Control of pulmonary surfactant secretion: an evolutionary perspective

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1Department of Physiology, University of Adelaide, South Australia, 5005; 2School of Biological Sciences, Macquarie University, New South Wales, Australia, 2109; and 3Quinnipiac College, Hamden, Connecticut 06410

Wood, Philip G., Olga V. Lopatko, Sandra Orgeig, Jean M. P. Joss, Allan W. Smits, and Christopher B. Daniels. Control of pulmonary surfactant secretion: an evolutionary perspective. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R611–R619, 2000.—Pulmonary surfactant, a mixture consisting of phospholipids (PL) and proteins, is secreted by type II cells in the lungs of all air-breathing vertebrates. Virtually nothing is known about the factors that control the secretion of pulmonary surfactant in nonmammalian vertebrates. With the use of type II cell cultures from Australian lungfish, North American bullfrogs, and fat-tailed dunnarts, we describe the autonomic regulation of surfactant secretion among the vertebrates. Adrenaline (Adr), but not epinephrine (Epi), stimulated total PL and disaturated PL (DSP) secretion from type II cells isolated from Australian lungfish. Both Epi and ACh stimulated PL and DSP secretion from type II cells of bullfrogs and fat-tailed dunnarts. Neither Epi nor ACh affected the secretion of cholesterol from type II cell cultures of bullfrogs or dunnarts. Pulmonary surfactant secretion may be predominantly controlled by the autonomic nervous system in nonmammalian vertebrates. The parasympathetic nervous system may predominate at lower body temperatures, stimulating surfactant secretion without elevating metabolic rate. Adrenergic influences on the surfactant system may have developed subsequent to the radiation of the tetrapods. Furthermore, ventilatory influences on the surfactant system may have arisen at the time of the evolution of the mammalian bronchoalveolar lung. Further studies using other carefully chosen species from each of the vertebrate groups are required to confirm this hypothesis.

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are believed to act on β2 receptors situated on the type II cell (3). The parasympathetic nervous system (PNS) does not appear to have any direct effects on the secretion of PS in mammals. Although ACh stimulates surfactant PL release in the isolated perfused rat lung, it is thought to act indirectly, causing a contraction of intrapulmonary smooth muscle, which in turn distorts the type II cell (17).

In contrast to mammals, changes in ventilatory pattern in the isolated lizard lung have no effect on PL release (28). However, both Epi and ACh trigger surfactant release in the isolated lizard lung (28) and from isolated lizard type II cells (29). Hence, it appears that both the sympathetic and parasympathetic branches of the autonomic nervous system may control surfactant secretion in the lizard (27, 29). The differences in the regulation of surfactant secretion between mammals and the lizard may result from the marked physiological differences between mammals and lizards. Perhaps the most important differences between these two groups with respect to the regulation of the surfactant system are metabolism, breathing pattern, and mode of ventilation. Lizards are ectothermic, have a highly variable breathing pattern incorporating variable-length nonventilatory periods that change markedly with changes in body temperature, and ventilate their lungs primarily by changing the volume of the large central airspace without significantly disturbing the respiratory region. Such a pattern may result in relatively irregular stimulation or distortion of the type II cells and hence poor surfactant secretion (28). Similar metabolic and ventilatory patterns are also characteristics of all other ectothermic vertebrates. These differences between mammals and nonmammals may, therefore, be indicative of differences in the regulation of the surfactant systems between these two groups. However, the processes controlling surfactant secretion in nonmammals have not been examined.

Here, type II pneumocytes were isolated and cultured from Australian lungfish, North American bullfrogs, and fat-tailed dunnarts. The Australian lungfish, *Neoceratodus forsteri*, is a primitive dipnoan that is found in selected rivers in southern Queensland, Australia. Although this species possesses lungs, in well-oxygenated water it relies primarily on fully developed gills for gas exchange and uses aerial breathing only under conditions of hypoxia or increased activity (10). Morphologically, the Australian lungfish closely resembles the stem ancestor of the terrestrial vertebrates. The bullfrog was studied as an air-breathing aquatic representative of the amphibians. The fat-tailed dunnart is a small, insectivorous marsupial found throughout the southern and central regions of Australia. Although dunnarts normally maintain a body temperature of 35–37°C, they also regularly enter torpor, a condition characterized by lowered body temperature and dramatic reductions in metabolic rate. Torpor in dunnarts is also associated with increased levels of surfactant lipid (15). This species is examined here as a representative of a primitive mammalian group that also undergoes large changes in body temperature and coincident changes in the surfactant system. These species were studied to create an evolutionary framework for the control of surfactant secretion among the tetrapods.

