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Dipalmitoylphosphatidylcholine is not the major surfactant phospholipid species in all mammals

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Lang, Carol J., Anthony D. Postle, Sandra Orgeig, Fred Possmayer, Wolfgang Bernhard, Amiya K. Panda, Klaus D. Jürgens, William K. Milsom, Kaushik Nag, and Christopher B. Daniels. Dipalmitoylphosphatidylcholine is not the major surfactant phospholipid species in all mammals. *Am J Physiol Regul Integr Comp Physiol* 289: R1426–R1439, 2005. First published July 21, 2005; doi:10.1152/ajpregu.00496.2004.—Pulmonary surfactant, a complex mixture of lipids and proteins, lowers the surface tension in terminal air spaces and is crucial for lung function. Within an animal species, surfactant composition can be influenced by development, disease, respiratory rate, and/or body temperature. Here, we analyzed the composition of surfactant in three heterothermic mammals (dunnart, bat, squirrel), displaying different torpor patterns, to determine: *1*) whether increases in surfactant cholesterol (Chol) and phospholipid (PL) saturation occur during long-term torpor in squirrels, as in bats and dunnarts; *2*) whether surfactant proteins change during torpor; and *3*) whether PL molecular species (molsp) composition is altered. In addition, we analyzed the molsp composition of a further nine mammals (including placental/marsupial and hetero-/homeothermic contrasts) to determine whether phylogeny or thermal behavior determines molsp composition in mammals. We discovered that like bats and dunnarts, surfactant Chol increases during torpor in squirrels. However, changes in PL saturation during torpor may not be universal. Torpor was accompanied by a decrease in surfactant protein A in dunnarts and squirrels, but not in bats, whereas surfactant protein B did not change in any species. Phosphatidylcholine (PC)16:0/16:0 is highly variable between mammals and is not the major PL in the wombat, dunnart, shrew, or Tasmanian devil. An inverse relationship exists between PC16:0/16:0 and two of the major fluidizing components, PC16:0/16:1 and PC16:0/14:0. The PL molsp profile of an animal species is not determined by phylogeny or thermal behavior. We conclude that there is no single PL molsp composition that functions optimally in all mammals; rather, surfactant from each animal is unique and tailored to the biology of that animal.

lung; temperature; surfactant proteins; electrospray ionization mass spectrometry; cholesterol

PULMONARY SURFACTANT is a complex mixture of lipids (90% by weight) and proteins (10% by weight) that reduces surface tension in the terminal air spaces, thus preventing pulmonary collapse (39). Phospholipids (PL) comprise 80–90% of the surfactant lipids, with phosphatidylcholine (PC) being the most abundant PL (70–85%). In homeothermic mammalian surfactant, the disaturated molecular species (molsp), dipalmitoylphosphatidylcholine (PC16:0/16:0) is traditionally considered to be the major contributor to surfactant surface activity (39). The anionic PLs, phosphatidylglycerol (PG) and phosphatidylinositol (PI) contribute ~10% to the total PL (39). The neutral lipid, cholesterol (Chol), comprises 10–20 mol% of the total lipid (39) and is thought to contribute to surfactant fluidity and thus, spreadability over the alveolar surface (39). There are four surfactant proteins (SP). SP-A and SP-D are hydrophilic, calcium-dependent, carbohydrate-binding proteins involved in lung defense and surfactant homeostasis (22). SP-B and SP-C are hydrophobic proteins (40) that facilitate the adsorption of lipids to the air-liquid interface and the formation and function of surface films in mammalian lungs (40, 44).

Temperature changes can alter the physical state and the packing density of the lipids at an air-water interface (29, 30), potentially altering surfactant surface activity. However, many small mammals allow their body temperature (T_b) to decrease during periods of inactivity when they enter a reduced metabolic state, known as torpor, without suffering from any apparent respiratory distress, or surfactant dysfunction (11, 29). These mammals are termed heterothermic. The impact of torpor on the surfactant system in heterothermic mammals is likely to vary with the depth, frequency, and length of a torpor bout. During the hibernating season, golden-mantled ground squirrels, *Spermophilus lateralis,* enter a much deeper and prolonged torpor than that reported for the "stress-induced torpor" of small marsupials (e.g., the marsupial fat-tailed dunnart, *Sminthopsis crassicaudata*), and the "daily torpor" of bats (e.g., Gould's wattled bat, *Chalinolobus gouldii*).

We have previously demonstrated that, while surfactant from torpid bats has inferior surface properties at 37°C, it has greater surface-tension lowering capabilities than surfactant from warm-active bats when measured at 24°C. Similarly, surfactant collected from warm-active and torpid dunnarts functions optimally at a temperature similar to the T_b of the animal at the time of sample collection (12, 29). We have previously suggested that the thermal differences in function

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between warm-active and torpid surfactants are related to changes in surfactant composition. Our recent work has demonstrated that heterothermic mammals (i.e., bats and dunnarts) can alter the surfactant composition, particularly the amount of Chol relative to PL, in response to decreases in T_b (12, 27, 46). However, the compositional responses to change are slow and do not fully explain the ability of their surfactants to function over a broad temperature range.

Here, we determined whether Chol also increases during the longer and deeper torpor experienced by ground squirrels. Furthermore, we determined the effect of torpor in bats, dunnarts, and squirrels, on PL molsp composition, using electrospray ionization-mass spectrometry (ESI-MS). The effect of torpor on SP-A and SP-B was also determined using ELISA methods. Contrary to our hypothesis that molsp composition would change during torpor, we did not observe any significant differences between warm-active and torpid surfactants in any of the three species examined. Therefore, we also analyzed the molsp composition of a further 9 mammals, which included placental vs. marsupial and heterothermic vs. homeothermic contrasts, to determine whether phylogeny or thermal behavior determines differences in molsp composition between mammals. This led us to conclude that the composition of surfactant PL in heterothermic mammals is already prepared for the thermoregulatory plasticity that the animals experience.

MATERIALS AND METHODS

Animals

All experiments were performed under approvals from the University of Adelaide Animal Ethics Committee (S/54/01) and the University of British Columbia Animal Ethics Committee and were in compliance with the "NIH Principles of Animal Care" and the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes."

Heterothermic eutherian/placental mammals. Adult golden-mantled ground squirrels, *S. lateralis,* were obtained from a wild-captive colony at the Department of Zoology, University of British Columbia, Canada. Squirrels were housed at $T_a = 22 \pm 1$°C under a 12:12-h light (L)-dark (D) cycle and fed laboratory chow supplemented with sunflower seeds ad libitum. In September and October (fall in the Northern Hemisphere), warm-active squirrels ($T_b = 35.2 \pm 0.5$°C; means ± SE, $n = 11$) were used. In late November (winter in the Northern Hemisphere), T_a was reduced to 5°C and the photoperiod altered (1L:23D). 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 they had been in a torpor bout for at least 4 days, without a period of arousal. The rectal T_b of each animal was recorded using a thermocouple. Torpid squirrels recorded T_b of 7.7 ± 0.2°C (mean ± SE, $n = 12$).

Gould's wattled bats, *C. gouldii*, are nocturnal, placental mammals that enter into daily torpor, throughout the year. During torpor, T_b is reduced to a few degrees above ambient temperature (T_a). In early March (fall in the Southern Hemisphere), Gould's wattled bats, *C. gouldii,* were trapped with harp traps in the southeast of South Australia. Gould's wattled bats were collected under South Australian National Parks and Wildlife permit W24091.The bats were held in a specially designed bat cage (0.5 m × 0.15 m × 0.35 m) with wire mesh on each long side and calico bags hanging from the center on which they could roost. Bats were given water ad libitum and fed mealworms (*Tenebrio molitor*) by hand for two days after capture. At sunrise on the third day, seven bats were placed individually in cloth bags that were tied so they hung vertically in a constant temperature cabinet. T_a was lowered to 12°C, over a 30-min period and the bats were checked after 1 h to ensure they had entered torpor. A T_a of 12 ± 1°C was used in order that the depth of the daily torpor bout (i.e., minimum T_b) in bats was comparable to the stress-induced torpor bout of dunnarts. Torpid bats are easily identifiable by their body posture, lack of alertness, and low T_b. All bats entered torpor under these conditions and were kept at $T_a = 12$°C for 8 h. Torpid bats had T_b of 12.4 ± 0.3°C (means ± SE, $n = 7$). Warm-active bats were kept at $T_a = 24$°C and killed in the evening when they were resting and had rectal T_b of 34.8 ± 0.34°C (means ± SE, $n = 7$).

The Etruscan shrew, *Suncus etruscus*, is the smallest mammal. It is a eutherian, heterothermic mammal that is capable of entering a stress-induced torpor, similar to that of dunnarts (see below), in response to low ambient temperatures and food shortages (19). Adult shrews (mass range 1.8–2.4 g) were caught in southern France in the area around Banyuls-sur-Mer. They were housed in a terrarium at room temperature (22°C) and fed mealworms and crickets. Water was available ad libitum. At this temperature, all shrews were at their warm-active body temperature range of 34–38°C (26). Only warm-active shrews ($n = 3$) were used in this study.

Heterothermic metatherian/marsupial mammal. Fat-tailed dunnarts, *S. crassicaudata*, are small heterothermic marsupial mammals from the family Dasyuridae, that enter torpor in response to food shortages or low T_a. Stress-induced torpor in dunnarts is similar in depth and duration to that observed in the daily torpor of bats. Adult, male dunnarts (mass range 10–20 g), were purchased from a captive-bred colony at the University of Adelaide. Animals were housed individually in standard small animal cages (50 cm × 20 cm × 20 cm). Dunnarts were kept at $T_a = 25 \pm 1$°C and exposed to a 8:16-h light-dark cycle that started at 9:00 AM and were fed daily with cat food. Water was provided ad libitum. The T_b of warm-active dunnarts was 35.5 ± 0.5°C (mean ± SE, $n = 25$).

