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Thermal acclimation of surfactant secretion and its regulation by adrenergic and cholinergic agonists in type II cells isolated from warm-active and torpid golden-mantled ground squirrels, *Spermophilus lateralis*

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Summary

Homeothermic mammals experience pulmonary surfactant dysfunction with relatively small fluctuations in body temperature. However, ground squirrels survive dramatic changes in body temperature during hibernation, when body temperature drops from 37°C to 0–5°C during prolonged torpor bouts. Using type II cells isolated from both warm-active and torpid squirrels, we determined the effect of assay temperature, autonomic agonists and torpor on surfactant secretion. Basal secretion was significantly higher in type II cells isolated from torpid squirrels compared with warm-active squirrels when assayed at the body temperature of the animal from which they were isolated (4°C and 37°C, respectively). A change in assay temperature significantly decreased surfactant secretion. However, the change in secretory rate between 37°C and 4°C was less than expected if due to temperature alone (Q_{10} range=0.8–1.2). Therefore, the surfactant secretory pathway in squirrel type II cells demonstrates some temperature insensitivity. When incubated at the body temperature of the animal from which the cells were isolated, the adrenergic agonist, isoproterenol, significantly increased surfactant secretion in both warm-active and torpid squirrel type II cells. However, the cholinergic agonist, carbamylcholine chloride, only increased secretion in torpid squirrel type II cells when incubated at 4°C. Torpor did not affect basal cAMP production from isolated type II cells. However, the production of cAMP appears to be upregulated in response to isoproterenol in torpid squirrel type II cells. Thus, at the cellular level, both the secretory and regulatory pathways involved in surfactant secretion are thermally insensitive. Upregulating basal secretion and increasing the sensitivity of type II cells to cholinergic stimulation may be adaptative characteristics of torpor that enable type II cells to function effectively at 0–5°C.

Key words: ground squirrel, *Spermophilus lateralis*, pulmonary surfactant, hibernation, torpor, alveolar type II cell, cholinergic, adrenergic.

Introduction

Pulmonary surfactant is a mixture of phospholipids (80–90%), neutral lipids (10%) and proteins, and is secreted into the alveolar airspace to reduce and vary surface tension in response to changes in lung volume (King, 1982). The most abundant phospholipid (PL) is phosphatidylcholine (PC) (79–85%) (King, 1982), and its disaturated form, dipalmitoylphosphatidylcholine (DPPC), containing two molecules of the fatty acid, palmitate, is the major contributor to surfactant surface activity (Possmayer, 1997). The surfactant components are synthesized in alveolar type II epithelial cells (ATII cells) and are stored in specialised secretory organelles known as lamellar bodies (Goerke, 1998; Wright and Dobbs, 1991). In mammals, lamellar bodies are secreted in response to signals from the autonomic nervous system (Chander and Fisher, 1990; Massaro et al., 1982), ventilation (Wirtz and Schmidt, 1992) and local biochemical factors (Chander and Fisher, 1990). In the aqueous layer that lines the alveolar epithelium, lamellar bodies unravel to release the surfactant components, which form a surface-active film at the air–liquid interface (Haagsman and Van Golde, 1991).

Homeothermic mammals, such as humans and rats, experience surfactant dysfunction and respiratory distress with small fluctuations in body temperature (Inoue et al., 1981; Meban, 1978; Peterson and Davis, 1986). However, heterothermic mammals such as fat-tailed dunnarts *Sminthopsis crassicaudata* and golden-mantled ground squirrels *Spermophilus lateralis* regularly experience rapid changes in body temperature when they enter a depressed metabolic state, known as torpor or hibernation, respectively (Geiser and Ruf, 1995). Temperature-induced changes in surfactant amount and/or composition have been observed during the stress-induced torpor of fat-tailed dunnarts *S.*

crassicaudata (Langman et al., 1996) and the daily torpor of the microchiropteran bats *Chalinolobus gouldii* (Codd et al., 2000b) and *Nyctophilus geoffroyi* (Slocombe et al., 2000). In dunnarts, after 8 h of torpor, there are increases in the relative amounts of PL, disaturated phospholipid (DSP) and cholesterol (CHOL) (Langman et al., 1996). These changes correlate with changes in surface activity and, therefore, enable dunnart surfactant to function effectively at torpid body temperatures (Lopatko et al., 1998). Similarly, total PL increased in lavage fluid collected from mildly cold-acclimated ground squirrels *Spermophilus richardsoni* (Melling and Keough, 1981). In contrast, surfactant PL decreased or did not change during torpor in the microchiropteran bats *N. geoffroyi* and *C. gouldii*, respectively (Codd et al., 2000b; Slocombe et al., 2000). The different responses of bats, dunnarts and squirrels probably reflect the different physiological states of the animals during torpor (Codd et al., 2000a).

During hibernation, ground squirrels enter a much deeper and more prolonged torpor than that reported for the stress-induced torpor of small marsupials and daily torpor of bats (Geiser and Ruf, 1995). The depth and duration of their torpor bouts makes squirrels an excellent model for studying the thermal dynamics of a mammalian surfactant system. Furthermore, ground squirrels enter hibernation readily under appropriate laboratory conditions. However, very little is known about the effects of torpor on the surfactant system in hibernators. During the hibernating season, golden-mantled ground squirrels *Spermophilus lateralis* are capable of reducing their body temperatures to as low as 0–5°C during torpor, and torpor bouts usually last for 10–14 days at a time (Milsom et al., 1999). After a torpor bout, the ground squirrels will spontaneously arouse, increasing their body temperature to 37°C for a brief period (h) before returning to a torpid state. Although hibernation is highly advantageous in terms of energy conservation, the long duration and depth of torpor bouts experienced by ground squirrels are likely to have marked effects on the composition, function and regulation of the surfactant system. Alternatively, given the different type of torpor and the annual regularity of the hibernating season, ground squirrels may have adopted novel and unique approaches for maintaining surfactant function at both warm-active (37°C) and torpid (0–5°C) body temperatures.