As it appears that the PNS has an important influence on the surfactant system in lizards but has no effect in eutherian mammals, this study also aims to identify the point at which the PNS ceases to influence surfactant secretion. To determine whether both adrenergic and cholinergic agonists act directly on the type II cells in these species, we examined the effects of autonomic neurotransmitters and receptor antagonists on the secretion of total PL, DSP, and Chol from primary cultures of type II pneumocytes isolated from the lungs of each species. These surfactant components were examined because they have previously been identified in the surfactant of representatives of all air-breathing vertebrates. They also undergo rapid changes in vivo, and are, therefore, likely to be precisely regulated.

**MATERIALS AND METHODS**

Animals. Seventeen lungfish (*Neoceratodus forsteri*, 100–150 g) were collected from breeding ponds at Macquarie University, New South Wales. Bullfrogs (*Rana catesbeiana*, 150–200 g) were obtained from commercial suppliers. Fat-tailed dunnarts (*Sminthopsis crassicaudata*, 10–15 g) were obtained from a breeding colony maintained at the University of Adelaide.

Isolation and culture of type II pneumocytes. Type II cells were isolated from lungfish, bullfrogs, and dunnarts using methods previously described (29), which employed modifications of the methods of Dobbs et al. (9). Briefly, animals were killed and the lungs were perfused via the pulmonary artery with a Ca2+- and Mg2+-free salt solution until free of blood. The lungs were filled with this solution and lavaged three times to remove alveolar macrophages. The lungs were removed from the body cavity and minced in the presence of DNAase type I. The tissue pieces were incubated at 37°C in collagenase type IV for 12–15 h. Cellular clumps were dispersed by trituration, and the resulting cell suspension was filtered and centrifuged. Type II cells were isolated by incubating the crude cell suspensions over IgG-coated bacteriologic petri dishes at 37°C for 90 min. During this time, macrophages and other immune cells adhere to the IgG, allowing the nonimmune cells, predominantly type II cells, to be decanted. The cell suspension was again centrifuged (150 g, 5 min) and the pellet resuspended in phosphate-free DMEM. The cells were counted using a hemocytometer and plated in six-well tissue culture trays (Becton Dickinson, Franklin Lakes, NJ) at 5 × 10^5 cells/well) in phosphate-free DMEM supplemented with 25 mM HEPES, 24 mM NaHCO3, 10% fetal bovine serum (Commonwealth Serum Laboratories, Adelaide, Australia), 25 µg/ml penicillin, 25 µg/ml streptomycin, 10 µg/ml amphotericin B, and 50 µg/ml gentamicin (Sigma Chemical, St Louis, MO). Cell viability was assessed by exclusion of the vital dye trypan blue. Differential cell counts were performed on air-dried smears using a modified Papanicolaou stain (14). For each species used, some modifications of this method were necessary to maximize cellular yield and viability.

*Neoceratodus forsteri*. Lungfish were killed by immersion in MS-222 (tricaine methanesulphonate) and subsequent decapitation. All solutions used were balanced to pH 7.4 at 37°C. On removal of the lungs from the thoracic cavity, they

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were minced with scissors in the presence of 100 μg/ml DNAase type I. This 10-fold increase in DNAase concentration was found necessary for the effective dissociation of the lungs. Cells were subsequently incubated and experiments undertaken at 37°C.

Rana catesbeiana. Bullfrogs were killed by pithing, and cells and tissue were incubated at all times at 30°C. All solutions used were balanced to pH 7.6 at 30°C and supplemented with 10 μg/ml polymixin B (Sigma). Cells were subsequently incubated, and experiments were undertaken at 30°C as the viability of frog cells at 37°C was severely compromised.

Sminthopsis crassicaudata. Fat-tailed dunnarts were killed with an overdose of pentobarbitone (150 mg/kg ip). All solutions used were balanced to pH 7.4 at room temperature. Cells were subsequently incubated, and experiments were undertaken at 37°C.

