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Control of alveolar surfactant in rats at rest and during prolonged hyperpnoea: pharmacological evidence for two tissue pools of surfactant

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1 Propranolol, atropine and indomethacin (i.p.) affect neither the amount (PLalv), nor the specific activity ($PLalv_{sp.act.}$) of alveolar surfactant-type phospholipids lavaged from the lungs of unanaesthetized rats, either at rest or made hyperpnoeic for 24 h with $5\%CO_2/13\%O_2/82\%N_2$.

2 Whereas salbutamol (280 $\mu g\,kg^{-1}$ body weight, i.p.) consistently increased PLalv and $PLalv_{sp.act.}$, pilocarpine (1.5, 3, 10 and 50 $mg\,kg^{-1}$, i.p.) and labetalol (1 and 5 $mg\,kg^{-1}$, i.p.) had no effect. The dose of pilocarpine reported by others to release surfactant (150 $mg\,kg^{-1}$) induced marked salivation, diarrhoea, chromodacryorrhoea and a three-fold increase in tidal volume.

3 In the isolated perfused lung of the rat, salbutamol (1.5 μM) consistently increased $PLalv_{sp.act}$, whereas pilocarpine (0.1 and 1 μM) had no effect on these variables.

4 In the isolated perfused lung, the maximum amount of surfactant that could be released by salbutamol (0.5 mM) was smaller than that which could be released in response to an increase in tidal volume (peak inflation pressure 28 cmH_2O).

5 When the concentration of salbutamol in the isolated perfused lung was adjusted to produce the same increase in PLalv as did a single simulated deep breath, the $PLalv_{sp.act}$ was significantly increased by salbutamol, but not by the simulated deep breath.

6 We conclude, that neither the autonomic nervous system nor the prostaglandin system is essential for the release of surfactant at rest or during hyperpnoea. Furthermore, we suggest that two pools of surfactant, with different release mechanisms, exist in lung tissue.

Introduction

Pulmonary surfactant is a complex mixture of lipids and proteins that is synthesized in the alveolar type II cell where it is stored in lamellar bodies. In response to a number of different stimuli, lamellar bodies are released into the alveolar hypophase where they unravel to form tubular myelin; this in turn gives rise to a phospholipid monolayer which greatly modifies surface tension at the gas-liquid interface and stabilizes the lung.

A well-documented stimulus for release of surfactant is an increase in tidal volume (Vt). This has been reported in both unanaesthetized (Nicholas *et al.*, 1982a, Power *et al.*, 1986) and anaesthetized animals (Oyarzun & Clements, 1978; Klass, 1979; Nicholas & Barr, 1983); both stimulatory and inhibitory factors may be involved *in vivo* (Nicholas & Barr, 1983). In the isolated perfused lung of the rat, release is directly related to the Vt value, once Vt exceeds the normal resting value (Nicholas & Barr, 1981); surfactant can even be released by a single simulated deep breath (Nicholas *et al.*, 1982b). Release in the isolated lung does not involve β-adrenergic, cholinergic, histaminergic, 5-hydroxytryptaminergic or prostaglandin systems. Importantly, it is not affected by tetrodotoxin (Nicholas & Barr, 1981), indicating that intrapulmonary neurones are not required for this response. We suggested that surfactant is released in response to direct distortion of the type II cell (Nicholas *et al.*, 1982a; Nicholas & Barr, 1983).

A wide range of drugs are reported to release surfactant (Hollingsworth & Gilfillan, 1984). Of particular interest are those drugs that act on effector sites for the autonomic nervous system. Specifically, β-

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adrenoceptor agonists are consistently shown to increase surfactant release in the foetal lung (Enhorning *et al.*, 1977), in adult lung slices (Gilfillan *et al.*, 1983) and in the isolated perfused lung (Nicholas & Barr, 1981; Brown & Longmore 1981; Massaro *et al.*, 1982). Furthermore, these drugs release surfactant from isolated alveolar type II cells (Dobbs & Mason, 1979; Mettler *et al.*, 1981) and β-adrenoceptors have been identified in these cells (Smith & Sidhu, 1984). There is much less concensus over the ability of drugs acting on muscarinic cholinoceptors to induce release. The apparent inability of these drugs to release surfactant from the isolated type II cells (Dobbs & Mason, 1979; Mettler *et al.*, 1981; Brown & Longmore, 1981) and the rat isolated perfused lung (Nicholas & Barr, 1981), coupled with the observation that in the rabbit foetus the action of pilocarpine could be blocked by β-adrenoceptor antagonists (Corbet, 1981), lead to the suggestion that, *in vivo*, these drugs acted via the sympathetic nervous system. Muscarinic cholinoceptors could not be identified on type II cells. Although type II cells certainly possess β-adrenoceptors, there is no convincing proof that these cells are innervated. Whereas salbutamol consistently releases a limited amount of surfactant and this in turn can be blocked by propranolol, the release induced by an increased Vt was much larger and could not be blocked (Nicholas & Barr, 1981). We suggested that two pools of tissue surfactant exist: a large pool that turns over slowly and is released in response to type II cell distortion, and a small pool that turns over rapidly and is under sympathetic nervous control (Nicholas *et al.*, 1982a; Nicholas & Barr, 1983). In the present paper we have further examined the possible role of the β-adrenergic, cholinergic and prostaglandin systems in control of release of surfactant in unanaesthetized resting rats and in rats with hyperpnoea induced by altering the inspired gas. In addition, we have used the isolated perfused lung preparation from the rat to test our hypothesis that two pools of tissue surfactant supply the alveolar compartment.