The low temperatures experienced by ground squirrels during torpor bouts are likely to also have a profound effect on the release of surfactant into the lung and the regulatory and secretory pathways controlling surfactant release. High temperatures increase metabolic rate and thus, may directly stimulate the rate of synthesis and/or secretion of lamellar bodies from type II cells (Chander and Fisher, 1990). Conversely, low temperatures decrease metabolic rate, and may therefore lower the rate of surfactant secretion. In ATII cells isolated from homeothermic rats, a decrease in incubation temperature to 5°C virtually abolishes surfactant secretion (Dobbs and Mason, 1979). Basal secretion is also significantly lower in type II cells isolated from warm-active dunnarts when incubated at 15°C compared to 37°C (Ormond et al., 2001). In dunnart type II cells, however, the decrease in the rate of surfactant secretion has a Q_{10} value of 1.3 (Ormond et al., 2001). The fact that this Q_{10} value is lower than 2, indicates that the secretory pathway in dunnart type II cells is relatively insensitive to temperature and must be regulated or altered in some way to counteract the kinetic effects of decreasing temperature (Ormond et al., 2001; Schmidt-Nielson, 1997). Therefore, we suggest that in ground squirrels, the composition, function and cellular biomechanics of the surfactant system must also be modified to enable efficient functioning at body temperatures of 0–5°C.

The sympathetic nervous system is an important regulator of surfactant release in mammals (Chander and Fisher, 1990). Adrenergic factors are released from the sympathetic nervous system (SNS) and bind to membrane-bound β-adrenergic receptors on type II cells to activate the signalling molecule, cAMP, and enhance surfactant secretion (Brown and Longmore, 1981; Dobbs and Mason, 1978; Wood et al., 1997). Adrenergic agonists stimulate surfactant secretion in type II cells isolated from homeothermic animals such as rat (Brown and Longmore, 1981; Chander and Fisher, 1990), chicken (Sullivan and Orgeig, 2001) and tammar wallaby (Miller et al., 2001), heterothermic animals such as fat-tailed dunnart (Ormond et al., 2001), and ectothermic animals such as bearded dragon, frog, lungfish (Wood et al., 2000) and crocodile (Sullivan et al., 2002). In type II cells isolated from bearded dragons and fat-tailed dunnarts, this response to isoproterenol did not change regardless of assay temperature (Ormond et al., 2001; Wood et al., 1999, 2000). Thus, the regulation of surfactant secretion by the β-adrenergic signalling pathway appears to be relatively temperature-insensitive in lizards and dunnarts. Sympathetic output, however, probably decreases markedly during torpor *in vivo* (Wood et al., 2000). Therefore, regulation by the parasympathetic nervous system (PNS) and cholinergic agonists, which do not increase metabolic rate, may be more important in controlling surfactant release during torpor (Wood et al., 2000).

Carbamylcholine chloride acts through membrane-bound muscarinic receptors to increase PC secretion in ATII cells isolated from fat-tailed dunnarts *Sminthopsis crassicaudata* (Ormond et al., 2001; Wood et al., 1999, 2000), and juvenile, unfurred (heterothermic) tammar wallabies *Macropus eugenii* (Miller et al., 2001). In direct contrast, type II cells isolated from homeothermic mammals such as humans and rats do not respond to cholinergic agonists (Dobbs and Mason, 1979). This suggests that heterothermic mammals may have a direct role for the parasympathetic nervous system in regulating surfactant secretion at low body temperatures. Type II cells isolated from the ectothermic bearded dragon *Pogona vitticeps* (T_b range: 15–40°C; T_b study=25°C) also respond to carbamylcholine chloride, but only at a relatively cold assay temperature of 18°C and not at 37°C (Wood et al., 1999). This switch in the response to cholinergic stimulation in lizard type II cells suggests that the cholinergic signalling pathway is highly sensitive to temperature changes. However, in fat-tailed

dunnarts, the response of isolated type II cells to carbamylcholine chloride remains the same, regardless of assay temperature (Ormond et al., 2001). This may enable dunnart type II cells to respond quickly to a physiological change in autonomic stimulation (adrenergic *vs* cholinergic) during the rapid entry and arousal from torpor (Ormond et al., 2001). Here, we characterise the effect of temperature on the control and release of surfactant from squirrel type II cells isolated from warm-active and torpid ground squirrels. Given the importance of the surfactant system to lung function, we hypothesize that squirrel type II cells will retain the ability to secrete surfactant even at very cold body temperatures. Upregulating their response to adrenergic and cholinergic stimulation may also increase the sensitivity of type II cells to autonomic control during torpor. Understanding how the surfactant system can remain functional over a range of temperatures has important consequences in hypothermic lung transplantation surgery and in the treatment of hypothermia and respiratory distress syndromes (Bernard, 1996; Erasmus et al., 1996; Osanai et al., 1991; Inoue et al., 1981; Meban, 1978).

Materials and methods

Animals

Adult golden-mantled ground squirrels *Spermophilus lateralis* Say 1823 were obtained from a wild-captive colony maintained in the Department of Zoology, University of British Columbia, Canada. Squirrels were housed in a controlled-environment chamber at T_a=22±1°C under a 12 h:12 h light:dark cycle. Squirrels were given water and fed laboratory chow supplemented with sunflower seeds *ad libitum* throughout the experiments, although they rarely ate during the hibernation season. In September and October (late summer), warm-active squirrels (T_b=35.2±0.5°C; mean ± S.E.M., *N*=11) were used in experiments. In late November, the temperature in the controlled-environment chamber was reduced to 5°C and the photoperiod altered (8 h:18 h L:D). Under these conditions, the squirrels entered hibernation within a few weeks. The length of torpor bouts was monitored by placing a wood chip on the back of each squirrel and recording whether it was present or absent each day. Torpid squirrels were used after at least 4 days of torpor without periodic arousal. The rectal body temperature of each squirrel was recorded using a thermocouple. Torpid squirrels recorded body temperatures of 7.7±0.2°C (mean ± S.E.M., *N*=12). Note that there was an overlap period where some squirrels were kept at summer conditions and some at winter conditions during November. Squirrels maintained under summer conditions did not enter hibernation.

Isolation of type II cells

Procedures for isolating type II cells were modified from the methods of Dobbs et al. (1986a), Wood et al. (1999, 2000) and Ormond et al. (2001). Animals were anaesthetised with an intraperitoneal injection of pentobarbitone sodium (50–150 mg kg^{-1} body mass). The trachea was cannulated and the ground squirrel placed on a ventilator set to a volume of 10 ml and a frequency of 20–30 breaths min^{-1} during the perfusion. The thorax was opened and the lungs were perfused, under gravity at 33 cmH_2O *via* the pulmonary artery, with a sterile saline solution containing 2 i.u. ml^{-1} of heparin sodium, until free of blood. The lungs were lavaged *via* the tracheal cannula with three separate 10 ml volumes of ice-cold saline. After lavaging, the lungs were excised from the squirrels, blotted with sterile gauze to remove excess water and weighed to determine wet lung mass. The lungs were then placed in a sterile 50 ml tube containing saline. Aseptic techniques were used from this point in the isolation procedure and carried out in a laminar flow hood. The lungs were rinsed twice with sterile phosphate-buffered saline containing 10 U ml^{-1} penicillin, 10 μg ml^{-1} streptomycin and 25 ng ml^{-1} amphotericin and transferred to new 50 ml sterile tubes containing 6 U ml^{-1} elastase (120 U per lung), 250 μg ml^{-1} DNase, 10 U ml^{-1} penicillin and 10 μg ml^{-1} streptomycin. The lungs were shaken continuously at room temperature for 30–40 min. The digested lungs were further dissociated mechanically by pipetting up and down with a 1 ml pipette for 2 min and filtered through a sterile 100 mesh filter (Sigma Chemical Company, St Louis, MO, USA) to remove any undigested tissue fragments and large contaminating cells. Cell suspensions were centrifuged at 200 ***g*** for 10 min at 22°C (Beckman GS-6R centrifuge) and the supernatant discarded.