To induce torpor, warm-active dunnarts were placed in clean cages without food, at $T_a = 20$°C overnight. In the morning, T_a was reduced to 12°C. After 1.5 h, the dunnarts were examined to determine whether they had entered torpor. Those dunnarts that had not entered torpor were fed and returned to the animal house ($T_a = 25$°C). Dunnarts in torpor were kept at $T_a = 12$°C for 8 h (standard length of torpor) and monitored regularly to ensure they did not arouse. Torpid dunnarts had T_b of 15.4 ± 0.5°C (mean ± SE, $n = 22$). Each day 1–2 dunnarts were warmed to $T_a = 22$°C after the 8-h torpor period, and they soon returned to a warm-active state, confirming that they were in torpor and that the protocol did not induce hypothermia.

Homeothermic eutherian/placental mammals. Aliquots of bronchoalveolar lavage fluid (BALF) stored at −80°C were analyzed for Sprague-Dawley rats ($n = 6$) (6), rabbit ($n = 6$) (41), pigs($n = 5$) (6) and for human subjects ($n = 6$). Conditions for collection of these samples have been reported previously, but these samples were reanalyzed for this study to ensure complete comparability of methodology and data analysis.

Homeothermic metatherian/marsupial mammals. One male Tasmanian devil, *Sarcophilus harrisii*, (age 13 yr; mass 13 kg) was obtained from a wildlife park in Sydney, Australia. This species is also a dasyurid marsupial and is a close homeothermic relative to the dunnart. Two southern hairy-nosed wombats, *Lasiorhinus latifrons*, were obtained from a captive colony kept at the University of Adelaide and originally trapped in southwestern South Australia. One male koala, *Phascolarctos cinereus*, (age ~1–2 yr) came from a South Australian wildlife park. Two tammar wallabies, *Macropus eugenii*, were obtained from a captive colony kept at the University of Adelaide. All had T_bs of 35–37°C.

Isolation of pulmonary surfactant. With the exception of the shrew, animals were anesthetized with an overdose of pentobarbitol sodium (50–150 mg/kg body wt) by injection (ip or via the femoral artery). For bats, squirrels and dunnarts, the lungs were lavaged via a surgically inserted tracheal cannula with three separate volumes (10 ml for

ground squirrel; ~1 ml for bats and dunnarts) of saline. Each volume was instilled and withdrawn three times. For the homeothermic marsupials, the trachea was surgically cannulated with a piece of appropriate sized tubing attached to a three-way valve. Using a 50-ml syringe, sterile, ice-cold saline was instilled into the lungs and then collected. In large animals, any bronchial mucus was decanted from the top of the lavage samples before further analysis. Saline from each of the lavage volumes was pooled and stored on ice, before being centrifuged at 150 *g* for 10 min at 4°C to remove cell debris. The supernatant was lyophilized and stored at −20°C until further analysis. Shrews were killed with an overdose of Halothane (Halothan, Hoechst). After death, lungs were perfused free of blood with Hank's saline via a catheter inserted into the right heart ventricle, while manually ventilating the lungs for 15 to 20 min with a 1-ml syringe attached to a tracheal cannula. Blood-free lungs were excised and stored in liquid nitrogen until analysis. For analysis of surfactant proteins in dunnart surfactant only, the supernatant was centrifuged at 40,000 *g* for 30 min at 4°C to separate the large aggregate subfraction (pellet) from the small aggregate subfraction (supernatant) and concentrate the apoproteins. The large aggregate subfraction contains dense structures and apoproteins that are responsible for the surface-activity of surfactant and hence maintain alveolar stability. Lighter, vesicular forms of surfactant that are undergoing recycling or clearance from the lung and are less surface-active, are present in the small aggregate fraction (8). The subfractions of dunnart surfactant were resuspended in 2 ml of deionized water for analysis of surfactant proteins.

Lipid Extraction and Biochemical Analysis of Squirrel Lavage

Lipids were extracted from lyophilized lavage fluid of squirrels using the method of Bligh and Dyer (7). Inorganic phosphorus (P_i) was measured by the method of Bartlett (1), and total PL was calculated by multiplying P_i by 25, because P_i comprises ~4% of PL (27). Disaturated phospholipid (DSP) and Chol were measured in the DSP and neutral lipid eluants, respectively, from aluminum oxide columns according to the adsorption chromatography (AC) method of Mason et al. (32) and as previously described for bats and dunnarts (12, 27). In this study, Chol and lathosterol (LaSL) in squirrel lavage were determined by gas chromatography as previously described (15). Molar units were calculated by dividing the mass of PL, DSP, Chol and LaSL by 770, 730, 386, and 386.7 g/mol, respectively.

Analysis of Phospholipid molsp

Total lipid extracts were obtained from lung tissue of shrews and from the lavage fractions of all other species, using the method of Bligh and Dyer (7). They were analyzed using ESI-MS on a triple-quadrupole tandem mass spectrometer (Quattro *Ultima*, Micromass, Manchester, England) equipped with an electrospray-ionization interface. Lipid extracts were dissolved in 25 μl of methanol:chloroform: water:NH_4OH (7:2:0.8:0.2, vol:vol) for single stage and tandem MS analysis of PC, PI, PG, phosphatidylserine and phosphatidylethanolamine, and were analyzed by nanospray ESI-MS. Dry heated nitrogen was used as both the cone and desolvation gas (70 and 450 l/h, respectively), and dry argon was used as the collision gas (3.5×10^{-3}/mbar). All data were recorded at mass resolution, as a signal average of 10–20 scans per collection, with a scan time of 2.5 to 12 s.

PC species were detected by positive ionization, while PI and PG were detected using negative ionization. After fragmentation with argon gas, PC molecules produced a fragment with $m/z = +184$, corresponding to the protonated phosphorylcholine headgroup, and precursor scans of the m/z 184 moiety provided diagnostic determination of PC. Collision gas-induced fragmentation of PI species generated a common dehydrated inositol phosphate fragment with $m/z = -241$, and precursor scans of this m/z 241 moiety provided diagnostic determination of PI. Identities of PG species were confirmed by precursor scans of glycerophosphate ($m/z = -153$). Data were acquired and processed using MassLynx NT software. After conversion to centroid format according to area, correction for [^{13}C] isotope effects and reduced response with increasing m/z values, the PL species in each class of PL were expressed as percentages of their respective totals present in the sample.

The acyl components of the predominant molsp present for each ion peak resolved was determined by analysis of fatty acyl fragments generated by collision gas-induced fragmentation under negative ionization. This enabled discrimination, for instance, between PC18:0/18:2 and PC18:1/18:1, which have identical masses. In addition, relative ion intensities of these fatty acyl fragments enabled their positional assignments to the *sn*-1 or *sn*-2 positions, for instance m/z 706 was predominantly PC16:0/14:0 and not PC14:0/16:0. PC was quantified from the parent scan of m/z = +184 under positive ionization mode, whereas all other PL classes were quantified from the negative ionization scans.

The percentages of disaturated phosphatidylcholine (DSPC), disaturated phosphatidylglycerol (DSPG) and disaturated phosphatidylinositol (DSPI) were calculated from the corrected mass spectrometry data by adding the mol% contribution of each of the individual disaturated species present in the samples. These values were compared with the % total disaturated phospholipid (%DSP/PL) obtained by AC chromatography (see *Lipid Extraction and Biochemical Analysis of Squirrel Lavage*). This latter method has been included for completeness, as both dunnart and bat surfactant compositions have previously been reported using this method (see Tables 1 and 2).

Surfactant Protein Analysis

Lavage samples from all three heterothermic species were resuspended in an appropriate volume of deionized water and diluted 1:2 in ELISA sample buffer for measurement in the surfactant protein ELISAs. Details of both the SP-A and -B ELISAs have previously been published (33). Lavage samples from pairs of bats were pooled. Human SP-A and ovine SP-B standards were a generous gift from Prof. Jeffrey Whitsett (Children's Hospital Medical Research Centre, Cincinnati, OH). SP-B standards were used in the concentration range 0, 5, 10, 25, 50, 75, and 100 ng/ml and the primary antibody was a polyclonal rabbit anti-sheep SP-B (Cat. # AB3780; Chemicon Australia, Boronia, Australia) at a dilution of 1:1,000. SP-A standards were used in the concentration range 0, 5, 10, 25, 50, 75, and 100 ng/ml. For the SP-A ELISA, we used a separate coating antibody (goat anti-rabbit SP-A antibody; 1:100 dilution; a gift from Prof. J. A. Whitsett). The primary detection antibody was rabbit anti-human SP-A (Cat # AB3420, Chemicon Australia) used at a 1:1,000 dilution. This antibody has previously been used in our laboratory to detect SP-A in the lung washings of a wide range of vertebrates (49). The secondary detection antibody for both SP-B and SP-A ELISAs was horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) at a dilution of 1:1,000.

Data Analysis

Statistical significance was determined, using SPSS software. Student's *t*-tests, assuming equal variance, with significance set at $P < 0.05$, were used in all analyses. Statistical analyses between lavage isolated from warm-active and torpid animals were only performed on molsp of PL if they contributed greater than 5 mol% to the PL component. All mol% values were arcsin transformed before analysis. Linear regressions were performed with SPSS to identify relationships between different molsp of PL and between animal groups.