Effects of autonomic neurotransmitters on surfactant lipid secretion from isolated type II cells. After incubation of the cells for 15 h, aliquots of the media were taken from each well [“time 0” (T₀)]. After a further 3 h, the media were again sampled [“time 1” (T₁)]. This first 3-h experimental period, which is before the addition of agonists (“predrug”), provides an indication of basal changes in media PL, DSP, and Chol content. Agonists (100 μM carbachol hydrochloride or 100 μM Epi hydrochloride) were added to each well, and the cells were incubated for a further 3 h, after which time the media were again sampled [“time 2” (T₂)]. This second experimental period (“postdrug”), after the addition of the agonists (or agonists/antagonists), provides an indication of changes in media PL, DSP, and Chol content in the presence of these agents. In all cases, between 10 and 20 μl of agonists or antagonists were added to 10–20 ml of media per well. Media aliquots from four wells at each time point were pooled, centrifuged (1,000 g, 10 min) to remove cellular debris, the supernatant decanted, and the total lipids extracted. For experiments using antagonists (lungfish and dunnarts: 1 mM propranolol hydrochloride, 100 μM atropine sulfate; bullfrogs: 1 mM alprendiol hydrochloride, 100 μM atropine sulfate), these were added 5 min before addition of the agonist. In all experiments in which Epi was used, sodium ascorbate (1 mM) was added to the media to prevent oxidation and inactivation of the Epi (3). For S. crassicaudata, a control group, into which no agonists or antagonists had been added, was also sampled throughout the 6-h experimental period. Due to a shortage of tissue, it was not possible to include control groups for either N. forsteri or R. catesbeiana. However, the experimental groups with specific blockers for Epi and ACh are in effect a control, because there is no effective agonist present throughout the 6-h period.

Surfactant lipid (PL, DSP, and Chol) secretion/uptake was calculated by multiplying the phosphorus content by 25, as phosphorus comprises ~4% of PL (7). The DSP were separated from the unsaturated PLs and the neutral lipids on aluminum oxide after reaction with osmium tetroxide (16). DSP content was determined using a phosphorus assay. Chol was quantified in the neutral lipid fraction using a high-pressure liquid chromatography system as previously described (29). It was not possible to measure the effects of the applied agonists on Chol secretion from cells of N. forsteri due to a shortage of sample material.

Fixation of cells for electron microscopy. Cell pellets from 6–10 individuals of all three species were fixed in 2% gluteraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide overnight. They were then stained en bloc in 1.5% uranyl acetate, dehydrated in 70, 80, 90, and 100% acetone and embedded in resin.

Statistical analyses. Percentage changes were arcsin transformed, and data were analyzed using one-way analyses of variance followed by paired or unpaired t-tests where appropriate. Statistical significance was assumed at P ≤ 0.05.

RESULTS

Electron microscopical examination of type II cells. Figure 1, A–C, depicts type II pneumocytes isolated from Australian lungfish, North American bullfrogs and fat-tailed dunnarts, respectively. Differential cell counts indicated that the cell populations were greater than 80% type II cells in each of the species used. Cell viability, as assessed by exclusion of the vital dye trypan blue, was found in each species to be >90%. In the case of the lungfish and dunnarts, the cells have large, membrane-bound osmiophilic lamellar bodies characteristic of mammalian type II cells (Fig. 1, A and C). In cells isolated from bullfrogs, the lamellar bodies appear as dense black masses (Fig. 1B). Exclusion of the vital dye trypan blue indicated that the cells remained healthy and functional over the 6-h sampling period.

Effects of autonomic neurotransmitters and antagonists on surfactant lipid secretion from isolated type II cells. For unknown reasons, propranolol interferes with the assay used to determine DSP, and, as such, it was not possible to measure DSP in those groups in which propranolol was used.

Neoceratodus forsteri. Addition of Epi did not affect media PL. Addition of ACh significantly elevated media PL content (t = 2.26, df = 11, P = 0.023; Fig. 2A). Addition of atropine abolished this increase (Fig. 2A). As seen from the ACh-atropine experimental group, media DSP significantly decreased over the 6-h experimental period (t = 2.157, df = 5, P = 0.042; Fig. 2B), reflecting cellular uptake of DSP from the media. This decrease was maintained in the presence of Epi (t = 2.93, df = 7, P = 0.011; Fig. 2B). Conversely, addition of ACh to the media abolished the decrease in media DSP, reflecting a stimulation of secretion (Fig. 2B).