The cell pellets were resuspended in an appropriate volume of DMEM + 10% foetal bovine serum (FBS) (containing 10 U ml^{-1} penicillin and 10 μg ml^{-1} streptomycin) and incubated on tissue culture plates for 1 h at the appropriate temperature (37°C for cells from warm-active squirrels and 4°C for cells from torpid squirrels) to allow any fibroblasts to attach to the plates. The plates were then gently ‘panned’ and rinsed twice with 5 ml of culture medium to remove unattached type II cells. All plate washes were collected and pooled. Cell suspensions were centrifuged at 200 ***g*** for 10 min at 22°C (Beckman GS-6R centrifuge) and the supernatant discarded. Sterile bacteriologic plates (size 60/15, Greiner Laboratories, Austria) were coated with 3–5 ml of bovine IgG solution (500 μg ml^{-1}, Sigma Chemical Corp., St Louis, MO, USA) and incubated at 22°C for a minimum of 3 h. IgG-coated plates were then washed twice with 5 ml of phosphate buffered saline (PBS, Sigma) and once with 5 ml of sterile culture medium (DMEM containing 20 mmol l^{-1} Hepes, 3.7 g l^{-1} sodium bicarbonate, 100 000 U l^{-1} penicillin, 100 mg l^{-1} streptomycin). Cell pellets were resuspended in 4–6 ml per plate of culture medium containing 250 μg ml^{-1} DNase and added to the bacteriological plates. Plates were incubated at the appropriate temperature (37°C for cells from warm-active squirrels, 4°C for cells from torpid squirrels) with 10% CO_2 for 1 h to allow macrophages to attach to the IgG-coated plates. After 1 h, the plates were examined to ensure that macrophages had attached to the plates. The plates were then gently ‘panned’ and rinsed twice with 5 ml of culture medium to remove unattached type II cells. All plate washes were collected and pooled. Final cell suspensions were examined using light and

electron microscopy to confirm cell type and purity. Type II cells have a cuboidal shape and contain lamellar bodies and microvilli. The ability to secrete PC also confirmed that these cells were type II epithelial cells. The final cell suspensions isolated by this method were >90% pure type II cells. The remaining proportion of cells consisted of macrophages and neutrophils that had not adhered to the IgG-coated plates. The occasional erythrocyte was also present.

Cell viability was measured by the exclusion of the vital dye, Trypan Blue (Dobbs et al., 1986b) using a haemocytometer (Neubauer improved, depth 0.1 mm, 0.0025 mm^2). Viable type II cells actively exclude the Trypan Blue dye and remain clear. Non-viable type II cells do not exclude the Trypan Blue dye and, therefore, appear blue. A total of 8 cell counts per cell suspension were performed to calculate percentage viability. Cell viability was determined using Trypan Blue on all freshly isolated cells at the time of plating for each experiment. In addition, cell viability was determined using a lactate dehydrogenase (LDH) cytotoxicity assay (Roche Diagnostics, GmbH, Germany) after overnight incubations at each of the temperatures and during the course of the secretion experiments. The assay was performed as per the manufacturer's instructions.

Microscopy of squirrel lung

Freshly isolated squirrel lungs were cut into 1 mm^3 pieces and fixed in 4% paraformaldehyde, 1.25% glutaraldehyde, 4% sucrose in 0.1 mol l^{-1} PBS, pH 7.2, at 4°C for 3–7 days. The fixed material was washed in 0.1 mol l^{-1} PBS and postfixed in 1% Osmium Tetroxide overnight. Tissue pieces were then stained *en bloc* in 1.5% uranyl acetate, dehydrated in 70, 80, 90 and 100% ice-cold acetone, embedded in Araldite resin and polymerized. Cut sections were mounted on grids and photographed using a transmission electron microscope (Philips CM 100 TEM).

Measurement of PC secretion

Fresh cell isolates from squirrels were centrifuged at 200 ***g*** for 10 min at 22°C (Beckman GS-6R centrifuge) and the cell pellet resuspended in culture medium containing 10% FBS (heat inactivated) to give a concentration of 3×10^6 cells ml^{-1}. 2 µl ml^{-1} of [methyl-^{3}H]choline chloride (specific activity 3.00 Tbq $mmol^{-1}$, 81.0 Ci $mmol^{-1}$, 1 µCi ml^{-1}, Amersham Pharmacia Biotech, Canada) was added to the final cell suspension. 100 µl of ^{3}H-labelled cell suspension were added to each well of fibronectin-coated plates to give a density of 3×10^5 cells $well^{-1}$ (0.6×10^6 cells cm^{-2}). Fibronectin coated plates (5 µg cm^{-2}) were prepared by adding 100 µl of fibronectin (25 µg ml^{-1}) to each well of a sterile, flat-bottomed 96-well tissue culture plate (Falcon, Becton Dickinson Labware, NJ, USA). The plates were incubated at room temperature for 45 min to allow the fibronectin to bind. The fibronectin solution was removed from the plates by aspiration, immediately before addition of the cell suspension. Plates were incubated for 22 h at the appropriate temperature with 10% CO_2. The type II cells attached to the fibronectin-coated plates during this time.