RESULTS

The Effect of Body Temperature on Molecular Species Composition

Surfactant composition in ground squirrels. Neither the amount nor the saturation of surfactant PL changed between

Table 1. *Composition of surfactant in whole lavage isolated from warm-active and torpid golden-mantled ground squirrels, Spermophilus lateralis, fat-tailed dunnarts, Sminthopsis crassicaudata and Gould's wattled bats, Chalinolobus gouldii*

	Warm-Active Squirrel, μmol/g WL	Torpid Squirrel, μmol/g WL	Warm-Active Dunnart, μmol/gDL	Torpid Dunnart, μmol/gDL	Warm-Active Bat, μmol/gDL	Torpid Bat, μmol/gDL
PL	0.45±0.05	0.55±0.06	28.35±3.79	49.91±2.29*	17.69±1.75	16.18±1.13
DSP	0.18±0.05	0.21±0.03	12.35±2.16	25.87±0.95*	7.92±1.10	7.33±0.70
Chol	0.05±0.01	0.10±0.01*	3.84±0.41	10.74±1.24*	0.66±0.09	0.82±0.08*
	nmol/gWL					
LaSL	0.13±0.06	7.29±1.45*				
				Mol%		
Chol/PL	11.47±1.41	18.57±1.19*	13.50±1.18	21.28±1.90*	3.19±0.60	4.99±0.80*
Chol/DSP	36.97±9.70	51.80±8.65	33.19±4.52	41.23±4.17*	6.62±1.32	10.59±2.27*
LaSL/Chol	0.26±0.14	7.35±1.65*				

All data are presented as means±SE; $n = 4–6$ for squirrel, $n = 6–9$ for dunnart, and $n = 4–6$ for bat. Phospholipid (PL) was measured using an inorganic phosphorus assay on extracted lipids (1). Disaturated phospholipid (DSP) and Cholesterol (Chol) were separated using adsorption chromatography and measured in eluants using a phosphorus assay (1) and gas chromatography, respectively. For bat and dunnart, we used our previously published μg/g dry lung (DL) values [Bat data from (12), where torpor body temperature (T_b) was ~25°C, Dunnart data from (27) in which torpor T_b was ~15°C] and converted these to molar units using the following molar masses: for PL-770, DSP-730, Chol-386, and lathosterol (LaSL)-386.7 g/mol. WL, wet lung. *Significant difference between warm-active and torpid animals.

warm-active and torpid ground squirrels (Table 1). The concentration of Chol [μmol/g wet lung (WL)] was higher in lavage collected from torpid than from warm-active squirrels and, consequently, the Chol:PL ratio was also higher in torpid surfactant. However, the Chol:DSP ratio, where DSP was measured after AC, did not change between warm-active and torpid squirrel surfactants (Table 1). Both the amount of LaSL (nmol/gWL) and the LaSL/Chol ratio were higher in surfactant from torpid than warm-active squirrels. Similar measurements for bats and dunnarts have already been reported (10, 12, 27, 46), and these data are also summarized in comparable form in Table 1.

Saturation of phospholipids. Estimates of saturation, as determined by the amount of DSPC per gram WL (ESI-MS) and DSP per gram WL (AC) did not change between warm-active and torpid squirrels (Table 1). There was also no difference in the percent saturation of PL, PC, PG, or PI between warm-active and torpid squirrel surfactant (Table 2). Moreover, there were no differences in %DSPC/PC and %DSPI/PI between lavage from warm-active and torpid bats (Table 2). However, there was a small decrease in %DSPG/PG in torpid bat surfactant. In lavage isolated from dunnarts, %DSPC/PC and %DSPI/PI, as determined using ESI-MS, did not change significantly between warm-active and torpid dunnarts. %DSPG/PG was significantly higher in torpid dunnart surfactant.

PC molecular species. The molsp of PC in surfactant isolated from warm-active and torpid squirrels, bats, and dunnarts are given in Fig. 1. There were no significant differences in the molsp of PC present between surfactant isolated from warm-active and torpid individuals in any of the three animal species.

In both warm-active and torpid ground squirrels, the composition of surfactant PC was dominated by the disaturated

Table 2. *Relative proportions of disaturated surfactant components in whole lavage isolated from warm-active and torpid golden-mantled ground squirrels, Spermophilus lateralis, Gould's wattled bats, Chalinolobus gouldii and fat-tailed dunnarts, Sminthopsis crassicaudata*

Animal	~T_b °C	DSPC/PC %	DSPG/PG %	DSPI/PI %	DSP/PL %
Squirrel					
Warm-active	35.2±0.5	42.4±4.8 (0.15)	14.34±2.5	3.6±1.1	39.6±13.0
Torpid	7.7±0.2	35.3±2.9 (0.10*)	18.6±2.0	3.4±1.6	39.1±5.5
Bat					
Warm-active	34.8±0.3	49.2±1.2 (0.08)	9.9±0.4	1.4±1.0	41.2±2.8†
Torpid	12.4±0.3	45.6±2.6 (0.09)	7.0±1.1*	0.5±0.3	43.0±3.3†
Dunnart					
Warm-active	35.5±0.5	39.1±1.7 (0.43)	25.6±0.5 (0.32)	0.3±0.1	40.2±2.6‡
Torpid	15.4±0.5	39.6±0.9 (0.56)	31.1±2.3*(0.42)	1.3±0.8	49.3±1.6*‡

All data are presented as means ± SE; $n = 4–6$ for squirrels, $n = 4–9$ for bats, and $n = 5–8$ for dunnarts. DSP/PL, disaturated phospholipid (separated using adsorption chromatography and measured using an inorganic phosphorus assay) as a fraction of total phospholipid (measured using an inorganic phosphorus assay); DSPC/PC, disaturated phosphatidylcholine as a fraction of total phosphatidylcholine [measured using electrospray ionization-mass spectrometry ESI-MS] includes PC14:0a/16:0, PC16:0/14:0, PC16:0a/16:0, PC16:0/16:0, PC16:0a/18:0, and PC16:0/18:0; DSPG/PG, disaturated phosphatidylglycerol as a fraction of total phosphatidylglycerol (measured using ESI-MS) includes PG16:0/16:0, PG16:0/18:0, PG18:0/18:0, and PG16:0/14:0 (present in dunnart only); DSPI/PI disaturated phosphatidylinositol as a fraction of total phosphatidylinositol (measured using ESI-MS) includes PI16:0/16:0, PI16:0/18:0, and PI18:0/18:0; in brackets: ratio of fluid/rigid molecular species that comprise the %DSPC or %DSPG estimate (i.e., for PC, fluid: PC14:0/14:0, PC16:0/14:0 + alkyl-acyl forms; rigid: PC16:0/16:0 and PC16:0/18:0 + alkyl-acyl forms; for PG, fluid: PG16:0/14:0 in dunnart only; rigid: PG16:0/16:0, PG16:0/18:0, and PG18:0/18:0) for warm-active and torpid animals. †data taken from (12)-Note: torpor T_b was ~25°C; ‡data taken from (27); n values and T_bs apply only to data collected in the present study; *Significant difference between warm-active and torpid animals using Student's unpaired t-tests assuming equal variance, $P < 0.05$.