Methods

Male Porton rats (210–270 g) were lightly anaesthetized with sodium methohexitone (40 mg kg^{-1} body weight, i.p.) (Eli Lilly, Australia). In most rats, an incision was then made at the hilum of the tail and a size 26 needle shaft, attached to a length of polyethylene tubing, was inserted into a lateral caudal vein. [Methyl-^{3}H]-choline chloride (20 μCi kg^{-1} body weight) (New England Nuclear, Boston, U.S.A.) was infused at 71 μl min^{-1} to a volume of 1 ml kg^{-1} body weight; the infusion took about 3.5 min. The needle was removed and the rat regained consciousness within 30 min.

In vivo *experiments*

First regimen The rat was given an i.p. injection of putative secretagogue antagonist or putative secretagogue, 1 or 4 h respectively after starting the choline infusion. Exactly 5 h after the start of the infusion, the rats were heavily anaesthetized with 160 mg kg^{-1} body weight i.p. of sodium methohexitone, a tracheal catheter was inserted and the lungs were ventilated at 60 breaths min^{-1} with air (Vt of 2.5–3.0 ml; end expired pressure of 2 cmH_2O). The vascular bed of the lungs was then perfused as previously described (Nicholas *et al.*, 1982a). The actual period of perfusion was about 3 min and was sufficient to flush blood from the vascular bed. The lungs were next removed and lavaged as described below.

Second regimen The test rats were made hyperpnoeic by placing them in a 6 compartment Plexiglas chamber maintained at 22°C and through which humidified gas (5% CO_2/13% O_2/82% N_2) was flushed at 51 min^{-1} for 24 h (Power *et al.*, 1986). A parallel series of control rats were placed in an identical chamber, but through which humidified medical grade air was circulated. The test rats were injected i.p. with one of: propranolol (5 mg kg^{-1} body weight in saline), atropine sulphate (3 mg kg^{-1} in saline) or indomethacin (15 mg kg^{-1} in dimethylsulphoxide [DMSO]). Control rats were injected with either saline or DMSO. The rats in the chambers were injected at time 0, 6, 18 and 21 h. At 19 h all rats were infused with [^{3}H]-choline, as above, and lungs were removed and lavaged (see below) at 24 h.

Impedance pneumograph The instrument was constructed as described previously (Power *et al.*, 1986), and was calibrated using an anaesthetized rat ventilated by positive pressure on a rodent respirator. In the present paper the pneumograph was used only to monitor breathing in one unanaesthetized rat following administration of pilocarpine at 150 mg kg^{-1} body weight, i.p.

In vitro *experiments*

Isolated perfused lung experiments One hundred and fifty min after the infusion of [^{3}H]-choline, the rat was again anaesthetized and the lungs were isolated and perfused as previously described (Nicholas & Barr, 1981). Briefly, they were ventilated with 5% CO_2/95% O_2 at 60 breaths min^{-1}, Vt of 2.5 ml and an end expired pressure of 2 cmH_2O. The thorax was opened and catheters placed in the main pulmonary artery via the right ventricle, and in the left atrium via the left ventricle. Without interrupting the circulation, the lungs were perfused at 10 ml min^{-1} with Krebs bicar-

bonate containing 4.5% bovine serum albumin (BSA Cohn fraction V). Finally the lungs were removed from the thorax and placed in a closed chamber saturated with water vapour at 37°C; the positive pressure ventilation continued. Putative secretagogues were placed in the 50 ml recirculating reservoir 30 min before lavage. A simulated deep breath was induced by increasing peak inflation pressure from 10 to 20 cmH_2O for a single breath. The lungs were hyperventilated by increasing peak inflation pressure (and hence Vt) to 20 cmH_2O and decreasing the end expired pressure from 2 to 0 cmH_2O for a 5 min period. We suggest it unlikely that either the drugs or the lavaging technique appreciably damaged the epithelium, for the levels of lactate dehydrogenase (LDH) in the lavage fluid remained very low and did not differ between the controls and test lungs. The viability of the isolated perfused lung preparations was checked by continually monitoring the perfusion pressure and the peak inflation pressure and by measuring the wet:dry lung weight ratio at the end of the experiments. If, during the perfusion, there was an increase in perfusion pressure of more than 5 mmHg, or of peak inflation pressure of more than 4 cmH_2O, or if the wet:dry lung weight ratio was above 5.5, the lung was discarded. In addition, we had previously used transmission electron microscopy to check that the lavaging method did not damage the lungs. We minimized the possibility that lavaging *per se* released surfactant by lavaging 22°C. Previously we have shown that if an isolated perfused lung was subjected to an increased Vt while maintained at 22°C, surfactant was not released (Nicholas & Barr, 1983).

Method of lung lavage The lungs were degassed in a vacuum dessicator at 0.5 atmospheres for exactly 1 min. They were then lavaged with three separate equal volumes of 0.15 M saline at 22°C (40 ml kg^{-1}, body weight), each volume being instilled and withdrawn 3 times. This yielded more than 85% of phospholipid recovered following 8-triple lavages. The total recovered lung lavage was centrifuged at 150 *g* for 5 min to remove macrophages and sloughed cells. Randomly selected lavages were then sampled for measurement of LDH as an indicator of possible epithelial damage, and the remainder was lyophilised. We used the LDH-L reagent optimised u.v. method (Reagents Applications Inc, CA) to measure LDH. The limit of detectability was 2.2 mu ml^{-1} and in the present experiments we consistently detected only trace amounts of LDH.