Rana catesbeiana. In each of the experimental groups, media PL content decreased in the 3 h before the addition of agonists, indicating cellular uptake of PL. Media PL increased markedly on addition of either of the agonists (Epi: t = 2.19, df = 6, P = 0.036; ACh: t = 3.34, df = 7, P = 0.006; Fig. 3A). The response to each agonist was abolished by addition of the respective
antagonists (Fig. 3A). Addition of either Epi or ACh increased media DSP content (Epi: $t = 2.06, df = 4, P = 0.03$; ACh: $t = 2.06, df = 4, P = 0.05$; Fig. 3B). Neither of the agonists used affected secretion of Chol (Fig. 3C).

Sminthopsis crassicaudata. No change in media PL content occurred in a control group into which no agonists or antagonists were added over the 6-h experimental period (T1/T0: 91.13 ± 3.63%; T2/T1: 103.45 ± 5.03%). In each of the experimental groups, media PL content decreased in the 3 h before the addition of agonists, indicating cellular uptake of PL. Media PL increased markedly on addition of either of the agonists (Epi: $t = 2.17, df = 5, P = 0.041$; ACh: $t = 4.12, df = 5, P = 0.005$; Fig. 4A). The response to each agonist was abolished by addition of the respective antagonists (Fig. 4A). No change in media DSP content occurred in a control group over the 6-h experimental period (T1/T0: 94.58 ± 2.80%; T2/T1: 100.96 ± 4.41%). Addition of either Epi or carbachol increased media DSP content (Epi: $t = 2.17, df = 4, P = 0.05$; ACh: $t = 2.07, df = 4, P = 0.05$; Fig. 4B). Although we were unable to measure the effect of propranolol in blocking the effect of Epi because this compound interferes with the DSP assay, the effect of atropine was to completely block the response of carbachol (Fig. 4B). Neither of the agonists used affected the secretion or uptake of Chol (Fig. 4C).

**DISCUSSION**

ACh (or its analog carbachol) and Epi stimulated total PL and DSP secretion from type II cells isolated from frogs and marsupials. In the case of the lungfish, only ACh stimulated PL secretion. Adrenergic fibers are not found in the lung of N. forsteri, and, in general, the adrenergic nervous system in all lungfish appears to be absent or at least poorly developed (10). The adrenergic nervous system appears to be represented only by the presence of catecholamine-containing chromaffin cells in the subendothelium of the atrium (5, 10). The primary function of these cells is thought to be in the adrenergic control of the heart. Although catecholamines released from the chromaffin cells may also affect other systems, lipid secretion by the type II pneumocytes of N. forsteri does not appear to be affected by adrenergic agents. Furthermore, N. forsteri relies predominantly on gills for gas exchange, air breathing very rarely. Such a discontinuous breathing pattern may be too irregular to provide a reliable mechanism for continued secretion.
It is, therefore, unlikely that changes in ventilation are responsible for continuing surfactant secretion in *N. forsteri*. The facts that neither ventilation nor the sympathetic nervous system are likely to affect secretion and only ACh affected the secretion of surfactant PL from *N. forsteri* pneumocytes suggest that the PNS may predominantly control surfactant secretion in the lungfish. Recent molecular phylogenetic analyses find lungfish to be the sister group either to all terrestrial vertebrates (30, 31) or to all other gnathostomes (22). *Neoceratodus forsteri* can be used, therefore, to infer possible evolution of organs and systems. For instance, the effects of ACh on cells isolated from this species suggest that the PNS is the phylogenetically primitive regulator of the surfactant system.

Both Epi and ACh stimulated release of surfactant PL from isolated bullfrog type II pneumocytes. The autonomic nervous system appears to be more highly developed in amphibians than in lungfish. Both adrenergic and cholinergic fibers are found in the frog lung (4). Although it is unclear whether the type II cells in the frog lung are directly innervated and the precise function of the nerve fibers in the lung is unknown, it appears that the surfactant system in bullfrogs, as in lizards, is under control of both the SNS and PNS. Sympathetic control of secretion may be mediated directly by innervation of the type II cells (6) or via fluctuations in the plasma levels of catecholamines. The surfactant system is also, at least in part, under parasympathetic control. As no source of circulating cholinergic agonists is known, parasympathetic control is likely to be mediated via direct innervation of the type II cells.