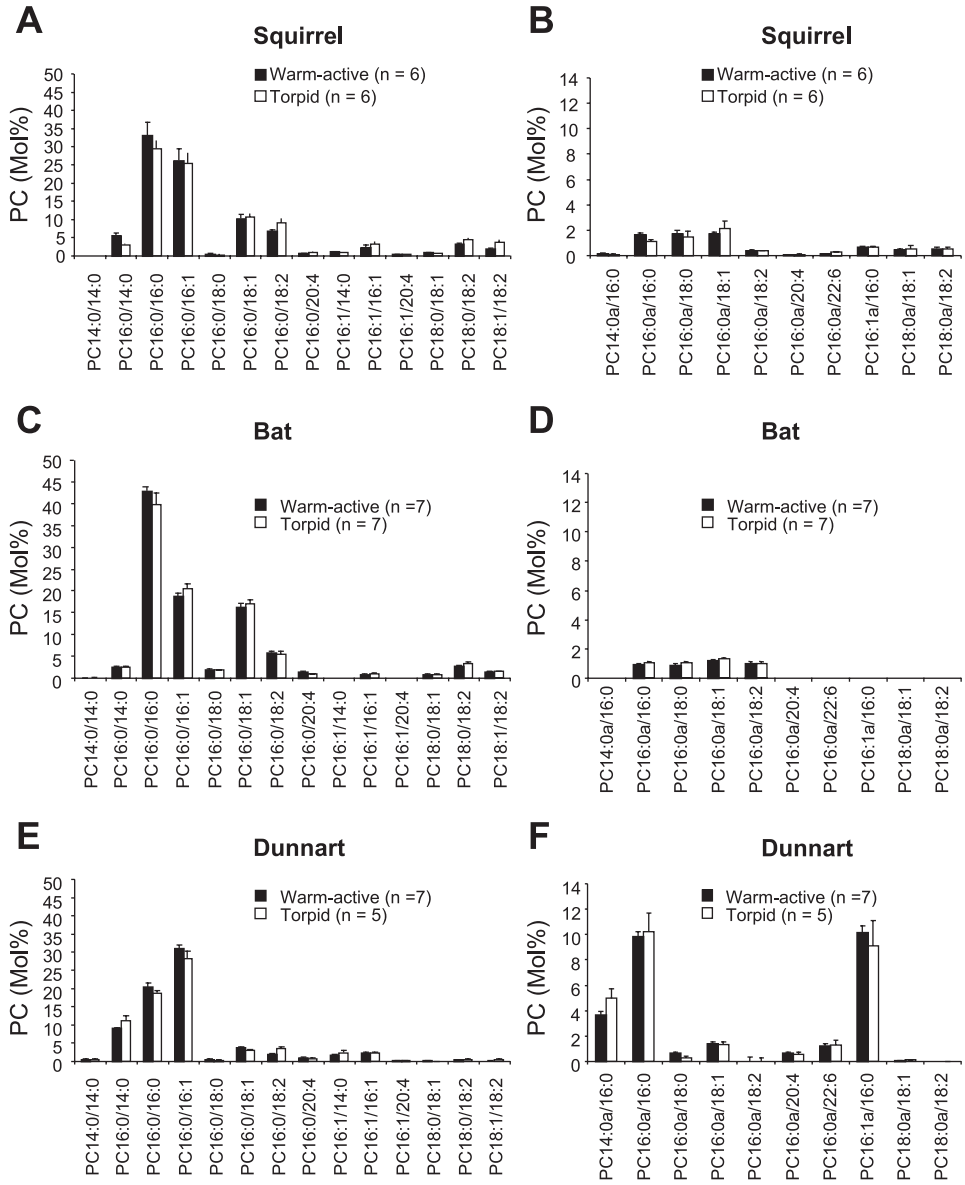

Fig. 1. The composition of individual molecular species of phosphatidylcholine (PC) in whole lavage fractions isolated from warm-active and torpid golden-mantled ground squirrels, *Spermophilus lateralis* (*A* and *B*), Gould's wattled bats, *Chalinolobus gouldii* (*C* and *D*) and fat-tailed dunnarts, *Sminthopsis crassicauda* (*E* and *F*). Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. In the lefthand panels (*A*, *C,* and *E*) all molecules are diacyl forms of molecular species (i.e., both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated by the letter "a", after the number of double bonds (y) (righthand panels *B*, *D,* and *F*). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as means ± SE. Palmitoylmyristoyl-PC, PC16:0/14:0; Dipalmitoyl-PC, PC16:0/16:0; Palmitoylpalmitoleoyl-PC, PC16:0/16:1; Palmitoylstearoyl-PC, PC16:0/18:0; Palmitoyloleoyl-PC, PC16:0/18:1; Palmitoylinoleoyl-PC, PC16:0/18:2; Palmitoylarachidonoyl-PC, PC16:0/20:4; stearoyloleoyl-PC, PC18:0/18:1; stearoyllinoleoyl-PC, PC18:0/18:2; oleollinoleoyl-PC, PC18:1/18:2. There were no significant differences between lavage isolated from warm-active and torpid animals in any of the three species (Student's unpaired *t*-test, assuming equal variance, significance at $P < 0.05$).

species, PC16:0/16:0, which contributed 30–35 mol% to the PC component (Fig. 1*A*). Other major PC components included the fluidizing species, PC16:0/16:1 (25 mol%), PC16:0/18:1 (10 mol%), PC16:0/18:2 (10 mol%), and PC16:0/14:0 (5 mol%). The molsp PC16:0/18:0, PC16:0/20:4, PC16:1/14:0, PC16:1/16:1, PC16:1/20:4, PC18:0/18:1, PC18:0/18:2, PC18:1/18:2 (Fig. 1*A*), and numerous alkyl-acyl-containing species (Fig. 1*B*) were present in small quantities (less than 5 mol% each) and made up the remaining proportion of PC species in the squirrel.

In both warm-active and torpid bats, the composition of surfactant PC was dominated by the disaturated species, PC16:0/16:0, which contributed 40–45 mol% to the PC component (Fig. 1*C*). The next major PC components present in bat surfactant were the unsaturated species, PC16:0/16:1 (20 mol%), PC16:0/18:1 (15–18 mol%), and PC16:0/18:2 (6 mol%). The molsp PC16:0/14:0, PC16:0/18:0, PC16:0/20:4, PC16:1/16:1, PC18:0/18:1, PC18:0/18:2, PC18:1/18:2 (Fig. 1*C*) and various alkyl-acyl forms (Fig. 1*D*) were present in small quantities (less than 4 mol% each) and contributed the remaining proportion of PC species.

Unlike all other mammalian surfactants previously studied, DPPC (PC16:0/16:0) was not the major component of dunnart surfactant (Fig. 1*E*). In lavage from both warm-active and torpid dunnarts, the composition of surfactant PC was dominated by the unsaturated species, PC16:0/16:1, which contributed 30 mol% to the PC component (Fig. 1*E*). PC16:0/16:0 was the second major component, comprising 16–18 mol%. Surfactant isolated from dunnarts also contained significant proportions of PC16:0/14:0 (12–15 mol%) (Fig. 1*E*) and the alkyl-acyl containing forms of PC16:0a/16:0 (10 mol%) and PC16:1a/16:0 (10 mol%) (Fig. 1*F*). The remaining PC component was made up of PC14:0/14:0, a species normally absent from surfactant, PC16:0/18:0, PC16:0/18:1, PC16:0/18:2, PC16:0/20:4, PC16:1/14:0, PC16:1/16:1, and the alkyl-acyl containing forms of PC14:0a/16:0, PC16:0a/18:0, PC16:0a/18:1, PC16:0a/20:4, and PC16:0a/22:6.

PG molecular species. The molsp of PG did not differ markedly between surfactants isolated from warm-active and torpid ground squirrels (Fig. 2A). In both warm-active and

torpid squirrels, PG composition was dominated by the unsaturated species PG16:0/18:1, which contributed 25 mol% to the PG component. PG16:0/16:1 comprised from 11–16 mol% and was lower in surfactant from torpid squirrels (11.5 ± 1.6 mol%, means ± SE, $n = 6$) compared with warm-active squirrel surfactant (16.3 ± 2.4 mol%, means ± SE, $n = 5$). PG18:1/18:1 comprised 16 mol% and PG16:0/16:0 and PG16:0/18:2, comprised 14 mol% each. The unsaturated species, PG18:1/18:2 comprised 6 mol%. The remaining PG component was made up of PG16:0/18:0, PG16:0/22:5, PG16:0/22:6, PG16:1/16:1, PG16:1/18:2, PG18:0/18:0, and PG18:0/18:1 (less than 5 mol% each) (Fig. 2*A*).

In surfactant from both warm-active and torpid bats, PG composition was dominated by the unsaturated species PG16:0/18:1, which contributed 40–45 mol% to the PG component (Fig. 2*C*). The other major PG components were PG18:1/18:1 (15–20 mol%), PG16:0/18:2 (12 mol%), PG18:0/18:1 (8–10 mol%), and the disaturated species, PG16:0/16:0 (8–10 mol%). There was a decrease in PG16:0/16:0 in torpid bat surfactant. The molsp PG16:0/16:1, PG16:0/20:4, PG16:1/18:2, and PG18:1/18:2 comprised less than 5 mol% each of the PG component (Fig. 2*C*).

In lavage from both warm-active and torpid dunnarts, PG composition was dominated by the unsaturated species PG16:0/18:1 and the disaturated species PG16:0/16:0, which contributed 20–25 mol% each of the PG component (Fig. 2*E*). In contrast to PC, there was a difference in some of the molsp of PG between lavage fractions of surfactant isolated from warm-active and torpid dunnarts (Fig. 2*E*). The proportions of PG16:0/14:0 and PG16:0/18:2 increased in the lavage fraction of surfactant isolated from torpid dunnarts. The proportion of PG16:0/18:1 decreased in torpid dunnart surfactant. PG16:0/16:1 comprised 18 mol% and PG16:0/18:2 comprised 12–15 mol%. The di-