After 22 h, the cells were examined under the microscope to assess viability and morphology. Cell viability was determined after the 22 h incubations in wells without radiolabel, using both Trypan Blue and a LDH cytotoxicity assay, and during the course of the secretion experiments. Stock solutions (1 mmol l^{-1}) of agonists (adrenergic agonist, isoproterenol and cholinergic agonist, carbamylcholine chloride) were prepared immediately prior to their use in sample medium (culture medium containing 1 mmol l^{-1} sodium ascorbate and 1% FBS) equilibrated to 4°C or 37°C. A total volume of 100 µl, prepared by the addition of 90 µl of sample medium and 10 µl of 1 mmol l^{-1} agonist solutions, was added gently to the experimental wells (three replicates of each). 90 µl of sample medium, followed by 10 µl of sample medium was added to control wells. 100 µmol l^{-1} concentrations of agonists were chosen from the literature (Brown and Longmore, 1981; Dobbs et al., 1986a). Plates were incubated for 30 min and 1 h at either 4°C or 37°C and in 10% CO_2. Following each experiment, the medium from each well was collected, each well was washed twice with 200 µl of culture medium and the washes pooled. 100 µl of 0.25% (w/v) trypsin/0.04% EDTA solution in sterile PBS was added to the wells. Media samples were centrifuged at 3800 ***g*** for 2 min in a capsule microcentrifuge (Tomy Seiko, Japan). The supernatant was transferred to fresh Eppendorf tubes, the cell pellet resuspended in 100 µl of fresh culture medium and centrifuged for a further 2 min to wash any secreted phospholipids from the cells. This last 100 µl wash was collected and added to the media samples. Once all the time points had been completed, all plates were incubated at 37°C until the trypsin/EDTA solution had removed all attached cells from the wells. The trypsin/EDTA solution from each well was added to the cell pellets (from medium spins) and each well was washed twice with 200 µl of culture medium and the washes also pooled. Samples were stored at –20°C until extracted. Lipids were extracted from the media and cell samples using the method of Bligh and Dyer (1959). Unlabelled L-α-phosphatidylcholine (250 µg) was added as a carrier molecule to the extractions to improve the recovery of radioactive lipids. Lipids in chloroform were transferred to 8 ml scintillation vials and evaporated in air (16 h). Lipids were reconstituted in 2 ml of ReadyOrganic™ liquid scintillation fluid, vortexed and the radioactivity counted on a liquid scintillation counter (Beckman LS 3801).

Data analysis

c.p.m. values were obtained for the medium and cell fractions of each sample. Results are expressed as the percentage secretion of incorporated ^{3}H-PC, which was calculated, for each sample, as follows:

$$\%\text{secretion} = \frac{\text{c.p.m. of media fraction}}{\text{total c.p.m.}} \times 100\,, \qquad (1)$$

where total c.p.m. = c.p.m. of medium fraction + c.p.m. of cell fraction.

Values for the percentage secretion were arcsin-transformed

and between-group differences in %PC secretion between warm-active and torpid groups, the presence or absence of agonist or the two incubation temperatures, were analysed using unpaired Student's *t*-tests (two-sample for means, assuming equal variance, $P<0.05$). For within-group comparisons (i.e. to determine if the agonist affected cells from one particular animal), the control and agonist-treated groups were from the same preparation of cells and incubated and treated in an identical manner and at the same temperature on the same plate. Therefore, the differences in PC secretion between control and agonist-treated wells were analyzed by paired *t*-tests (two-sample for means).

Q_{10} measurements were calculated for each time point at 37°C and 4°C from the mean of the %PC secretion values obtained in each experiment, using the following equation (Schmidt-Nielson, 1997):

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2-T_1}}, \quad (2)$$

where R_2=% secretion at 37°C, R_1=% secretion at 4°C, T_2=37°C and T_1=4°C.

cAMP production

The cAMP assay was adapted from McKinley and Hazel (2000). Fresh cell isolates were incubated overnight in DMEM + 10% FBS on bacteriological plates. After 22 h, the cells were centrifuged at 200 ***g*** and washed twice with PBS. After the final spin, cells were resuspended in PBS containing 0.83 mg ml^{-1} theophylline at 22°C and counted using a haemocytometer. The cell suspension was diluted to approximately 1×10^6 cells ml^{-1}. 300 µl of cell suspension were sampled into Eppendorf tubes (3×10^5 cells per tube). 30 µl of sample medium was added to the control tubes and 30 µl of 1 mmol l^{-1} isoproterenol (prepared in PBS containing theophylline) was added to each Eppendorf tube. The tubes were incubated at either 37°C or 4°C for 15 min. After 20 min, 60 µl of 6% (w/v) trichloroacetic acid (TCA) was added to each tube and the tubes plunged immediately into liquid nitrogen to stop the reaction. The samples were thawed and neutralised with 200 µl of 2 mol l^{-1} $KHCO_3$. The samples were centrifuged for 2 min at 17 000 ***g*** in an Eppendorf centrifuge and the supernatant collected. The samples were then stored at –80°C for a maximum of 21 days. cAMP was measured using a direct cAMP ELISA kit (Amersham Pharmacia Biotech, Canada). cAMP production (pmole $cell^{-1}$) was calculated from the data. Data were analysed using paired and unpaired *t*-tests assuming equal variance. Statistical significance was assumed at $P<0.05$.

Results

Descriptive statistics

Warm-active and torpid ground squirrels had mean rectal body temperatures of 35.2±0.5°C (mean ± S.E.M., N=11) and 7.7±0.2°C (mean ± S.E.M., N=12), respectively. The wet lung mass recorded for the warm-active and torpid squirrels used in these secretion studies were 3.88±0.31 g (mean ± S.E.M., N=6) and 3.49±0.25 g (mean ± S.E.M., N=6), respectively. There was no significant difference between recorded wet lung mass for warm-active and torpid squirrels.

Cell yield, purity and viability

When analysed using Trypan Blue, yields of $34.5\pm3.3\times10^6$ (mean ± S.E.M., N=15) and $29.3\pm3.3\times10^6$ (mean ± S.E.M., N=10) viable alveolar type II cells were obtained from each warm-active and torpid squirrel, respectively. Freshly isolated warm-active squirrel cell suspensions were 96.6±0.4% (mean ± S.E.M., N=16) viable. Freshly isolated torpid squirrel cell suspensions were 97.85±0.33% viable (N=12). Type II cell suspensions from warm-active squirrels were 93.4±0.5% (mean ± S.E.M., N=10) viable after overnight incubations at 37°C. Type II cell suspensions from torpid squirrels were 98.9±0.6% viable (mean ± S.E.M., N=10) after overnight incubations at 4°C. As determined by the LDH assay, cell viability remained above 90% after overnight incubations and for the duration of all experiments, at both temperatures. Both the incubation temperature and the presence or absence of agonists had no effect on cell viability, as measured by the exclusion of the vital dye, Trypan Blue and the LDH cytotoxicity assay.

Microscopy

An electron micrograph of the alveolar epithelium of the lung of a golden-mantled ground squirrel *Spermophilus lateralis* is shown in Fig. 1. The photograph shows an alveolar type II cell, located in a crevice between alveoli, and demonstrates the cuboidal shape, microvilli and presence of large osmiophilic lamellar bodies in these cells.