The central Australian agamid lizard *Pogona vitticeps* appears to undergo a temperature-induced alternation in the autonomic control of the surfactant system with changes in body temperature (27, 28). Although changes in ventilation have no effect on surfactant PL secretion in the isolated lizard lung, both the SNS and PNS may control surfactant secretion in *P. vitticeps* (28). Higher body temperatures are associated with an increase in plasma levels of the surfactant secretagogues Epi and norepinephrine (27). In the warm, alert animal, release of Epi and norepinephrine into the circulation from the adrenal gland may facilitate surfactant release. However, these neurotransmitters also increase metabolic rate. Stimulating surfactant release via these two neurotransmitters may be inappropriate as body temperature decreases, and the animal needs to conserve energy. At low body temperatures, the PNS may, therefore, predominate, triggering surfactant release without unduly affecting metabolic rate (27). Such an alternation in the relative dominance of the SNS and PNS with changes in body temperature may also occur in other heterothermic animals. The fat-tailed dunnart, *Sminthopsis crassicaudata*, is a small (10–15 g) Australian dasyurid marsupial that under-
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Significant difference from predrug [in large reductions in body temperature (from low ambient temperature. Torpor is characterized by going torpor during periods of low food availability or

Fig. 4. Effect of Epi and Epi-Prop and ACh and ACh-At on total surfactant PL secretion (A), DSP secretion (B), and Chol secretion (C) from type II cells isolated from Smnithopsis crassicaudata. Data interpretation as in Fig. 2. * Significant difference from predrug (in A: P = 0.041, P = 0.005 (ACh); in B: P = 0.05 (Epi), P = 0.05 (ACh)).

line stimulated PL and DSP secretion from type II cells isolated from S. crassicaudata. Although the adrenergic stimulation of surfactant secretion has been widely investigated in mammals, cholinergically mediated surfactant secretion is less well documented. Cholinergic agents have been found to stimulate PL secretion in the isolated perfused rat and rabbit lung (17, 20), but not from isolated rat type II cells (3). In vivo, ACh may stimulate some secretion by triggering a contraction of smooth muscle within the lung, which in turn distorts the type II cells, or by stimulating the release of catecholamines from the adrenal medulla (17). Therefore, the effects of these agents are believed to be mediated indirectly, and the PNS is generally not believed to affect surfactant secretion in eutherian mammals. However, carbamylcholine triggered secretion from isolated dunnart type II cells by acting directly on cholinergic receptors on the cells. The mechanisms for the cholinergic stimulation of secretion may persist in the dunnart to enable continued surfactant secretion during bouts of low body temperature.

Although muscarinic receptors are found on the type II cells of rats, the function of these receptors are unknown (13). They do not, however, appear to be involved in the movement of ions across the cell membrane or in the control of the fluid balance of the hypophase (11). Secretion of surfactant in response to a number of stimuli (i.e., ATP, mechanical stretch of the type II cell) is preceded by an increase in intracellular Ca2+ that is thought to activate many intermediary steps in the movement of lamellar bodies to the plasma membrane (23, 24). Similarly, stimulation of muscarinic receptors on isolated dunnart type II cells causes an increase in intracellular Ca2+, but whether this increase is sufficient to trigger surfactant secretion is not known (13). It is possible that the muscarinic receptors found on mammalian type II cells may have been retained for the control of surfactant secretion at lower body temperatures. Whereas their function in homeothermic mammals remains unknown, the metabolic cost of producing and maintaining these receptors and their associated signal transduction mechanisms must be considerable. The presence of muscarinic receptors on mammalian type II cells suggests that the PNS has some role in regulating the cellular activities (surfactant related or otherwise) of the mammalian type II cell. Furthermore, as an in vivo source of circulating ACh is not apparent, the activities of the muscarinic receptors on the type II cells must be triggered by parasympathetic cholinergic nerves adjacent to, or in close proximity to, the type II cells.

In the dunnart and the bullfrog, surfactant Chol remained unchanged after stimulation with either adrenergic or cholinergic agonists. A similar response was found from isolated type II cells (29) and in isolated perfused lungs (27, 28) of the lizard Pogona vitticeps. The Chol component of surfactant is capable of rapid changes in vivo in response to changes in body temperature in lizards (7). Similarly, a reduction in body temperature during torpor in the dunnart, S. crassicaudata, is associated with the production of a surfactant
enriched in Chol and DSP (15). In vivo increases in surfactant Chol with decreases in body temperature may result in a more surface-active surfactant that remains fluid and functional as body temperature decreases (7, 15). Whereas it appears that Chol is a dynamic component of surfactant, titrated rapidly and independently of the PL component, its secretion was unaffected by any agonists used in any of the species examined, which suggests that the secretion of Chol may not be under autonomic control.