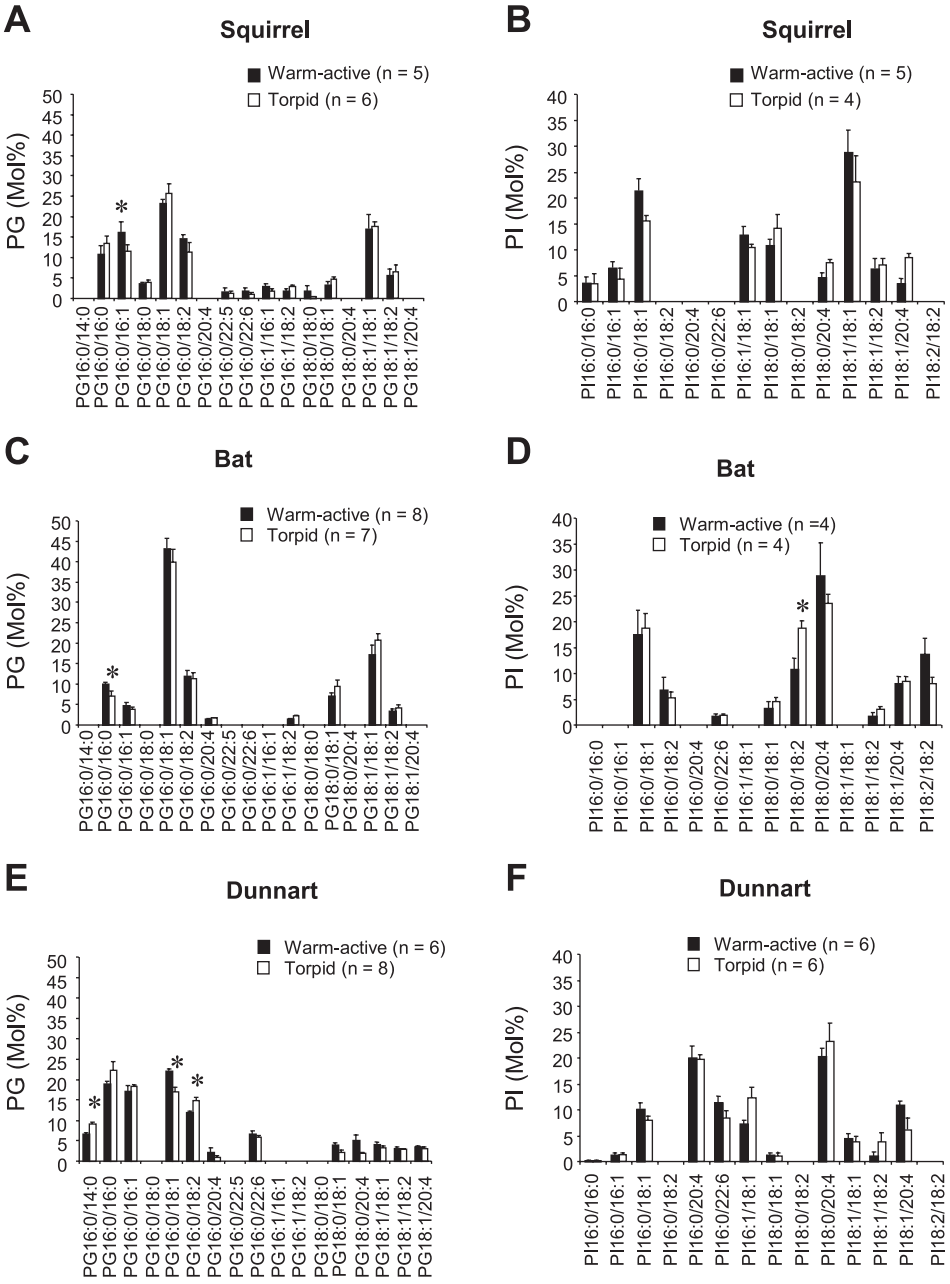

Fig. 2. The composition of individual molecular species of phosphatidylglycerol (PG) (*A*, *C*, and *E*) and phosphatidylinositol (PI) (*B*, *D*, and *F*) in whole lavage fractions isolated from warm-active and torpid golden-mantled ground squirrels, *Spermophilus lateralis* (*A* and *B*), Gould's wattled bats, *Chalinolobus gouldi* (*C* and *D*) and fat-tailed dunnarts, *Sminthopsis crassicaudata* (*E* and *F*). Data presentation, nomenclature, and abbreviations are as for Fig. 2. *Significant difference between lavage isolated from warm-active and torpid animals, measured only for those molecular species >5mol% (using Student's unpaired *t*-test, assuming equal variance, significance at $P < 0.05$).

saturated species, PG16:0/14:0 comprised 6–10% of surfactant PG. The remaining PG component was made up of PG16:0/20:4, PG16:0/22:6, PG18:0/18:1, PG18:0/20:4, PG18:1/18:1, PG18:1/18:2 and PG18:1/20:4 (less than 5 mol% each) (Fig. 2*E*).

PI molecular species. In both warm-active and torpid squirrels, the composition of surfactant PI was dominated by the molsp PI18:1/18:1, which comprised 30% of the PI component (Fig. 2*B*). The PI species, PI16:0/18:1 comprised 15–20 mol% of the surfactant PI component, while PI16:1/18:1 and PI18:0/18:1 comprised 12 mol%. PI16:0/16:0, PI16:0/16:1, PI18:0/20:4, PI18:1/18:2, PI18:1/20:4, and PI18:2/18:2 made up the remaining PI component (less than 5 mol% each) (Fig. 2*D*). In addition, PI18:0/22:6 and PI20:1/20:4 were present at these low levels (data not shown).

In both warm-active and torpid bats, the composition of surfactant PI was dominated by the unsaturated species, PI18:0/20:4 (25 mol%) and PI16:0/18:1 (20 mol%) (Fig. 2*D*). The other major components of PI in bat surfactant include PI18:0/18:2 (10–20 mol%), PI18:2/18:2 (8–14 mol%), PI18:1/20:4 (8 mol%) and PI16:0/18:2 (5–7 mol%). There was an increase in PI18:0/18:2 in surfactant isolated from torpid bats. PI16:0/22:6, PI18:0/18:1, and PI18:1/18:2 made up the remaining PI component (less than 5 mol% each). Other long-chain species present (less than 3%) that are not shown included PI18:0/20:3, PI18:0/22:6, and PI18:0/22:5. The disaturated species, PI16:0/16:0 was present in negligible quantities (Fig. 2*D*).

As for PC, there were no differences in the molsp of PI present in surfactant isolated from warm-active and torpid dunnarts. In dunnart lavage, surfactant PI was dominated by the unsaturated species PI18:0/20:4 (20–25 mol%) and PI16:0/20:4 (20 mol%) (Fig. 2*F*). The next major components of PI in dunnart surfactant include PI16:0/18:1 (8–10 mol%), PI16:0/22:6 (8–12 mol%), PI16:1/18:1 (7–12 mol%), and PI18:1/20:4 (5–10 mol%). In addition, a species peculiar to dunnarts, PI18:2/20:4 was present at about 10 mol% but is not shown. Other species that make up the remainder of the PI component include PI16:0/16:1, PI18:0/18:1, PI18:1/18:1, PI18:1/18:2, and PI18:1/18:2 (less than 5 mol%). Other long-chain species that are not shown include PI18:0/22:6, PI20:1/20:4, and PI20:0/20:4 (less than 2 mol%). PI16:0/16:0 was present only in very low quantities (less than 1 mol%) (Fig. 2*F*).

Surfactant proteins. Compared with warm-active animals, the ratio of SP-A/PL was lower in whole surfactant isolated from torpid squirrels and in large aggregate surfactant fractions isolated from torpid dunnarts. However, in bat surfactant, the ratio of SP-A/PL did not differ between surfactant isolated from warm-active and torpid bats. The ratio of SP-B/PL did not differ between whole lavage isolated from warm-active and torpid squirrels and bats or between large aggregate surfactant fractions isolated from warm-active and torpid dunnarts. There were no differences in the proportion of SP-A or SP-B relative to PL, between the small aggregate fractions isolated from warm-active and torpid dunnarts (Table 3).

Table 3. *Surfactant protein A (SP-A) and SP-B in surfactant isolated from warm-active and torpid golden-mantled ground squirrels, Spermophilus lateralis, fat-tailed dunnarts, Sminthopsis crassicaudata and Gould's wattled bats, Chalinolobus gouldii*

Animal	T_b, °C	SP-A, ng/mol PL	SP-B, ng/mol PL
Squirrel			
Warm-active	35.2±0.5	92.2±16.5	15.0±2.7
Torpid	7.7±0.2	43.2±7.2*	17.5±2.6
Dunnart LA			
Warm-active	35.5±0.5	87.5±18.2	500.5±87.5
Torpid	15.4±0.5	43.3±6.3*	342.0±37.5
Dunnart SA			
Warm-active	35.5±0.5	35.4±12.2	8.7±3.1
Torpid	15.4±0.5	43.6±6.7	13.6±1.6
Bat			
Warm-active	34.8±0.3	561.0±229.9	305.6±78.6
Torpid	12.4±0.3	626.3±131.4	426.6±137.7

Values are means ± SE; $n = 6–8$ for squirrels, $n = 7–8$ for dunnarts, and $n = 3–4$ for bats. Whole lavage surfactant fractions were used for bat and squirrel measurements. Pairs of bat samples were pooled for protein measurement. In dunnarts, large aggregate fractions were used for protein measurement in order to concentrate proteins. Dunnart LA, large-aggregate fraction of dunnart surfactant; Dunnart SA, small-aggregate fraction of dunnart surfactant. Statistical significance was determined using Student's *t*-tests, assuming equal variance; significance was set at $P < 0.05$. *Significant decrease in lavage isolated from torpid animals compared to warm-active animals.

Molecular Species Comparisons in Mammals

PC molecular species. Figure 3 illustrates the molsp profile of PC for all warm-active mammals collected here. The PC profile demonstrated extensive diversity between individual heterothermic and homeothermic mammal species (Fig. 3). In particular, the proportion of PC16:0/16:0 varied widely, ranging from 49 mol% in human surfactant, through 21, 19, and 16 mol% in the wombat, Etruscan shrew, and the dunnart, respectively, to a very low value of 2 mol% in the Tasmanian devil. Surfactants with a low content of PC16:0/16:0 were generally enriched in the monounsaturated PC16:0/16:1 species. In the wombat, Etruscan shrew, and the dunnart, PC16:0/16:1 was present at 23, 23, and 28 mol%, respectively, making it the dominant component in the surfactant of these three species. In the Tasmanian devil, PC16:0/16:1 was relatively low (~5%), concomitant with an extremely low concentration of total diacyl PC species (16 mol%) in this surfactant. However, unlike the other mammals, the Tasmanian devil had a surfactant enriched in plasmanyl ether PC (alkyl-acyl species) at the expense of the analogous diacyl species, such that PC16:0a/16:1 constituted 45% of the PC in this surfactant.

As the physical properties of plasmanyl and phosphatidylcholine species are very similar (24), the sum of their respective 16:0/16:0 species are plotted against several major fluidizing species (Fig. 4). There were statistically significant inverse relationships between the saturated species PC16:0/16:0 and the two fluid species PC16:0/16:1 and PC16:0/14:0 (Fig. 4, *A* and *B*), but there was no relationship between PC16:0/16:0 and PC16:0/18:1 (Fig. 4*C*). Another pattern that emerges, despite the tremendous variability between species, is that virtually all PC molecular species were shorter-chain molecules, with the fatty acid palmitate (C16:0) rather than stearate (C18:0) or oleate (C18:1) at their *sn*-1 position, and were characterized by a very low content of polyunsaturated fatty acids (Fig. 3).