Phospholipid analysis Lipids were extracted from the lyophilised lavage fluid and lung tissue by the method of Bligh & Dyer (1959) and the phosphorus content was measured by the method of Bartlett (1959). We did not correct for recovery, which was consistently in excess of 91%. Total phospholipid was calculated by multiplying phosphorus content by 25. We have expressed PLalv as mg phospholipid g^{-1} drug lung in all cases. The specific activities of the phospholipids in the alveolar compartment ($PLalv_{sp.act}$) and that in the lung tissue ($PLtiss_{sp.act.}$) were expressed as d.p.m. μg^{-1} of phosphorus. In some cases d.p.m. per μg of phosphorus in the lipid fraction extracted from alveolar lavage is expressed as a percentage of the d.p.m. per μg of phosphorus in the lipid fraction extracted from lung tissue (d.p.m.PLalv/d.p.m.PLtiss × 100). The methods involved in radioisotope counting were as previously described (Nicholas *et al.*, 1982a).

Drugs and statistical analysis Salbutamol and labetalol were gifts from Allen & Hanburys (Australia). All other drugs were obtained from Sigma Chemical Company (St Louis, Missouri, U.S.A.). One-way analyses of variance and unpaired Student's *t* tests were used to test for significance of differences between group means.

Results

Effect of putative secretagogues on alveolar phospholipids in vivo

In resting unanaesthetized rats, doses of 1 mg or 5 mg kg^{-1} body weight of labetalol i.p. affected neither PLalv, nor d.p.m.PLalv/d.p.m.PLtiss (Figure 1). In contrast, salbutamol (280 $\mu g\,kg^{-1}$, i.p.) significantly increased both PLalv ($P < 0.001$) and d.p.m.PLalv/d.p.m.PLtiss ($P < 0.001$). The time course of effect of this dose of salbutamol is illustrated in Figure 2. Doses of pilocarpine of 1.5, 3.0 and 10.0 mg kg^{-1} body weight i.p., had no effect. A dose of 50 mg kg^{-1} induced a milder salivation and diarrhoea, with little change in Vt and no change in PLalv or d.p.m.PLalv/d.p.m.PLtiss. A dose of pilocarpine of 150 mg kg^{-1} body weight i.p. was administered to only one rat. It induced excessive salivation, chromodacryorrhoea, diarrhoea, a three fold increase in Vt and marked increases in the frequency and depth of sighs as monitored by the impedance pneumograph (Figure 3).

Effect of putative secretagogue antagonists on alveolar phospholipids in vivo

Indomethacin (15 mg kg^{-1} body weight, i.p.) affected neither PLalv nor d.p.m.PLalv/d.p.m.PLtiss; it depressed $PLalv_{sp.act.}$. Propranolol (5 mg kg^{-1} body weight, i.p.), atropine sulphate (3 mg kg^{-1}, body weight, i.p.) and DMSO (1 ml kg^{-1}, body weight, i.p.) affected neither PLalv, nor d.p.m.PLalv/d.p.m.PLtiss (Table 1). Propranolol (3 mg kg^{-1}) when administered

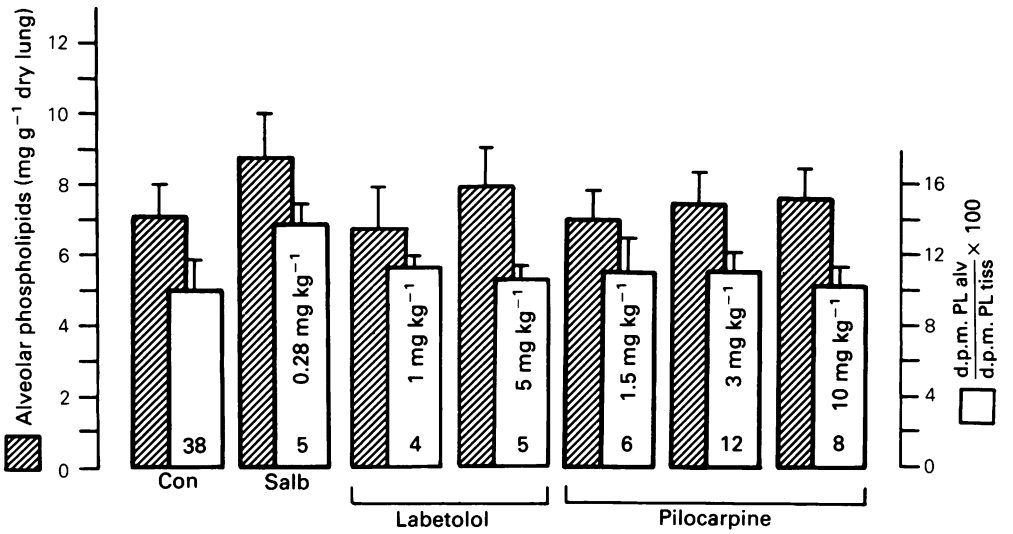

Figure 1 Effect of putative secretagogues on the amount of phospholipid that could be lavaged from rat lung and on the radioactivity of that phospholipid expressed as a percentage of the radioactivity of the phospholipids remaining in the lung tissue. Rats were infused with 20 μCi [methyl-^{3}H]-choline chloride per kg and 4 h later were given i.p salbutamol (Salb, 280 μg kg^{-1} body weight), labetalol (1 or 5 mg kg^{-1}), pilocarpine (1.5, 3 or 10 mg kg^{-1}) or saline (Con). After another hour the rat was heavily anaesthetized, the pulmonary vasculature flushed free of blood and the lung degassed and lavaged. Results are expressed as mean with s.d. shown by vertical lines and the number of rats shown at the base of each histogram. Salbutamol increased both the total alveolar phospholipids and the d.p.m.PLalv/d.p.m.PLtiss ($P < 0.001$). Neither labetalol nor pilocarpine significantly affected either variable.

