effect of reactive blue 2 on C-kinase agonist 12-O-tetradecanoylphorbol 13-acetate (TPA)- and β-adrenoceptor agonist (terbutaline)-stimulated surfactant phospholipid secretion.

Methods

Animals

Pathogen-free male Sprague-Dawley rats (200–250 g) were obtained from Charles River (Wilmington, MA).

Isolation and culture of Type II cells

Type II cells were isolated from rat lungs by a modification of the method described by Dobbs *et al.* (1986). Rats were maintained under sterile guard hooded cages and allowed food and water *ad libitum* before the experiment. Rats were then anaesthetized with sodium pentobarbitone and lungs perfused via the pulmonary artery with buffer A (NaCl 125 mM, KCl 5 mM, Na_2HPO_4 2.5 mM, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 17 mM,

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glucose 1 mg ml^{-1}, gentamycin 10 µg ml^{-1}, penicillin 20 u ml^{-1}, pH 7.4) containing 3% bovine serum albumin (w/v). Lungs were then lavaged as previously described (Rice & Singleton, 1986) and incubated with elastase solution (40 ml, 0.4 units ml^{-1} in buffer A) for 20 min at 37°C (Dobbs *et al.*, 1986). Tissue was minced and filtered through progressively smaller Nitex gauze as described and washed in buffer A. Cells were then resuspended in Dulbecco's Modified Eagle's medium and incubated on 100 mm bacteriological plastic Petri dishes which had been precoated with 500 µg ml^{-1} IgG for 3 h at room temperature. Following a 1 h incubation at 37°C in 5% CO_2 in air, the plates were 'panned' as described previously (Dobbs *et al.*, 1986) and the unattached cells removed and collected by centrifugation. Isolated cells were resuspended in Dulbecco's Modified Eagle's medium (GIBCO, Grand Island, NY, U.S.A.) containing 10% foetal calf serum and utilized for experiments following 18 h in culture at 37°C in 5% CO_2. This procedure routinely yielded 40×10^6 cells per rat. For release experiments, cells were diluted in plating medium to 0.5×10^6 cells ml^{-1}. Cell suspension (1 ml) was placed in each well of a 24 well tissue culture plate which had been precoated with collagen (Collaborative Research, Lexington, MA, U.S.A.) and prelabelled by addition of 1 µCi of [^{3}H]-choline, (specific activity, 80.0 Ci mmol^{-1}). The plating efficiency at 18 h was generally 40–50%. Non-adherent cells were removed from the wells by washing before the assay. Cultures contained $89 \pm 2\%$ viable Type II cells as determined by fluorescence staining with phosphine 3R.

Secretion of phosphatidylcholine

Secretion of phosphatidylcholine (PC) by cultured Type II cells was determined as previously described (Rice & Singleton, 1986). Briefly, cells incubated overnight were washed 3 times with Dulbecco's Modified Eagle's medium containing 40 mM HEPES and 3 mg ml^{-1} bovine serum albumin, pH 7.4, 37°C (buffer B). Cells were allowed to equilibrate for 30 min and at the end of this time, agents were added and [^{3}H]-PC released was determined after 3 h. Medium was aspirated and the cells were washed with 0.5 ml of fresh medium. The two samples of medium were then combined and centrifuged at 9000 *g* for 5 min to pellet the cells. Supernatant was removed and lipid extracted according to Folch with addition of 1 mg of egg-PC as a carrier (Folch *et al.*, 1975).

Cells remaining in the wells were extracted with 1 ml ethanol × 2 and fractions obtained as for the medium. Samples containing lipid were dried overnight and radioactivity determined with a β-scintillation counter after addition of 5.0 ml of Scintiverse II to each sample. Dipalmitoyl [^{14}C]-phosphatidylcholine was used as an internal standard to follow recoveries of [^{3}H]-PC which were generally $95 \pm 1\%$. The amount of [^{3}H]-PC secretion was calculated as the percentage of total [^{3}H]-PC present in the medium relative to the amount present in cells (i.e., c.p.m. in medium/(c.p.m. in medium + c.p.m. in cells)). The amount of [^{3}H]-PC released following the 30 min preincubation was subtracted for all samples.