PG and PI molecular species. The most notable features of surfactant PG and PI were their low content of disaturated species (PG, 9–28%; PI, 1–15%) relative to PC, the domination of mono- and diunsaturated species and the wide variations of composition between mammal species (Fig. 5). Sur-

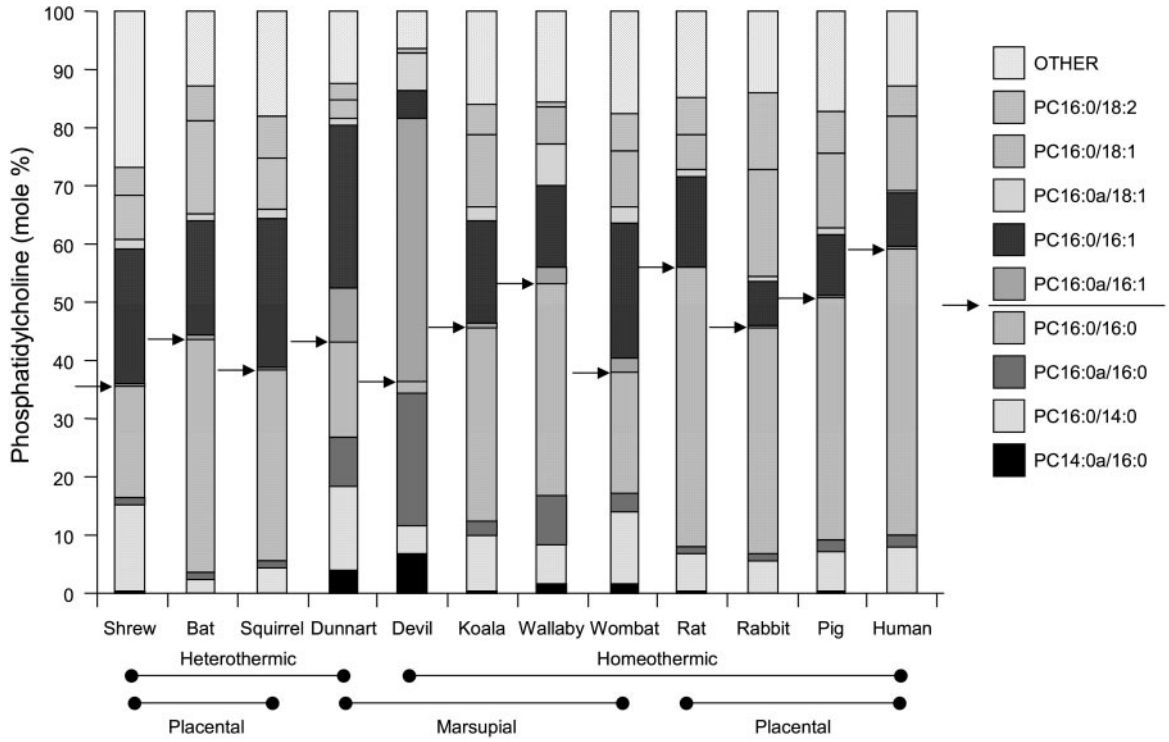

Fig. 3. Comparison of molecular phosphatidylcholine (PC) composition for each mammal studied. Molecular species are designated by the abbreviation in the format A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the PL, respectively; while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e., both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated by the letter "a", after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Black arrows mark the proportion of disaturated species. The category "other" includes the following molecular species: PC16:0/18:0; PC16:1/14:0; PC16:1/16:1; PC16:0a/22:6; PC18:0a/18:1; PC18:0a/20:4; PC18:0a/18:0; PC16:0a/18:0; PC16:1/18:2; PC18:0/18:1; PC16:0/20:4; PC18:1:18:2; PC18:0/18:2; PC16:0/22:6; PC18:1/20:4; PC18:0/20:4; PC18:0/20:2; and PC18:0/20:1; PC18:0/22:6.

factant PG molsp were generally dominated by PG16:0/18:1, which was the most abundant molsp in 9 of the 12 animals listed, varying from ~20% in the dunnart and squirrel to 42% in the koala (Fig. 5*A*). Only in the rat, Etruscan shrew, and Tasmanian devil, was PG16:0/18:1 not the dominant species, making up 12, 9, and 7%, respectively. In fact, the Tasmanian devil had the most unusual PG molsp composition, with negligible amounts of PG16:0/16:0 but significant proportions of PG18:0/18:0 and PG16:0a/18:1, two species that are either not observed or are poorly represented in most other mammalian surfactants (Fig. 5*A*). As far as the heterothermic species are concerned, dunnart and Etruscan shrew PG was dominated by PG16:0/16:0, PG16:0/16:1, PG16:0/18:2, and PG16:0/18:1, whereas squirrels and bats had high concentrations of the unsaturated species PG16:0/18:2, PG16:0/18:1, and PG18:0/18:2, with lower concentrations of PG16:0/16:1 (Fig. 5*A*).

Surfactant PG and PI from the same animal typically exhibited very different molecular compositions (Fig. 5, *A* and *B*). In particular, PI species, relative to the PG species from the same surfactant, demonstrated either greater levels of arachidonic acid (20:4)-containing species (e.g., PI16:0/20:4 or PI18:0/20:4), as well as other long-chain unsaturated species with more than 2 double bonds (e.g., PI16:0/22:6 and PI18:0/20:3). In addition, the PI component included greater levels of alkyl-acyl species compared with the PG component. Disaturated diacyl PI levels (mainly PI16:0/16:0) were very low (0–5% total).

DISCUSSION

Surfactant composition during torpor. We demonstrate that torpor in bats, dunnarts, and ground squirrels is not accompanied by any major compositional changes in surfactant PL, involving either PL saturation or molsp composition of PC, PG, or PI. Using AC, we have previously reported an increase in the proportion of DSP during torpor in dunnarts (27). However, using AC, PL saturation (i.e., DSP/PL ratio) did not change during torpor in either ground squirrels (Table 1) or bats (12). Moreover, in this study, using ESI-MS, we did not observe any differences in PC saturation (i.e., the sum of PC14:0/14:0, PC16:0a/16:0, PC16:0/16:0, PC16:0a/14:0, PC16:0/14:0, PC16:0a/18:0, and PC16:0/18:0 components) between warm-active and torpid bats, dunnarts, or squirrels (Table 2). Hence, the increase in DSP observed previously in dunnarts is probably an artifact of the AC technique. Whereas the technique of ESI-MS is sensitive, precise and state-of-the art, AC chromatography has recognized limitations, in that some monounsaturated species may contaminate the DSP eluate.

However, although both %DSP (AC) and %DSPC (ESI-MS) calculations were performed here to match those reported in previous studies, from a functional perspective, not all the disaturated components have the same physical properties. For example, PC16:0/16:0 and PC16:0/18:0 are rigid PL species,

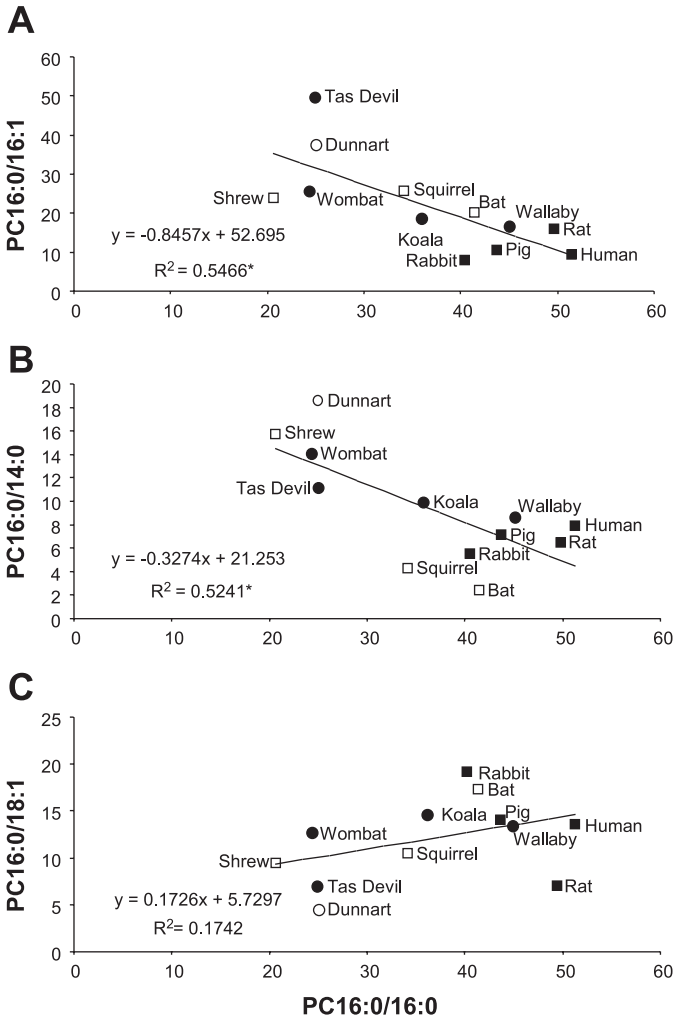

Fig. 4. Comparison of the major molecular species of PC between homeothermic (solid symbols) and heterothermic (open symbols), placental (squares) and marsupial (circles) mammals. Relationships are plotted of PC16:0/16:0 vs. PC16:0/16:1 (*A*), PC16:0/14:0 (*B*), and PC16:0/18:1 (*C*). In each case, the molecular species include both the diacyl and the alkyl-acyl forms (e.g., PC16:0/16:0 and PC16:0a/16:0). *Significant inverse relationship between PC16:0/16:0 and PC16:0/16:1 species ($F = 9.973$, $P = 0.010$) and PC16:0/16:0 and PC16:0/14:0 ($F = 12.850$, $P = 0.005$). There was no significant relationship between PC16:0/16:0 and PC16:0/18:1 ($F = 1.368$, $P = 0.269$).

while PC16:0/14:0 and PC14:0/14:0 are fluidic PL species at 37°C. Hence, it is perhaps more informative to examine the ratios of individual or groups of PL and, particularly, the ratio of fluid/rigid components that traditionally, and currently, are included in the estimates of lipid saturation (e.g., both %DSP and %DSPC measures calculated in this study). When we calculated the ratio of fluid/rigid components of disaturated PC in squirrel, dunnart, and bat surfactant (shown in brackets in Table 2), there was only a significant difference in the ratio between surfactant isolated from warm-active and torpid squirrels. Therefore, it seems unlikely that changes in PL saturation are a necessary adaptation to torpor in heterothermic mammals.