Surfactant secretion

The effect of temperature on phosphatidylcholine secretion

Basal secretion after 0.5 and 1 h of incubation was significantly higher when cells were incubated at a temperature matching the body temperature of the squirrel from which they were isolated (37°C or 4°C), compared to the alternative assay temperature (4°C or 37°C, respectively) (Fig. 2). In type II cells isolated from warm-active squirrels, basal secretion was 1.6- and 2.2-fold higher at 0.5 and 1 h, respectively, at an assay temperature of 37°C compared to 4°C. In type II cells isolated from torpid squirrels, basal secretion was 1.8- and 1.7-fold higher at 0.5 and 1 h, respectively, at an assay temperature of 4°C compared to 37°C. Consequently, agonist-stimulated secretion was also significantly higher at an assay temperature of 4°C than at 37°C in torpid squirrel cells at 0.5 and 1 h time points. The Q_{10} values obtained for the process of surfactant secretion in type II cells isolated from warm-active and torpid squirrels are shown in Table 1. For cells from both warm-active and torpid squirrels, the presence of agonists had no effect on the Q_{10} value of surfactant secretion. Cells from torpid squirrels had a significantly lower Q_{10} value than that observed in cells from warm-active squirrels (1.23 *vs* 0.86); however, both Q_{10} values fell well below a Q_{10} of 2, which

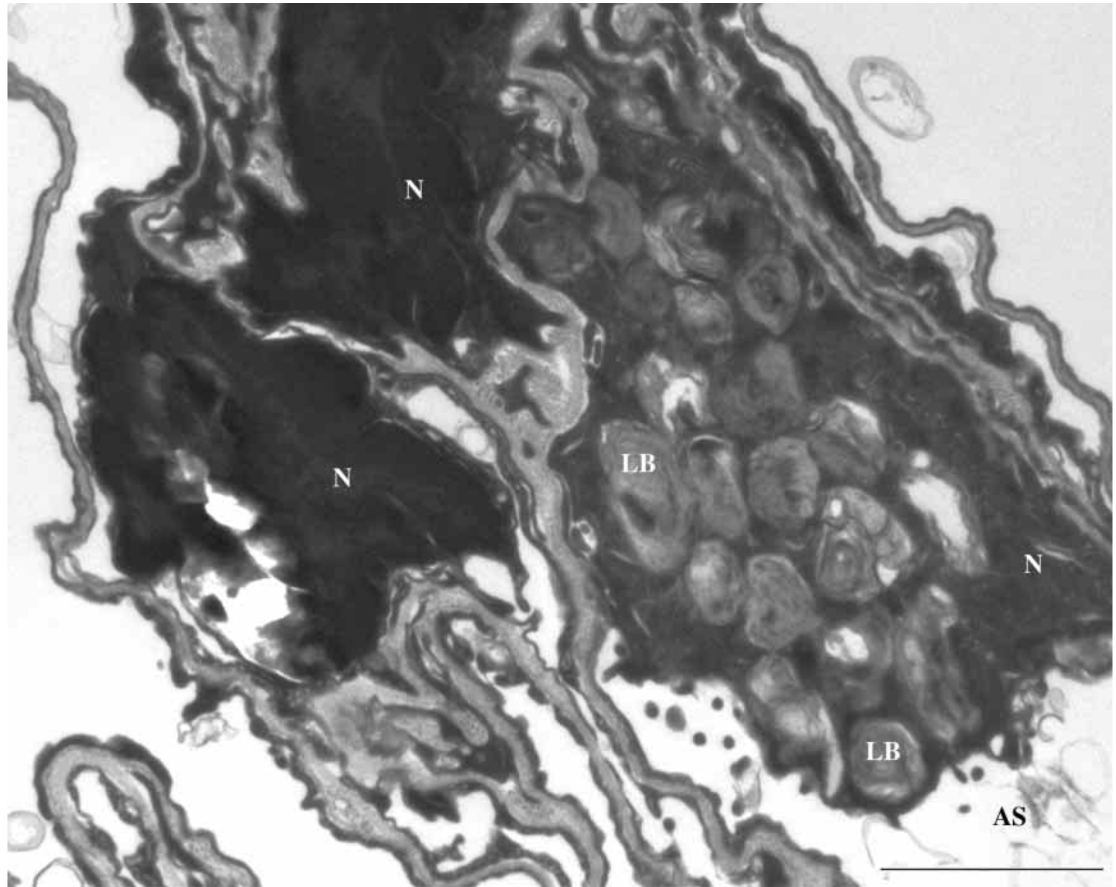

Fig. 1. Electron micrograph of an alveolar type II epithelial cell in lung tissue isolated from the golden mantled-ground squirrel *Spermophilus lateralis*. Scale bar, 2 µm. AS, air space showing surfactant myelin material; LB, lamellar body; N, nucleus.