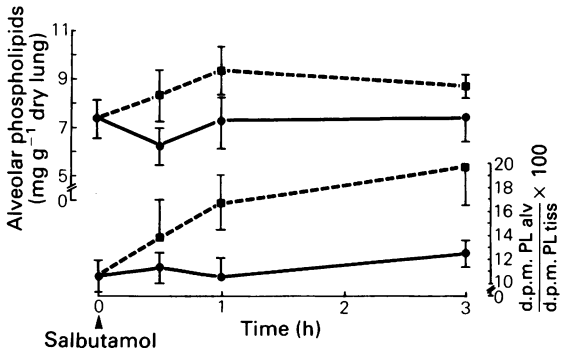

Figure 2 Time course of effect of salbutamol on amount of phospholipid that can be lavaged from rat lung and on the radioactivity incorporated in that phospholipid expressed as a percentage of that in phospholipid remaining in the lung tissue. Rats were given either salbutamol (■) (280 μg kg^{-1}) i.p. or an equivalent volume of saline (●). At various times afterwards, the rats were heavily anaesthetized, the pulmonary vasculature flushed of blood and the lung degassed and lavaged. In each case the rats had been infused exactly 5 h previously with 20 μCi [methyl-^{3}H]-choline chloride via a lateral caudal vein. Each point represents the mean of 4 rats with s.d. shown by vertical lines. Whereas salbutamol did not significantly affect alveolar phospholipids at any time point, it increased d.p.m.PLalv/d.p.m.PLtiss after 1 ($P < 0.025$) and 3 h ($P < 0.01$).

7 h beforehand was sufficient to block the increase in PLalv induced by salbutamol (control: 7.4 ± 0.64 [6 rats], salbutamol 8.3 ± 0.59 [8 rats], propranolol: 7.4 ± 0.59 [5 rats], propranolol plus salbutamol 7 h later: 7.1 ± 0.77 [8 rats] expressed as mean ± s.d. mg g^{-1} dry lung).

Following 24 h hyperpnoea induced by altering the inspired gas, there was a marked increase in PLalv, but no change in either $PL_{sp.act.}$ or d.p.m.PLalv/d.p.m.PLtiss (compare Tables 1 and 2). Propranolol, atropine and indomethacin each failed to prevent the increase in PLalv induced by hyperpnoea and did not modify $PL_{sp.act.}$ or d.p.m.PLalv/d.p.m.PLtiss (Table 2).

Effects of drugs on alveolar phospholipids in vitro

Salbutamol (1.5 μM) added to the perfusate of the isolated perfused lung for 30 min, significantly increased the harvest of PLalv and $PLalv_{sp.act.}$ ($P < 0.001$) (Figure 4). However, there was no significant further increase when lungs were exposed to 0.5 mM. The latter, very large concentration, resulted in a yield of PLalv of 8.5 ± 0.5 mg g^{-1} dry lung, and this can be considered to be the maximal response to salbutamol. In contrast, pilocarpine had no effect in concentrations of either 0.1 μM or 1.0 μM. Increasing Vt so that peak inflation pressure increased from 10 to 20 cmH_2O, markedly increased PLalv and d.p.m.