In dunnarts, bats, and squirrels, there were no changes in the molecular composition of the major PL component, PC, between lavage fluid isolated from warm-active and torpid animals. Although, small, but statistically significant, differences were observed in one or two of the molecular components that comprise greater than 5 mol% of PG and PI in each animal species, none of these changes were consistent in either directionality or across the animal species examined. PG and PI are two of the more variable components of surfactant (13), and the acidic headgroups are thought to be more important to surfactant function than the precise molsp compositions (41). In addition, because the observed changes are relatively small, they are not necessarily reflected in the overall saturation of surfactant PL. Although it is possible that these minor PL components do play a role in signaling events related to surfactant metabolism, these changes probably do not have any functional significance in terms of surface activity. Hence, as for PL saturation, changes in molsp composition, do not appear to be a necessary adaptation for torpor in bats, dunnarts, or ground squirrels. It is possible that the lack of changes in PL composition we observed here, is related to the metabolism of lipid components. In C57 mice, the turnover and biological half-life of DSPC flux between lamellar bodies and the lavage fluid has been estimated at 5.9 h and 16 h, respectively (21). While, in newborn and adult sheep, the half-life of DSPC in the whole lung is 45 and 54 h, respectively (23). In rats, PC has also been reported to have a half-life of 10 h in the intracellular surfactant pool and 15 h in the extracellular surfactant pool (16). Hence, it is possible that the 8-h duration of torpor in dunnarts and bats is too short for changes in surfactant lipid composition to occur. Moreover, given the long half-lives for surfactant PC turnover and the relatively short duration of torpor bouts, it is perhaps not surprising that dunnarts and bats do not use PC molsp alterations as a mechanism for maintaining surfactant function during torpor. However, we also did not observe any changes in surfactant lipid composition in ground squirrels, which experience a deep torpor of at least 96 h duration, prior to surfactant collection. Hence, given the longer torpor period relative to PC half-life, it should have been possible to observe a change, if it had occurred. Nevertheless, it is possible that the lack of molsp alterations in squirrels may be due to a decrease in metabolic rate, and thus, a decreased turnover of surfactant lipids during torpor, but this remains to be confirmed.

This study supports our previous observations (10, 27) that torpor is accompanied by changes in Chol composition. In ground squirrels, as in all heterothermic mammals studied, the amount of Chol increased during torpor (Table 1). In addition, both the amount of LaSL and the ratio of LaSL/Chol increased significantly in lung lavage fluid isolated from torpid ground squirrels. LaSL is a precursor of Chol and is commonly used as a marker of Chol synthesis or metabolism in plasma and other tissues (28). To our knowledge, this study is the first to demonstrate the presence of LaSL in lung lavage fluid. However, whether it originates from type II cells or plasma is unknown. Assuming that LaSL is a valid marker for cholesterol synthesis in torpid as well as active animals, the observed increase in LaSL/Chol may indicate that Chol synthesis is significantly higher in the lung during torpor in ground squirrels. Hass and Longmore (1980) concluded that type II cell endogenous Chol synthesis accounts for less than 1% of the total Chol used by the lung for surfactant synthesis. If this is the case, then the uptake of Chol from the blood may also significantly increase during torpor in ground squirrels and contribute to the increases in Chol that we have observed.

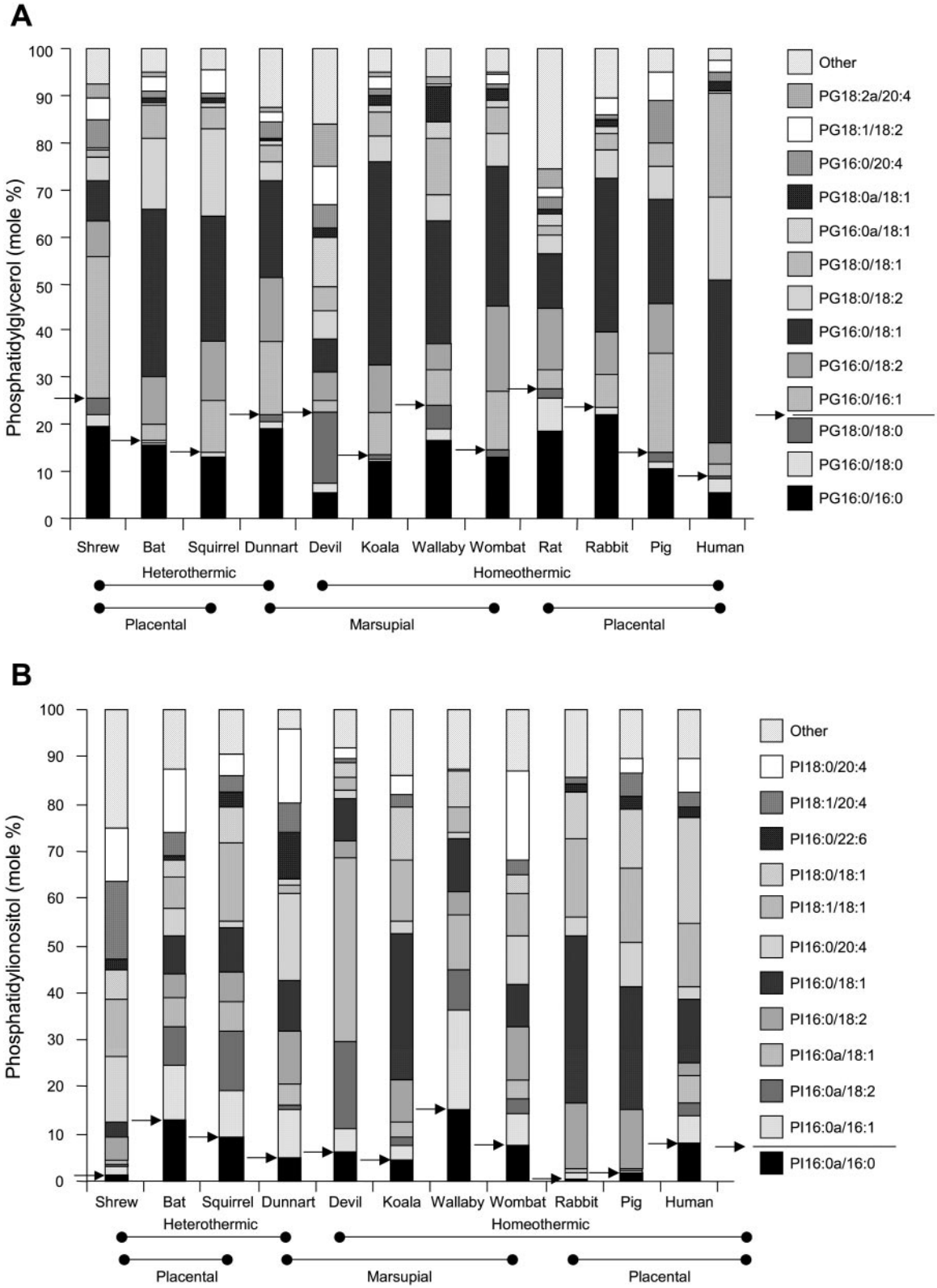

Fig. 5. Comparison of molecular PG (*A*) and PI (*B*) composition for all mammals studied. Figure presentation and abbreviations are as for Fig. 4. Black arrows mark the proportion of disaturated species. The category "other" includes the following species: PG16:0a/16:0; PG16:1/16:1; PG16:0/18:2; PG18:0a/20:3; PG18:0a/20:0; PG18:0/20:4; PG18:1a/20:4; PI16:0/16:0; PI18:0a/18:1; PI18:1/18:2; PI18:0/20:3; and PI18:0/22:6.

Levels of SP-B, relative to PL, did not change between surfactant isolated from warm-active and torpid mammals, and this implies that SP-B levels in warm-active animals are sufficient for adequate function at the lower temperatures, during torpor. It has previously been shown that in fast breathing neonatal mammals, SP-B is increased (43). However, given that breathing parameters are reduced during torpor, such that there is no increase in air-liquid interface dynamics, there would not be a requirement for more SP-B. In contrast, SP-A decreased significantly in surfactants isolated from torpid dunnarts and squirrels. The decrease in SP-A was not observed in bats during torpor, suggesting that this response may not be a general phenomenon. Because of the diverse roles of SP-A in the lung, the decrease in SP-A could be due to many different mechanisms, including the down-regulation of SP-A gene expression. Gene expression has been observed to decrease for some proteins during torpor and increase for others. Low temperatures can also destabilize the hydrophobic interactions required to maintain proper protein conformation and thus increase the potential for protein denaturation (47).

Surfactant function. The above findings suggest that irrespective of the type of torpor pattern displayed by an animal (i.e., daily torpor, stress torpor, or hibernation), the ability to maintain a functioning surfactant system at cold T_b during torpor bouts does not require specific alterations in surfactant PL composition. Hence, it is likely that the PL composition that is optimized for warm T_b is critical and cannot be altered for the special circumstance of torpor. Under the classical model of surfactant function, the primary role of PL in surfactant is to lower the surface tension, by forming a tightly packed, liquid-condensed film at the air-liquid interface that effectively excludes water molecules. In bats (11) and dunnarts (29), surfactant isolated from both warm-active and torpid animals is capable of effectively reducing surface tension at the appropriate T_b. This paper demonstrates that they do this without any major changes in PL molsp composition. However, warm-active and torpid surfactant does not function as effectively when measured at different assay temperatures, that is, cold and warm T_b, respectively (11, 29). The physical structure of warm-active and torpid surfactant films at an air-liquid interface, are also known to differ (36). It is therefore apparent that despite the identical PC molsp, pulmonary surfactant is somehow altered during, or for, torpor via other components or biophysical interactions.