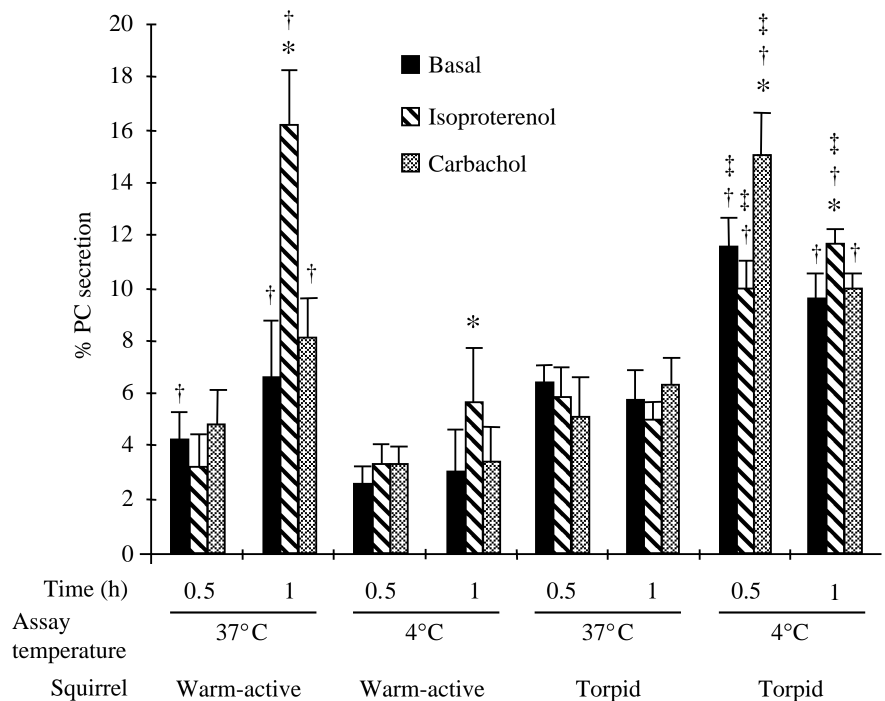

Fig. 2. Regulation of surfactant phosphatidylcholine (PC) secretion by the adrenergic agonist, isoproterenol (100 µmol l^{-1}) and the cholinergic agonist, carbamylcholine chloride (Carbachol, 100 µmol l^{-1}), in alveolar type II cells isolated from warm-active and torpid ground squirrels. Both warm-active and torpid cells were assayed at 37°C and 4°C for 0.5 and 1 h. *Significant increase above basal secretion for each experimental group (paired *t*-test, $P<0.05$). †Significant difference in secretion between matching experimental groups measured at the two different assay temperatures, i.e. 0.5 h/torpid/4°C *vs* 0.5 h/torpid/37°C (*t*-test, $P<0.05$). ‡Significant difference in % secretion between warm-active cells assayed at 37°C and torpid cells assayed at 4°C (*t*-test, $P<0.05$). Bars represent % of incorporated [^{3}H]choline secreted as phosphatidylcholine (mean ± S.E.M.) for *N* experiments after 0.5 and 1 h; for warm-active animals, *N*=4; for torpid animals, *N*=5. % secretion = % c.p.m. measured in the medium divided by the total cpm (cells + medium)×100 measured for each sample.

Table 1. *Q_{10} values for basal and agonist-stimulated secretion of phosphatidylcholine (PC) from type II cells isolated from warm-active and torpid ground squirrels* Spermophilus lateralis

	PC secretion (Q_{10})		
Incubation time	Basal	Isoproterenol	Carbachol
Warm-active squirrel cells			
0.5 h	1.15	1.00	1.12
1 h	1.35	1.36	1.09
Mean	1.25±0.1*	1.18±0.18*	1.11±0.02*
Torpid squirrel cells			
0.5 h	0.87	0.88	0.76
1 h	0.89	0.79	0.89
Mean	0.88±0.01*	0.84±0.04*	0.83±0.06*

Q_{10} values were calculated from the mean rates of secretion given in Fig. 2.

*Significant difference between the Q_{10} values obtained for cells isolated from warm-active and torpid squirrels (*t*-test, $P<0.05$).

There were no significant differences between the Q_{10} values obtained for basal and agonist-stimulated secretion ($P>0.05$), nor between incubation times ($P>0.05$).

suggests that surfactant secretion in squirrel type II cells is insensitive to temperature.

Temperature and the control of phosphatidylcholine secretion by neurochemicals

In type II cells isolated from warm-active squirrels, the adrenergic agonist, isoproterenol, significantly increased surfactant secretion above basal levels after 1 h at 37°C, but not after 0.5 h at 37°C (Fig. 2). When warm-active squirrel type II cells were incubated at 4°C, isoproterenol significantly increased surfactant secretion above basal levels after 1 h. Torpid squirrel type II cells did not respond to the adrenergic agonist, isoproterenol, after 0.5 or 1 h at an assay temperature of 37°C; however, isoproterenol did significantly increase surfactant secretion after 1 h at an assay temperature of 4°C. The cholinergic agonist, carbamylcholine chloride, did not significantly increase surfactant secretion above basal levels in type II cells isolated from warm-active ground squirrels, at assay temperatures of either 37°C or 4°C (Fig. 2). Moreover, type II cells isolated from torpid ground squirrels did not respond to carbamylcholine chloride when incubated at 37°C; however, carbamylcholine chloride did significantly increase surfactant secretion above basal levels in torpid squirrel cells when the cells were incubated at 4°C for 30 min.

cAMP production

Basal cAMP production was unaffected by either the state of the squirrel from which the cells were isolated, or by the temperature at which the assay was performed (Fig. 3). Isoproterenol significantly increased cAMP production in type II cells isolated from both warm-active and torpid squirrels at both 37°C and 4°C assay temperatures. When assayed at the body temperature of the squirrel from which the cells were isolated, isoproterenol-stimulated secretion was significantly higher in type II cells isolated from torpid ground squirrels compared to warm-active squirrels.

Discussion

The effect of temperature on basal secretion from type II cells

Our laboratory has successfully modified the cell culture method of Dobbs et al. (1986a) to isolate mammalian and non-mammalian alveolar type II cells using different digestive enzymes (Miller et al., 2001; Ormond et al., 2001; Sullivan and Orgeig, 2001; Sullivan et al., 2001; Sullivan et al., 2002; Wood et al., 1999, 2000). In this study, Trypan Blue, microscopy and LDH cytotoxicity assays confirmed the health and viability of the squirrel type II cells during the isolation and assay procedures at both 4°C and 37°C. Warm-active squirrel type II cells assayed at 37°C appeared to secrete 2–3 times the amount of phosphatidylcholine (6% after 1 h) than was secreted from isolated rat type II cells in culture (2% after 1.5 h) (Brown and Longmore, 1981; Dobbs and Mason, 1979). Furthermore, torpid squirrel type II cells assayed at 4°C secreted even higher basal levels of phosphatidylcholine (10%

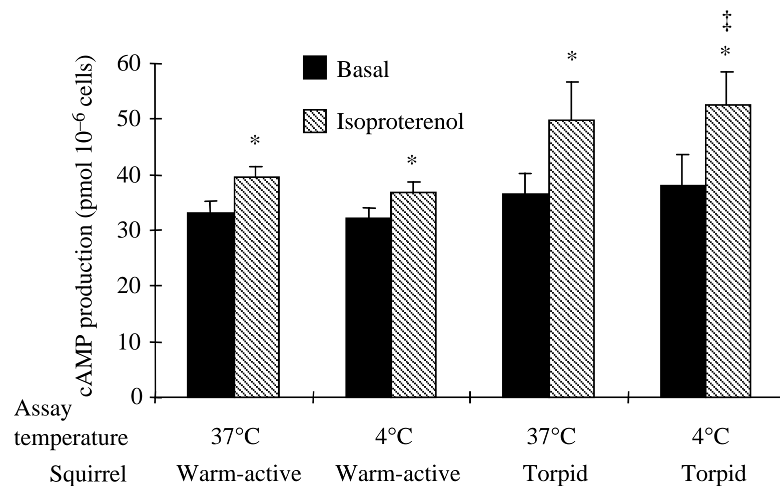

Fig. 3. The production of cAMP (pmoles 10^{-6} cells) in alveolar type II cells isolated from warm-active and torpid ground squirrels and assayed at two assay temperatures (37°C and 4°C). cAMP production was calculated using a direct cAMP ELISA kit (Amersham Pharmacia, Sydney, Australia). Bars represent the amount of cAMP produced (mean ± S.E.M.) in N=5 experiments in all cases. *Significant increase above basal cAMP production for each experimental group; †significant difference between warm-active cells assayed at 37°C and torpid cells assayed at 4°C (*t*-test, $P<0.05$).