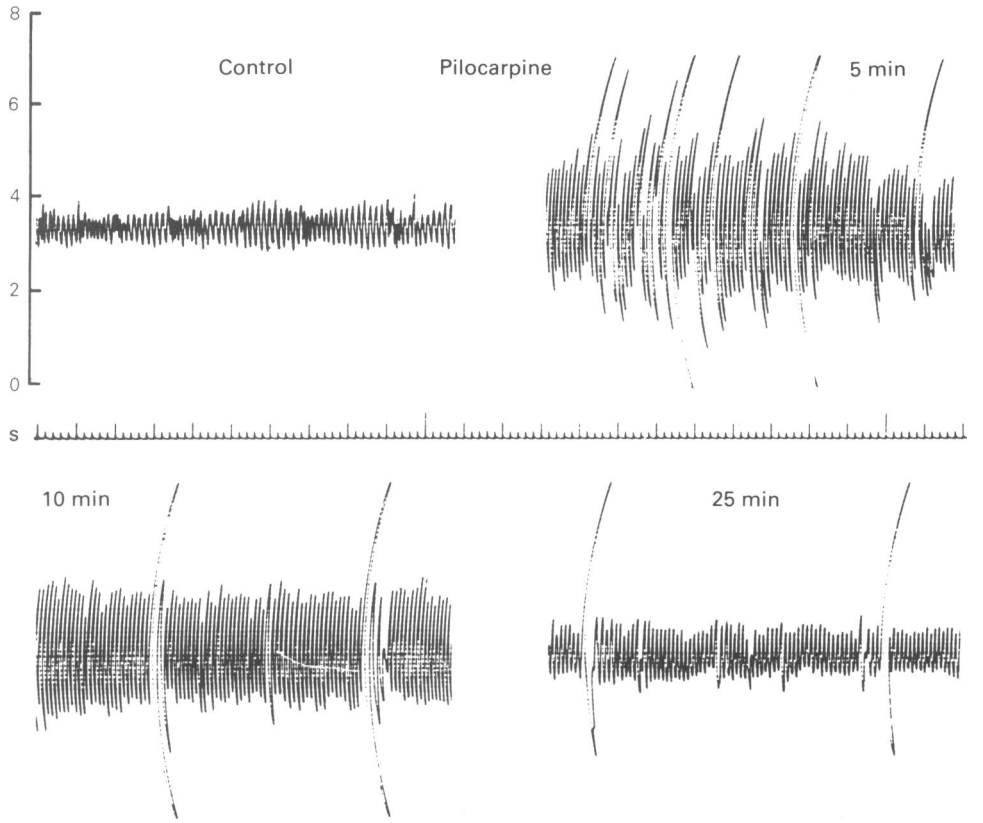

Figure 3 Effect of pilocarpine (150 mg kg^{-1} body weight, i.p.) on respiration in the unanaesthetized rat. The rat was lightly anaesthetized and three electrodes from the impedance pneumograph were inserted under the skin. Three hours after recovery, the rat was injected with pilocarpine hydrochloride and the pattern of breathing monitored for a further 30 min. The ordinate scale is expressed in arbitrary units, with 2.5 units equivalent to a tidal volume of 4 ml; there is a linear relationship between these units and tidal volume up to a tidal volume of 7 ml.

Table 1 Effect of putative secretagogue antagonists of surfactant phospholipids in the resting unanaesthetized rat

	PLalv (mg g^{-1} dry lung)	$PLalv_{sp.act.}$ (d.p.m.μg^{-1} P)	*d.p.m.PLalv/d.p.m. PLtiss* (%)
Saline	8.2 ± 1.35 (14)	273 ± 35.6 (14)	12.4 ± 2.2 (13)
Propranolol	8.8 ± 1.35 (5)	300 ± 24.5 (5)	12.0 ± 1.8 (5)
Atropine	8.6 ± 1.46 (9)	305 ± 43.5 (8)	10.9 ± 2.1 (9)
Indomethacin	9.6 ± 2.08 (10)	231 ± 29.3 (11)*	11.0 ± 3.1 (11)
DMSO	8.8 ± 1.38 (14)	314 ± 56.4 (14)	10.7 ± 2.1 (13)

Results expressed as mean ± s.d. The numbers of rats are in parentheses. PLalv is the total phospholipid lavaged from the alveolar compartment. $PLalv_{sp.act}$ is the specific activity of that phospholipid. Rats were placed in a chamber through which humidified medical grade air was passed. At each of 0, 6, 18 and 21 h they were injected i.p. with one of: saline 1 ml kg^{-1} body weight, propranolol 5 mg kg^{-1}, atropine 3 mg kg^{-1}, indomethacin 15 mg kg^{-1} or dimethylsulphoxide 1 ml kg^{-1} (DMSO). At 19 h they were infused with 20 μCi kg^{-1} [methyl-^{3}H]-choline chloride and at 24 h the lungs were lavaged. *$P < 0.01$. PLalv: phospholipids lavaged from alveolus; PLtiss: phospholipids remaining in lung tissue; sp.act.: specific activity; P: phosphorus.

Table 2 Effect of putative secretagogue antagonists on surfactant phospholipids in the unanaesthetized rat made hyperpnoeic for 24 hours

	PLalv (mg g^{-1} dry lung)	$PLalv_{sp.act.}$ (d.p.m.μg^{-1} P)	*d.p.m.PLalv/d.p.m. PLtiss* (%)
Control	13.2 ± 1.55 (14)	228 ± 41.4 (15)	10.9 ± 1.86 (14)
Propranolol	14.6 ± 1.29 (7)	204 ± 19.6 (6)	10.9 ± 1.73 (7)
Atropine	12.8 ± 2.54 (11)	246 ± 52.7 (11)	10.8 ± 2.24 (11)
Indomethacin	15.2 ± 3.64 (11)	213 ± 28.7 (11)	11.4 ± 3.63 (10)
DMSO	14.0 ± 2.49 (11)	201 ± 37.1 (10)	10.1 ± 2.39 (9)

Results are expressed as mean ± s.d. Numbers are in parentheses. PLalv is the total phospholipid lavaged from the alveolar compartment. $PLalv_{sp.act.}$ is the specific activity of that phospholipid. Rats were placed in a chamber through which humidified 5% CO_2/13% O_2/82% N_2 circulated. At each of 0, 6, 18 and 21 h they were injected i.p. with one of the following: saline 1 ml kg^{-1} body weight, propranolol 5 mg kg^{-1}, atropine 3 mg kg^{-1}, indomethacin 15 mg kg^{-1}, dimethylsulphoxide 1 ml kg^{-1} (DMSO). After 19 h all rats were infused with 20 μCi kg^{-1} [methyl-^{3}H]-choline chloride and at 24 h the lungs were lavaged. Abbreviations as for Table 1.