Summary. On the basis of this preliminary evolutionary framework, the following working hypothesis can be derived. The PNS may represent the phylogenetically primitive control mechanism of the surfactant system. It is retained as the predominant control mechanism in Australian lungfish and is also functional in the control of surfactant secretion in other nonmammalian vertebrates and in the heterothermic marsupial Sminthopsis crassicaudata (Fig. 5). Sympathetic control of the surfactant system occurs in the frog, the lizard, and mammals and may have evolved subsequent to the evolution of the terrestrial vertebrates. Furthermore, the relative dominance of the SNS and PNS may alternate with changes in body temperature. In the warm, alert animal, release of Epi and norepinephrine into the circulation from the adrenal gland may facilitate surfactant secretion. At low body temperatures, the PNS may predominate, triggering surfactant PL release without unduly affecting metabolic rate. Similarly, heterothermic mammals and ectothermic vertebrates may also be subject to a temperature-induced alternation in the sympathetic/parasympathetic control of surfactant secretion. However, the temperature-dependent mechanisms of surfactant secretion have only been determined in the lizard and need to be elucidated for the other vertebrate groups.

Most ectothermic vertebrates have highly discontinuous breathing patterns with variable-length nonventilatory periods. Such a pattern may not ensure regular mechanical stimulation of the type II cells and subsequent surfactant release (28). Alternatively, the alveolar type II cells may not receive adequate distortion due to their location within the lung. In lizards, the walls of the faveoli “unfold” rather than inflate and stretch in the manner of mammalian alveoli (28). Furthermore, in reptilian (and bird) lungs, the vascularized region does not change volume significantly, whereas the saccula portion act as bellows. Thus a lizard can change its tidal volume by means of the airsac region. Although the effects of changes in ventilation on surfactant secretion are currently only known for lizards (28) and mammals (18, 20), the discontinuous breathing patterns of most nonmammals suggest that ventilation may not affect secretion in these groups. Ventilatory influences on surfactant secretion may, therefore, have developed subsequent to, or at the time of, the evolution of the bronchial alveolar lung (Fig. 5).
Perspectives

Virtually nothing is known about the factors that control the secretion and composition of pulmonary surfactant in nonmammalian vertebrates. In mammals, secretion is triggered primarily by changes in ventilatory pattern and input from the SNS. In most nonmammals, however, secretion may be controlled predominantly by the autonomic nervous system. In heterothermic animals, the PNS may predominate over the SNS at lower body temperatures, stimulating surfactant secretion without elevating metabolic rate. The control of the surfactant system in the unique avian lung, a structure in which the respiratory tissue does not distort during ventilation, remains unknown.

The regulation of the system may have changed with the evolution of the autonomic nervous system and the development of the bronchoalveolar lung. The PNS may represent the primitive control mechanism, whereas adrenergic influences on the surfactant system may have developed subsequent to the radiation of the tetrapods. Furthermore, the highly variable discontinuous breathing patterns of most nonmammalian vertebrates suggest that ventilatory influences on the surfactant system may have arisen at the time of the evolution of the mammalian bronchoalveolar lung.

The authors gratefully acknowledge Dennis Opheim, Greg Joss, Frank Hemmings, Tim Butler, and Michael Adams for invaluable technical advice and assistance. We also thank Kate Davidson for performing the cholesterol analysis and Sonya Johnstone for assistance in preparing the manuscript.

The majority of the work was funded by Australian Research Council (ARC) grants to C. B. Daniels and S. Orgeig, an ARC Postdoctoral Research Fellowship to S. Orgeig, and a Univ. of Adelaide Postgraduate Research Scholarship and George Murray and Univ. of Adelaide Overseas Travel grants to P. G. Wood. Lungfish experiments were funded in part by ARC Grant A19700437 to M. P. Joss. Experiments on bullfrogs were funded in part by ARC Grant A19700437 to J. M. P. Oyarzun, M. J. Oyarzun, and J. A. Clements. Surfactant release in excised rat lung is stimulated by air inflation. J. Appl. Physiol. 51: 905–910, 1981.


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