after 1 h) than warm-active type II cells at 37°C (Fig. 2). High levels of phosphatidylcholine secretion have been observed in type II cells isolated from heterothermic fat-tailed dunnarts *Sminthopsis crassicaudata* (10% after 1 h) (Ormond et al., 2001) and juvenile tammar wallabies *Macropus eugenii* (12–18% after 4 h) (Miller et al., 2001). Hence, a higher rate of basal phosphatidylcholine secretion may be an adaptation to the temperature fluctuations experienced by heterothermic mammals. In addition to reducing the intrapulmonary pressure required to inflate compressed lungs (Possmayer, 1997), larger amounts of surfactant may be required during torpor to prevent the adhesion of alveolar surfaces, which are in contact with each other during long non-ventilatory periods (Daniels et al., 1998).

PC secretion from type II cells isolated from torpid squirrels incubated at 4°C is almost double that measured from type II cells isolated from warm-active squirrels incubated at 37°C. Therefore, despite a dramatic drop in body temperature during torpor, the release of surfactant from type II cells in torpid squirrels is maintained and even upregulated to levels above those of type II cells from warm-active squirrels. Such an observation suggests that squirrel type II cells and, particularly, their pathways of surfactant secretion, are highly adapted to cope with temperature fluctuations. Furthermore, the upregulation of surfactant secretion during torpor highlights the importance of surfactant function to the lung even when ventilatory rate and tidal volume are markedly reduced. The Q_{10} values obtained from type II cells isolated from both warm-active and torpid squirrels and incubated at both 37°C and 4°C are given in Table 1. In all experimental groups, the Q_{10} values were significantly below 2, which confirms that the process of surfactant secretion in squirrel type II cells is highly insensitive to changes in incubation temperature. We reported a similar observation (Q_{10}=1.2) in type II cells isolated from warm-active dunnarts and incubated at 37°C and 18°C (Ormond et al., 2001). In addition, the Q_{10} value of 0.85 obtained for type II cells isolated from torpid squirrels is significantly lower than the value of 1.2 obtained for type II cells isolated from warm-active squirrels. This may indicate that during torpor, additional modifications occur at the cellular level to promote the secretion of surfactant from squirrel type II cells. Such modifications may include thermal acclimation of plasma membranes (Hazel and Zerba, 1986), thermal modification of proteins or enzymes (Storey, 1997) or the upregulation of receptors and signalling molecules (Van Breukelen and Martin, 2002) involved in the surfactant secretory and regulatory pathways within the cell.

Although the process of surfactant secretion from squirrel type II cells is relatively insensitive to temperature, short-term changes in assay temperature significantly decreased surfactant secretion. PC secretion from type II cells isolated from warm-active squirrels was significantly higher at an assay temperature of 37°C than at 4°C. Conversely, PC secretion from type II cells isolated from torpid squirrels was significantly higher at an assay temperature of 4°C than at 37°C. Hence, basal surfactant secretion was significantly higher when type II cells were incubated at a temperature similar to the body temperature of the squirrel from which they were isolated, whether 37°C or 4°C. The decreases in surfactant secretion from warm-active squirrel type II cells incubated at 4°C compared to 37°C could be attributed to a decrease in cellular metabolic rate at 4°C. A similar decrease in PC secretion was observed in warm-active dunnart type II cells incubated at 18°C compared to 37°C (Ormond et al., 2001). We observed a significant increase in PC secretion in type II cells isolated from torpid squirrels and incubated at 4°C, however, and this suggests that squirrel type II cells undergo a process of thermal acclimation, in preparation for or during torpor, which results in the upregulation of surfactant secretion. Therefore, the upregulation of surfactant secretion from squirrel type II cells appears to be an adaptive characteristic of torpor.

Control of phosphatidylcholine secretion by an adrenergic agonist

Warm-active squirrel type II cells appear to respond to isoproterenol at both warm and cold incubation temperatures. This is similar to observations we have previously made in type II cells isolated from bearded dragons and warm-active dunnarts incubated at 18°C and 37°C (Wood et al., 1999). In the present study, the stimulatory response of squirrel type II cells to isoproterenol after 1 h at 37°C (270% after 1 h) is similar to that observed in rat type II cells at 37°C (300% after 1.5 h) (Dobbs and Mason, 1979). In type II cells isolated from torpid squirrels, the response to isoproterenol is small (125% after 1 h at 4°C) compared to that observed in warm-active squirrel cells (270% after 1 h at 37°C). Although the relative roles of the sympathetic and parasympathetic nervous systems during torpor are not yet clear, it is generally accepted that during the deep torpor of hibernators the activity of the sympathetic nervous system is dramatically reduced, if not eliminated (Milsom et al., 1999). Hence, adrenergic agonists may not be an important regulatory mechanism of surfactant secretion during deep torpor. Consequently, squirrel type II cells may downregulate their response to adrenergic stimulation by decreasing receptor number or the activity of enzymes in the β-adrenergic stimulatory pathway. Decreasing receptor number, however, could potentially impair the initiation of arousal, which is accompanied by a large increase in sympathetic activity (Milsom et al., 1999). Hence, in order to enable a rapid response to the return of sympathetic activation, it seems more likely that receptor number on alveolar type II cells is maintained during torpor. Furthermore, we observed an increase in the production of cAMP in response to isoproterenol in torpid squirrel type II cells (Fig. 3), which indicates that isoproterenol is binding to at least some β-adrenergic receptors in the plasma membranes of torpid cells. A decrease in cellular metabolic rate and/or the activity of enzymes involved in β-adrenergic receptor signalling could also account for the smaller response to adrenergic stimulation we observed in torpid squirrel type II cells; however, we have observed that torpid squirrel type II

cells have significantly higher levels of basal PC secretion than warm-active squirrel type II cells, when assayed at the body temperature of the animal from which the cells were isolated. Therefore, the upregulation of basal secretion may be an adaptive response to the decrease in adrenergic stimulation, and hence, agonist-stimulated surfactant secretion, during torpor. Alternatively, the decrease in the response of torpid type II cells to adrenergic stimulation may be due, at least in part, to the upregulation of basal secretion during torpor, and therefore a lower reserve capacity to respond to adrenergic agonists.