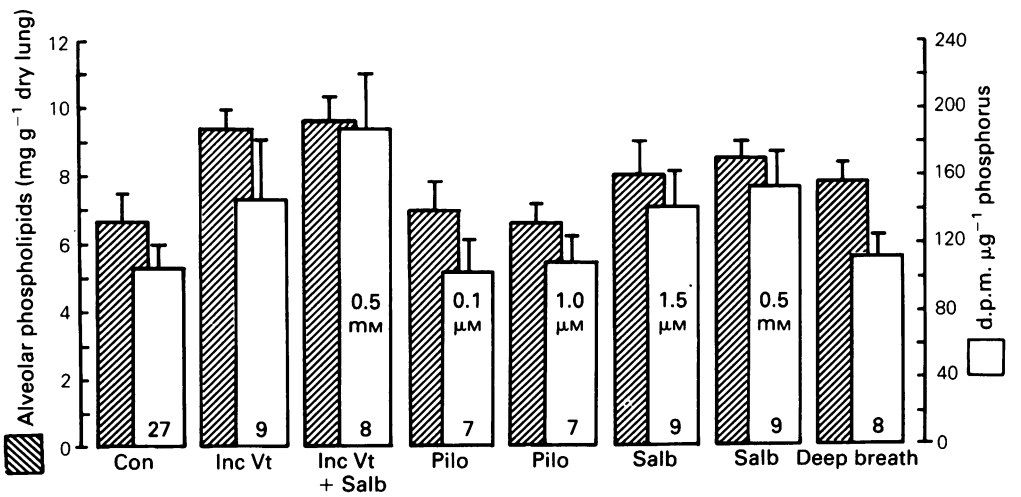

Figure 4 Effect of putative secretagogues on alveolar phospholipid secretion by the rat isolated perfused lung. Rats were infused with 20 μCi [methyl-^{3}H]-choline chloride kg^{-1} body weight. After 150 min the rat was heavily anaesthetized, the lung was removed and set up as an isolated perfused preparation. Drugs were added to the 50 ml reservoir of Krebs bicarbonate containing 4.5% BSA and which was recirculating at 10 ml min^{-1}. Tidal volume was elevated by increasing the peak inflation pressure from the control value of 10 cmH_2O to 20 cmH_2O and the end expired pressure from the control value of 2 cmH_2O to O cmH_2O for a 5 min period. Frequency was maintained at 60 min^{-1}. A simulated deep breath was induced by increasing the peak inflation pressure from 10 to 20 cmH_2O for a single breath. All other experiments were carried out under the control conditions. Results are expressed as mean ± s.d., and the numbers of lungs used and the concentration of drug in the reservoir are listed in each histogram. The alveolar phospholipids and their specific activities were significantly increased above controls by an increased Vt ($P < 0.001$), an increased Vt + salbutamol (Salb) ($P < 0.001$), both concentrations of salbutamol ($P < 0.001$), and a single simulated deep breath ($P < 0.001$). An increased Vt + salbutamol increased specific activity above that with an increased Vt alone ($P < 0.025$). Both concentrations of salbutamol significantly increased the specific activity above that found with a single simulated deep breath ($P < 0.005$). Pilo = pilocarpine.

PLalv/d.p.m.PLtiss. Although addition of 0.5 mM salbutamol did not further increase PLalv above that found with the increased Vt, it significantly increased $PLalv_{sp.act.}$ (increased Vt: 146 ± 35, $n = 9$; increased Vt + salbutamol: 190 ± 33, $n = 8$, mean ± s.d. in d.p.m.μg^{-1} phosphorus ($P < 0.025$)). When the peak inflation pressure was increased even further, to 26 cmH_2O for 15 min, a yield of 11.96 ± 1.32 mg phospholipid g^{-1} dry lung ($n = 7$) resulted. While this is well in excess of that recovered following 0.5 mM salbutamol, it is probably still submaximal. Finally, whereas a single simulated deep breath produced an increase in PLalv similar to that induced by salbutamol (0.5 mM), salbutamol but not the simulated deep breath, caused a significant increase in $PLalv_{sp.act.}$ (simulated deep breath: 112 ± 12, $n = 8$; salbutamol: 154 ± 20, $n = 9$, mean ± s.d. in d.p.m.μg^{-1} phosphorus) ($P < 0.001$) (Figure 4).

Discussion

Validation of methods

We used total PLalv as an index of alveolar surfactant for reasons previously discussed (Power *et al.*, 1986). Although we speak of increases in PLalv as reflecting release of surfactant from the alveolar type II cell, the possibility remains that, in experiments lasting hours, the increase may also be due to a depressed removal of surfactant from the alveolar compartment. However, in the isolated perfused lung experiments, the time-course is so short (30 min) that even complete inhibition of surfactant removal from the alveolar compartment could not result in the increases in PLalv found. The advantage of using the isolated perfused lung is that it allows precise control over perfusate gases, ventilatory pressures, temperature, pH and drug concentrations, while removing the lung from all extrapulmonary neural and humoral influences.

In the present experiments we used [^{3}H]-choline as the phospholipid precursor, as we had found that about 90% of the label subsequently detected in the lung was as phosphatidylcholine (Johnson *et al.*, 1979), the principal phospholipid in surfactant. We chose to lavage the lungs 5 h after the infusion of choline, for at this time $PLalv_{sp.act.}$ is about 60% of maximal labelling which occurs at between 8 and 12 h (Power *et al.*, 1986). We had found that the presence of a radiolabel in PLalv was a sensitive indicator of total amount of PLalv present (Nicholas *et al.*, 1982b). We also hoped that if indeed there were two pools of tissue surfactant, the radiolabel might assist our detecting the one that turns over more rapidly and hence is more readily labelled (Nicholas *et al.*, 1982a).