Lactate dehydrogenase activity was determined in each sample as a measure of cytotoxicity by adding an aliquot (0.3 ml) to 0.5 ml of 250 µM 3-[N-morpholino]propane sulphonic acid, pH 7.0, and 0.1 ml of freshly prepared 10 mM NADH. The reaction was initiated by adding 0.1 ml of sodium pyruvate and the enzyme activity determined by following the decrease in absorbance at 340 nm. Total activity was determined by treating sample plates with 0.1% Triton X-100.

Statistical analyses

Kruskall-Wallis one-way analysis of variance by ranks (Kruskall & Wallis, 1952) or Friedman two-way analysis of variance by ranks (Friedman, 1937) for non-parametric data were utilized as appropriate. Analysis of variance for both parametric and non-parametric data was performed on an IBM PC XT microcomputer using commercially available statistical packages.

Materials

Methyl [^{3}H]-choline chloride and dipalmitoyl [^{14}C]-phosphatidylcholine were purchased from New England Nuclear (Boston, MA, U.S.A.). Chloroform, methanol and Scintiverse II were from Fisher Chemical Co. (Cincinnati, OH, U.S.A.). ATP, TPA and terbutaline were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Elastase was obtained from Cooper Biomedicals (Malvern, PA, U.S.A.).

Results

ATP potently stimulated [^{3}H]-phosphatidylcholine secretion, as previously observed. This ATP-stimulated [^{3}H]-phosphatidylcholine secretion was inhibited by reactive blue 2 in a dose-dependent fashion (Figure 1). The IC_{50} (concentration producing 50% of the maximal inhibition) for inhibition of ATP-induced [^{3}H]-PC secretion by reactive blue 2 was $\sim 1.5 \times 10^{-4}$ M.

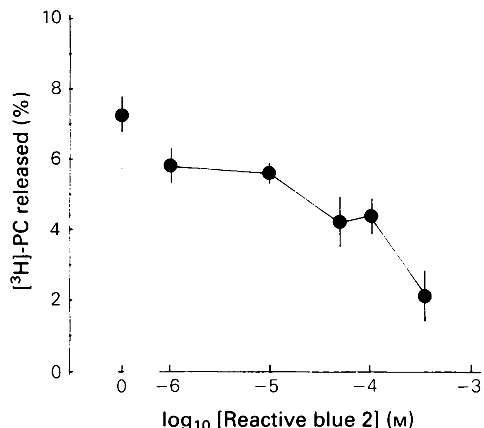

Figure 1 The effect of reactive blue 2 on ATP-induced [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to ATP (10 μM). This concentration of ATP was previously shown to give maximal stimulation of [^{3}H]-PC release (Rice & Singleton, 1986). Reactive blue 2 was present at the indicated concentrations for 30 min before the addition of ATP. Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean ($n = 6$ experiments, 3 replicates in each) and vertical lines show the s.e.mean. Reactive blue 2 significantly inhibited ATP-induced [^{3}H]-PC release ($P = 0.0001$).

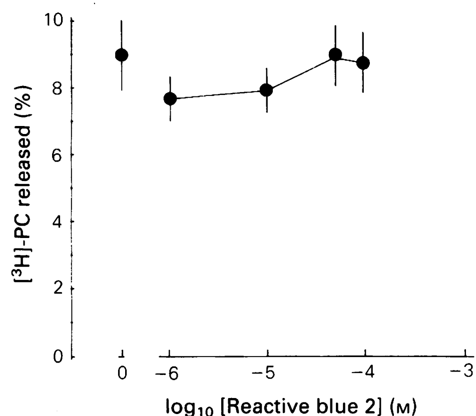

Figure 2 Effect of reactive blue 2 on 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to TPA (100 nM). This concentration of TPA was previously shown to give maximal stimulation of [^{3}H]-PC release (Dobbs & Mason, 1978). Release of [^{3}H]-PC is expressed as stated in Methods. Data represent the mean ($n = 4$ experiments, 3 replicates in each). Vertical lines show the s.e.mean. Reactive blue 2 did not have a significant effect on TPA-induced [^{3}H]-PC release at the concentrations tested.

To examine the specificity of the reactive blue 2 inhibition, we also tested reactive blue 2 for its ability to inhibit β-adrenoceptor agonist (terbutaline)-induced surfactant phospholipid secretion and 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced surfactant secretion mediated by C-kinase. Reactive blue 2 had no significant effect on TPA-induced surfactant secretion (Figure 2) nor did it significantly inhibit terbutaline-induced surfactant secretion (Figure 3).