Chol is predicted to broaden the phase-transition temperature of the surfactant mixture, thus, increasing its fluidity (35). Hence, increases in Chol may explain the differences in surfactant function previously observed. Although the mechanisms by which Chol may enhance surfactant function at cold T_b have not been examined, Chol does have a role in the redistribution of PL (9, 18, 34) and/or in altering the structure of the surfactant film (3, 17). Whether the decrease in SP-A can contribute to the differences in function we have previously observed in warm-active and torpid surfactant, is not known. However, it is likely that the amounts of SP-A required for protecting surfactant, by enhancing adsorption and preventing inhibition by serum proteins, is less than the amounts of SP-A we observed in torpid surfactants (unpublished observations), and thus this aspect of surfactant function is likely to be maintained in both warm-active and torpid animals.

Comparative Biology of Mammalian Surfactant

Phosphatidylcholine. The PL saturation of surfactant did not differ between the groups of homeothermic and heterothermic animals (Table 2). However, there was extensive diversity in the individual molsp composition of their PC (Fig. 3). Despite its classically accepted critical role in surfactant function, the proportion of PC16:0/16:0 varied extensively. In fact, to our knowledge, this is the first study to describe a mature surfactant that does not have PC16:0/16:0 as the predominant PC molsp. Here, we report four mammalian species, the wombat, Etruscan shrew, dunnart, and Tasmanian devil that had low concentrations of PC16:0/16:0, i.e., 21, 19, 16, and 2 mol%, respectively. This discovery demonstrates clearly that a high concentration of PC16:0/16:0 in surfactant cannot be essential to support respiration in a healthy alveolar lung in all mammalian species. Moreover, this observation is in agreement with a recent study, where surfactant from newborn piglets displayed a function superior to that from adult pigs, although it contained less PC16:0/16:0, but more PC16:0/16:1 and PC16:0/14:0 (42). Finally, we are confident that contamination by bronchial mucus is unlikely to have contributed to this diversity of surfactant PC composition. All evidence suggests that airway surfactant for instance originates in the alveolus (4) and there is a direct correlation between the PC molsp compositions of induced sputum and BALF samples obtained from healthy human volunteers (51).

Coincident with the low surfactant content of PC16:0/16:0 in certain mammal species, there was also an enrichment in the monounsaturated PC16:0/16:1 species, with the highest value of 28 mol% measured in the dunnart. Although the surfactant of the Tasmanian devil had only 5% PC16:0/16:1, it did have 45% of its alkyl-acyl derivative, PC16:0a/16:1. The analysis of PC species distributions (Fig. 3) shows that some marsupial surfactant, especially that from the Tasmanian devil, is enriched in plasmanyl ether (alkyl-acyl) PC at the expense of the analogous diacyl species. The reason for this is not clear, but it is not likely to be an adaptation against inflammatory-associated hydrolysis, as both lipid types are equally good substrates for group IIA secretory phospholipase A_2 (unpublished observation). Hence, as the diacyl and alkyl-acyl species appear to have similar properties (24), we combined the two groups of species in our molsp correlational analyses (Fig. 4). In addition to the inverse relationship between PC16:0/16:0 and PC16:0/16:1, we also described a highly significant inverse correlation between PC16:0/16:0 and another fluid molsp, namely, PC16:0/14:0. Inverse relationships between PC16:0/16:0 and PC16:0/14:0, as well as with PC16:0/16:1 have previously been described in relation to development within a species [e.g., between newborn and adult pigs (42)] and in relation to differences in respiratory rate between, as well as within species (6). Hence, a molecular substitution pathway in alveolar type II cells between the saturated, nonfluid PC16:0/16:0 and the more fluid species PC16:0/16:1 and PC16:0/14:0 is not uncommon and is presumably related to differences in lung function associated with a host of physiological variables (e.g., development or respiratory rate) (6). Clearly, the present study indicates, that among the mammals, there is generally a trade-off between the saturated nonfluid PC16:0/16:0 and the major fluidizing components, PC16:0/16:1 and PC16:0/14:0 (Fig. 4).

The range and overlap in values between the various groups of mammals, that is, homeothermic/heterothermic and placental/marsupials (Fig. 4), shows clearly that virtually any combination of these three molecular structures can be used as the basis for a functional surfactant. In general, marsupial surfactants were enriched in PC16:0/16:1 and PC16:0/14:0, but had a lower PC16:0/16:0 content than the placental mammals (Fig. 4). However, the differences observed in dunnart surfactant compared with other heterothermic and homeothermic placental mammals do not appear to be phylogenetically determined (i.e., closely related species did not have more similar surfactant profiles). For example, the composition of dunnart surfactant shared very few similarities with the composition of its closest nonheterothermic relative, the carnivorous Tasmanian devil, *Sarcophilus harrisii.* Hence, differences in surfactant composition of marsupials, and particularly that of dunnarts, cannot be specifically related to being an Australian dasyurid marsupial. Moreover, the relative contributions of molsp in different species can also not be solely attributed to the thermal behavior or metabolic strategy, that is, whether a species is heterothermic or homeothermic, as the heterothermic species (shrew, bat, squirrel, dunnart) were all as different and as similar to each other as they were from homeothermic species. Hence, it appears that the molsp profile of a particular animal species is a mosaic determined by the entire gamut of physiological characteristics that determine the biology of that species.

Despite their varied compositions, PC from all these surfactants displayed common compositional features that were clearly distinguished from those of typical mammalian cell membrane PC and from other surface phospholipids such as epithelial lining fluid from the ear (37). Virtually all PC species were shorter-chain molecules, with the fatty acid palmitate (C16:0), rather than stearate (C18:0) or oleate (C18:1) at their *sn*-1 position, and were characterized by very low contents of polyunsaturated fatty acids. These observations imply that, irrespective of the precise details of their PC compositions, all the animals studied must have essentially comparable mechanisms for packaging and secretion of shorter-chain disaturated and monounsaturated PC species into the functional surfactant pool.

Phosphatidylglycerol and phosphatidylinositol. PG and PI represent 8–12% of total PL in all mammalian pulmonary surfactants examined to date (2, 14). Here, the most notable features of surfactant PG and PI were their low content of disaturated species relative to PC and their wide variations of composition. Unlike the relatively ordered differences in surfactant PC molsp composition between the various mammalian species, no similar patterns were apparent for either PG or PI compositions (Fig. 2), apart from both being dominated by mono- and diunsaturated species. Although it is the case for PC, the targeting of anionic PLs to alveolar surfactant is apparently not restricted to fatty acids with 16 or less carbon units. The only molsp that appears to represent a significant proportion (20–40 mol%) in most surfactants, is PG16:0/18:1.

Surfactant PI molsp profiles showed considerably more difference between surfactants than either PG or PC, as well as a greater proportion of more unusual molsp (e.g., long-chain unsaturated species with more than two double-bonds and alkyl-acyl species). Total disaturated diacyl PI levels (mainly PI16:0/16:0) were very low (1–10% total). However, some marsupials had significant quantities of PI16:0a/16:0. The relatively high levels of unsaturated PI species and the high relative levels of arachidonic acid (20:4) and docosahexaenoid acid (22:6) containing species were surprising. However, normal tissue PIs are rich in PI18:0/20:4, and PI levels are relatively low in adult surfactants (~5%). Consequently, no particular molsp will contribute a very high proportion of total PL, and it is therefore unlikely that a particular PI molsp is critical for surfactant function.

Differences in PL molsp Compositions—a Fine-Tuning Mechanism?

Mechanisms resulting in the observed differences in molecular composition appear to be fine-tuning mechanisms because firstly, the % saturation of PL in mammalian surfactants is fairly constant (typically, 40–50%) and secondly, regardless of the precise molsp composition, the sum of PC16:0/14:0, PC16:0/16:1, and PC16:0/16:0, is 60–70% in all animals studied. Even in the Tasmanian devil, the alkyl-acyl forms of these components contribute >70% to surfactant PC.

The similarities in the extent to which molecular PC composition varies during development and in heterothermic mammals, suggests that similar molecular mechanisms for regulating the packing, synthesis, and secretion of surfactant PL are present in all mammalian type II cells. Dunnarts, squirrels, and bats may alter these common mechanisms as an adaptive strategy to fine-tune their surfactant composition for efficient function at both warm and cold T_b. However, rat and human type II cells may alter these same mechanisms in response to developmental changes in lung structure and function. Common responses to differences in lung function that are emerging include variations in PC16:0/16:0, PC16:0/16:1, and PC16:0/14:0.

The present data suggest that there is not a single molsp PL composition that can function optimally in all mammals. Rather, there is a spectrum of different molecular compositions within mammals, such that the molecular PL composition of each animal species is unique and optimized to the physiology of that animal. Similar concepts of molecular adaptation have previously been suggested by others. Heterothermy represents one factor that may influence the molecular species composition of surfactant, as well as the cholesterol concentration. Other factors include development (6, 25), disease (31), and differences and alterations in alveolar size (14) and respiratory rate (6, 42). The combination of all these factors ultimately leads to differences in the composition of surfactant at the neutral lipid, PL, and protein levels and also at the molsp PL level enabling the surfactant to function if not optimally, at least effectively, at all physiological variables experienced by an animal in its daily or seasonal cycle, behavior, or development.

Perspective

The data presented here are of significant value for comparative and general physiology, as well as clinical pulmonology. In intensive care medicine PC16:0/16:0-enriched surfactants are still used in therapy, despite being of limited success. The relevance of PLs other than PC16:0/16:0 to the biophysical and immunoregulatory functions of surfactant should be an important revision of our thinking. Hence, the data presented here

may contribute to the future design of synthetic therapeutic surfactants that are better adapted to clinical needs.

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