Although we had shown that a single simulated deep breath (approximately × 2.5 Vt, peak inflation pressure increased from 10 to 20 cm H_2O) increases PLalv in the rat isolated perfused lung (Nicholas *et al.*, 1982b), we have no information whether this is also the case *in vivo*. However, with this possibility in mind, the rats were handled very gently and kept in a quiet area. Any rats that became agitated or were difficult to anaesthetize were discarded.

Control of surfactant in resting rats

The putative secretagogue antagonists we chose had all been reported to inhibit surfactant release in hyperpnoeic anaesthetized rabbits (Oyarzun & Clements, 1977; 1978). However, using doses similar to those reported, we could show no effect of propranolol, atropine or indomethacin in resting unanaesthetized rats. Although these drugs did not inhibit the surfactant system, this does not necessarily mean that β-adrenoceptor agonists, muscarinic cholinoceptor agonists and prostaglandins are not normally involved in surfactant homeostasis. It is possible that in the presence of these drugs, the unanaesthetized rat may, for example, sigh more frequently, or otherwise change its pattern of breathing. This cannot be tested until a more satisfactory method is devised for monitoring breathing in small animals for prolonged periods.

Salbutamol consistently elevated PLalv. The fact that this effect could be blocked by propranolol confirms that salbutamol is acting on β-adrenoceptors, and that the dose of propranolol adopted in our experiments is sufficient to block these receptors. In contrast, pilocarpine had no effect in doses of 1.5, 3 or 10 mg kg^{-1} body weight i.p. Both Goldenberg *et al.* (1969) and Massaro *et al.* (1982) have reported that pilocarpine releases surfactant in unanaesthetized rats. However, the dose they administered was 150 mg kg^{-1}, or about one thousand times that given to humans. Goldenberg *et al.* (1969) reported that this dose caused 'excessive lacrimation and salivation as well as intermittent malodorous fecal discharges. Some rats released mucinous fluid from their nostrils'. We suggest that these authors were observing pilocarpine poisoning rather than the specific effect of muscarinic cholinoceptor stimulation. Certainly when we administered this very large dose of pilocarpine, we observed excessive salivation, chromodacyorrhoea and diarrhoea, accompanied by marked increases in Vt and in the frequency of sighing; in our experience the respiratory effects were of sufficient magnitude to release surfactant. However, the effects were so severe that we used only one rat and did not actually measure PLalv. When we reduced the dose to 50 mg kg^{-1} body weight, there was less salivation and diarrhoea and little effect on respiration; PLalv was not increased. Massaro *et al.* (1982) reported that the effect of 150 mg kg^{-1} pilocarpine could be blocked by both

atropine and propranolol, suggesting that pilocarpine may be acting via the sympathetic nervous system. In fact, it seems that pilocarpine could not act directly on the type II cell, as Dobbs & Mason, (1979), Mettler *et al.* 1981 and Brown & Longmore (1981) could not find any evidence for cholinoceptors mediating the release of surfactant from isolated type II cells; hence any action of acetylcholine-like agents *in vivo* must be indirect. This conclusion is borne out by the results of Corbet (1981) who reported that in foetal rabbits the response to pilocarpine was blocked by propranolol. However, he used an even larger dose of 250 mg kg^{-1} body weight. It was important to determine whether pilocarpine can release surfactant in the isolated perfused lung. Here it seems that our past (Nicholas & Barr, 1981) and present results are at variance with those of Brown & Longmore (1981) and Massaro *et al.* (1982). Brown & Longmore (1981) found that if 0.2 μM pilocarpine was included in the medium, together with 50 μCi of [1-^{14}C]-palmitate and the lung perfused for 3 h there was a 2 fold increase in sp.act. of alveolar disaturated phosphatidylcholine (DSPC) expressed as d.p.m.$nmol^{-1}$; they do not report total alveolar DSPC. Certainly 3 h are sufficient to increase rate of synthesis and this could explain the increase in specific activity. Massaro *et al.* (1982) found that whereas 500 μM pilocarpine did not affect PLalv, 1 μM induced a 3 fold increase expressed as % d.p.m.DSPCalv/d.p.m.DSPCtiss after 30 min of reperfusion. Again, total alveolar DSPC was not reported. Whereas these authors demonstrated that 0.5 mM isoprenaline decreased the lamellar body volume density, and that 0.5 mM pilocarpine had no effect, they did not report whether 1 μM pilocarpine affected this variable. In our experiments we could show no effect of either 0.1 or 1.0 μM pilocarpine in the perfusate on PLalv or $PLalv_{sp.act}$ over a 30 min period. Previously we were unable to show any effect of 10 μM pilocarpine (Nicholas & Barr, 1981). There are a number of differences in experimental design, which include the choice of radiolabelled molecule, the anaesthetic, the periods of perfusion and contact with drug and the way of expressing the results. At this stage we do not know if these explain the differences in results. Certainly our results do not support the hypothesis that pilocarpine releases surfactant via an intrapulmonary mechanism.