Control of phosphatidylcholine secretion by a cholinergic agonist

Carbamylcholine chloride did not appear to stimulate surfactant secretion very effectively in the squirrel, and this finding is consistent with observations made in homeothermic mammalian type II cells. While carbamylcholine chloride can stimulate surfactant secretion *in vivo*, it does not appear to act directly on type II cells isolated from homeothermic mammals (Dobbs and Mason, 1979). However, a significant increase in response to carbamylcholine chloride was observed in torpid squirrels at an assay temperature of 4°C after 1 h and this finding is consistent with observations made in type II cells isolated from heterothermic mammals and ectothermic animals. Type II cells isolated from dunnarts *S. crassicaudata,* bearded dragons *P. vitticeps*, frogs *Rana catesbeiana* and Australian lungfish *Neoceratodus forsteri* respond directly to cholinergic agonists (Wood et al., 1999, 2000). Furthermore, in isolated bearded dragon type II cells, carbamylcholine chloride only stimulated cells that were incubated at cold assay temperatures (18°C) and not at warm assay temperatures (37°C) (Wood et al., 1999). Similarly, in the present study, carbamylcholine chloride only stimulated surfactant secretion in torpid squirrel cells incubated at 4°C, and not at 37°C. Furthermore, warm-active squirrel type II cells did not respond to carbamylcholine chloride at either incubation temperature. Hence, the response to carbamylcholine chloride appears to be highly temperature sensitive in squirrel and lizard type II cells and appears to be only operational at cold temperatures. The switch in the response of type II cells to cholinergic factors at low incubation temperatures may be due to temperature-sensitive cholinergic receptors or enzymes in the cholinergic signalling pathway. Alternatively, low temperatures may reduce the activity of enzymes, such as acetylcholinesterase, which break down acetylcholine and, therefore, may lead to a relative increase in the amount of acetylcholine interacting with the cholinergic receptors on type II cells (Wood et al., 1999).

In this study, type II cells isolated from warm-active squirrels did not respond to cholinergic stimulation, which suggests that parasympathetic control of surfactant secretion, through interactions with muscarinic receptors on type II cells, is not crucial in warm-active animals. However, it should be noted that parasympathetic control of surfactant secretion from type II cells may still occur indirectly in warm-active animals *in vivo*. Wood et al. (1997) postulated that the parasympathetic nervous system can also stimulate surfactant secretion *in vivo via* the stimulation of receptors on pulmonary smooth muscle and the subsequent distortion of type II cells (mechanical stimulation). In ground squirrels, the entrance into torpor is controlled by the parasympathetic nervous system, which regulates the initial change in heart rate that occurs before body temperature falls (Milsom et al., 1999). The failure of warm-active squirrel type II cells to respond to cholinergic agonists, at both warm and cold assay temperatures, suggests that parasympathetic control may not be important in controlling surfactant secretion during entry into torpor. Indeed, as body temperature begins to fall, parasympathetic tone appears to be progressively withdrawn (Milsom et al., 1999). Some authors believe that during deep torpor, parasympathetic influence is at a minimum (Lyman and O'Brien, 1963) or completely absent (Milsom et al., 1993). There is also evidence, however, to suggest that both the sympathetic and parasympathetic nervous systems are reduced in proportion to body temperature (Q_{10}=3) (Milsom et al., 1993). Furthermore, although the activity of the vagus nerve is low during torpor, other studies support the conclusion that the parasympathetic system still plays some role in cardiovascular control (Milsom et al., 1999). In this study, torpor increased the sensitivity of squirrel type II cells to cholinergic agonists, and this supports observations that some parasympathetic tone is retained during deep torpor. Increasing the sensitivity of type II cells to direct cholinergic stimulation during torpor may enable some regulation of surfactant secretion, despite a reduced autonomic output.

cAMP production

We observed no differences in basal cAMP production between type II cells isolated from torpid squirrels and warm-active squirrels assayed at 4°C and 37°C, respectively. Hence, ground squirrels are still able to maintain cAMP levels during torpor at 4°C. Furthermore, an acute temperature change for the assay period (from 37°C to 4°C in warm-active squirrel cells or from 4°C to 37°C in torpid squirrel cells) had no effect on cAMP production. This is probably due to the short incubation time (15 min) of the cAMP assay which, under culture conditions, may not have been long enough to enable the type II cells to register the temperature change. Isoproterenol significantly increases cAMP levels in type II cells isolated from warm-active squirrels and torpid squirrels at both assay temperatures; however, isoproterenol-stimulated cAMP production is significantly higher in type II cells isolated from torpid ground squirrels compared to cells isolated from warm-active squirrels when assayed at the temperature that matched the body temperature of the squirrels from which the cells were isolated. This indicates that the squirrel type II cells may be upregulating their response to isoproterenol during torpor without increasing basal cAMP levels. Furthermore, isoproterenol-induced cAMP production does not appear to correspond directly to isoproterenol-stimulated PC secretion. cAMP is an important indicator of β-adrenergic receptor activity; however, there is no evidence to suggest that increases

in cAMP are directly linked to increases in surfactant secretion. In fact, this study suggests that cAMP is not directly involved in the stimulation of PC secretion, and the activities of other signalling molecules may play a role here.

Conclusions

In this study, we observed that alveolar type II cells isolated from torpid squirrels demonstrate an increased basal secretion of pulmonary surfactant. These findings are supported by previous observations that surfactant PL increases during cold-acclimation in ground squirrels (Melling and Keough, 1981). The process of surfactant secretion from squirrel type II cells is highly resistant to temperature changes, as demonstrated by Q_{10} values of 0.85–1.2. Furthermore, when assayed at the temperature matching the body temperature of the animal from which they were isolated, type II cells from torpid squirrels demonstrated a higher basal surfactant secretion than those isolated from warm-active squirrels. Therefore, the upregulation of surfactant secretion from squirrel type II cells appears to be an adaptive characteristic of torpor, as does the response of type II cells to cholinergic stimulation. Increasing the sensitivity of type II cells to cholinergic (parasympathetic) stimulation during torpor may enable surfactant secretion to be regulated or enhanced, even when autonomic output is low. The response of squirrel type II cells to cholinergic stimulation during torpor, but not euthermia, supports observations that although parasympathetic tone may be significantly reduced during deep torpor, it is still important in regulating some physiological processes.

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