None of the agents utilized for these experiments produced a statistically significant release of lactate dehydrogenase above control levels which were 1–2% of total cellular lactate dehydrogenase released after a 3 h incubation.

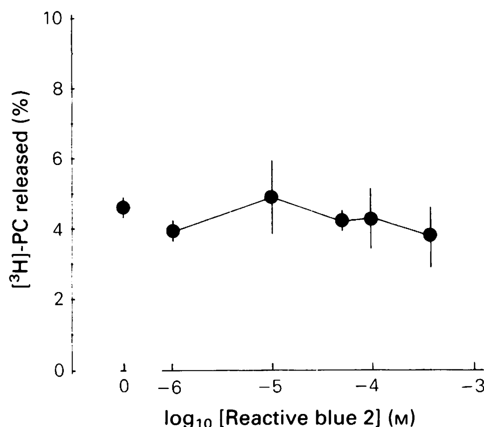

Figure 3 The effect of reactive blue 2 on terbutaline-induced [^{3}H]-phosphatidylcholine ([^{3}H]-PC) release. Release of [^{3}H]-PC was determined after a 3 h exposure of Type II cells to terbutaline (10 μM). This concentration of terbutaline was previously shown to give maximal stimulation of [^{3}H]-PC release (Dobbs & Mason, 1979). Reactive blue 2 was present at the indicated concentrations for 30 min before the addition of terbutaline. Data represent the mean ($n = 4$ experiments, 3 replicates in each). Vertical lines show s.e.mean. Reactive blue 2 had no significant effect on terbutaline-induced [^{3}H]-PC secretion at the concentrations tested.

Discussion

Exogenous ATP is a potent stimulus for surfactant phospholipid secretion from isolated alveolar Type II cells (Rice & Singleton, 1986; Gilfillan & Rooney, 1987). Previous work suggested the receptor on the Type II cell mediating this effect was a P_{2y}-purinoceptor based on rank order of agonist potency (Rice & Singleton, 1987). The present work supports this hypothesis.

Reactive blue 2 has been purported to be a specific inhibitor of P_{2y}-purinoceptors in rabbit and rat portal vein (Reilly *et al.*, 1987), rabbit mesenteric

artery (Burnstock & Warland, 1987), canine endothelium (Houston *et al.*, 1987) and rat caecum (Manzini *et al.*, 1986). The present work supports the specificity of reactive blue 2 at P_{2y}-purinoceptors, since reactive blue 2 potently inhibited surfactant phospholipid secretion induced by ATP, while reactive blue 2 did not have an effect on surfactant phospholipid secretion induced by the phorbol ester TPA or the β-agonist terbutaline which act via protein kinase C (Sano *et al.*, 1985) and cyclic AMP-dependent protein kinase (Rice *et al.*, 1985), respectively. The IC_{50} which we observed for reactive blue 2 inhibition of ATP-induced surfactant secretion was 1.5×10^{-4} M. This compares favourably with an IC_{50} of 4×10^{-4} M previously obtained for inhibition of junction potentials of the rat caecum by reactive blue 2 (Manzini *et al.*, 1986).

While inhibition of ATP-induced surfactant phospholipid secretion by reactive blue 2 could be the result of cytotoxicity, this does not appear probable for two reasons. Reactive blue 2 at similar concentrations had no effect on surfactant phospholipid secretion induced by other secretagogues (terbutaline and TPA) and release of a cytosolic marker (lactate dehydrogenase) was no different in control cells compared to cells exposed to various concentrations of reactive blue 2. The concentrations of reactive blue 2 utilized for the present study are also less than concentrations used in previous studies when cytotoxicity was also not noted (Manzini *et al.*, 1986; Reilly *et al.*, 1987; Burnstock & Warland, 1987).

In conclusion, we demonstrated that reactive blue 2 is a specific inhibitor of ATP-induced surfactant phospholipid secretion. Previous work supports the hypothesis that ATP-induced surfactant secretion is mediated via a P_{2y}-purinoceptor (Rice & Singleton, 1987), consistent with the present data. The present work also supports the previous observations of Burnstock and colleagues (Manzini *et al.*, 1986; Reilly *et al.*, 1987; Burnstock & Warland, 1987; Houston *et al.*, 1987) suggesting reactive blue 2 is a specific inhibitor of P_{2y}-purinoceptors. Further work to design a more potent antagonist of P_{2y}- purinoceptors based on the structure of reactive blue 2 may prove useful.

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