The α/β-adrenoceptor antagonist, labetalol, was reported to accelerate foetal lung maturation in man (Michael, 1980). Furthermore, this drug increased both lung compliance and PLalv of the rabbit foetus at day 27 of gestation (term: 31 days) (Nicholas *et al.*, 1978). In addition to its antagonist properties, labetalol is reported to be a partial β-adrenoceptor agonist (Carey & Whalley, 1979), so possibly it was stimulating the β-adrenoceptors on the type II cell. In the present study with adult rats, despite using a range of perfusate concentrations of labetalol in the isolated lung, and a range of doses *in vivo*, we were unable to show any change in PLalv. We cannot explain the apparent effect of labetalol in the foetus.

Control of surfactant in hyperpnoeic rats

Previously we had shown that swimming increases both PLalv and $PLalv_{sp.act.}$ in rats (Nicholas *et al.*, 1982a). However, whereas both propranolol and atropine depressed $PLalv_{sp.act.}$, they had no effect on PLalv. Although swimming consistently increased Vt, the model is complex and involves activating widespread neuroendocrine events. Furthermore, it is limited to relatively brief periods and the magnitude of increase of Vt could not be titrated. More recently we have increased Vt by altering the composition of inspired gas (Power *et al.*, 1986). In the present experiments we have modified the gas mixture from one containing 4% CO_2/10% O_2/86% N_2 to one containing 5% CO_2/13% O_2/82% N_2. Whereas both mixtures approximately doubled Vt and frequency of breathing, the latter better maintained normal blood gases and involved minimal stress. Rats maintained normal food and water consumption and showed a normal rate of increase in body weight.

Twenty four hours hyperpnoea consistently produced large increases in both PLalv and $PLalv_{sp.act.}$. We have recently found that this is associated with an increase in the activity of cholinephosphate cytidylyltransferase, the rate-limiting enzyme in the synthesis of DPPC (Nicholas *et al.*, 1987a). Propranolol, atropine and indomethacin, drugs reported to inhibit release of surfactant, had no effect on release in our experiments. There are a number of considerations in evaluating these results. First, it is possible that hyperpnoea affects both release and reuptake of alveolar surfactant, which makes it difficult to state empirically that a drug does or does not affect release. Second, the dose and frequency of dosing may have been inadequate. Of the three drugs used, we can only be sure that propranolol was providing adequate blockade of the appropriate receptor, although the dose of atropine used was effective in swimming rats. We have no way of testing whether the dose of indomethacin was adequate, as we have never been able to show an effect on the surfactant system. Finally, as mentioned above, it is possible that although the β-adrenoceptor system, muscarinic cholinoceptors or the prostaglandin receptors may be involved normally, when they are blocked, other mechanisms come into play. For example, the rats may sigh more frequently. Again, this possibility cannot be tested until an improved method is available for monitoring respiration. We conclude that our present experiments do not support a role for the β-adrenoceptors or muscarinic cholinoceptors, or for the prostaglandins in the increased PLalv associated

with prolonged hypernoea.

The presence of two pools of tissue surfactant

Previously we proposed that the alveolar compartment is supplied by two pools of tissue surfactant (Nicholas *et al.*, 1982a; Nicholas & Barr, 1983; Power *et al.*, 1986; 1987). The first is relatively small, turns over rapidly and is under the control of the sympathetic nervous system. The second is larger and is released in response to an increase in Vt. Whether there are two different types of type II cells (Nicholas *et al.*, 1982a) is unknown. Very recently we have isolated two subfractions from the lamellar body fraction, each of which contains the full spectrum of surfactant phospholipids. However, while one contains the 35 kD protein, the other contains the 15 kD protein of surfactant. DSPC in one is radiolabelled more rapidly than that in the other (Power *et al.*, 1988). We also have evidence that the two fractions can be released differentially (Nicholas *et al.*, 1987b). We believe that the present work provides additional evidence for the two-pool hypothesis.

When we adjusted the concentration of salbutamol in the perfusate of the isolated perfused lung so that the increase in PLalv matched that produced by a single simulated deep breath, the $PLalv_{sp.act.}$ was significantly greater with salbutamol; this is consistent with PLalv being derived from different tissue pools. This same phenomenon was apparent when a large concentration of salbutamol was included in the perfusate of a hyperventilated isolated perfused lung: total PLalv did not increase, but $PLalv_{sp.act.}$ increased 30%. Therefore, it seems that although salbutamol consistently releases surfactant, the maximum amount released is smaller and appears to emanate from a tissue pool that is more highly radiolabelled than that released by increasing Vt. An alternative explanation is that salbutamol also increased reuptake of alveolar surfactant (Fisher *et al.*, 1985), possibly favouring the phospholipids that had been in the alveolus for longer and were less radiolabelled. We think that this is unlikely within the 30 min duration of these experiments.

In conclusion, we have shown in unanaesthetized rats that although stimulation of β-adrenoceptors can induce consistent increases in PLalv, these are not as large as those induced by increased Vt. We have been unable to release surfactant with pilocarpine, despite using a range of doses up to 50 mg kg^{-1} body weight, and suggest that previous reports of release with 150 mg kg^{-1} actually reflect a secondary phenomenon of release stimulation by increased Vt. In resting and hyperpnoeic rats, PLalv is not influenced by propranolol, atropine or indomethacin. Finally, we have shown in the rat isolated perfused lung that salbutamol appears to release surfactant from a different tissue pool from a simulated deep breath. We could find no evidence that muscarinic cholinoceptors mediate release of surfactant.

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