Effects of SNP, ouabain, and amiloride on electrical potential profile of isolated sheep pleura

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Recently, indirect evidence was provided to support an active electrolyte transport by mesothelial cells (1, 22, 23). Agostoni and Zocchi (1) showed the occurrence of a solute-coupled liquid absorption from the pleural space by using inhibitors for the Na+/Cl−, Na+/H+, and Cl−/HCO3− double exchange or for the Na+/K+ pump. Other experimental data (8) suggest the presence of both tracheal-vascular and vascular-pleural electrical potentials in rat lung and suggested that the potentials were generated by active transport of both Na+ and Cl− across these barriers. Evidence for a small, active transport of Na+ from the serosal to the interstitial side of the dog parietal pleura in vitro was found by D’Angelo and colleagues (5). Other authors suggested that both the visceral and parietal pleura behave as highly permeable membranes, allowing free movement of water and large proteins (13, 19).

Results from different studies (13–15, 19) support the hypothesis that pleural capillaries determine the rate of liquid and solute movement across the whole pleural membrane and that no water or solute flux takes place in the absence of oncotic or hydrostatic driving forces. More evidence for this theory is the benign postpartum pleural effusion, which is attributed to the increase in hydrostatic pressure and decrease in the colloid osmotic pressure (11). For water and solute to be filtered or reabsorbed by the pleural capillaries, it must pass through both the capillary endothelium and the pleural mesothelial tissue. Whereas it has been assumed that pleural tissue offers little resistance to fluid movement across either the parietal or the visceral pleura, the permeability of sheep pleural tissue is unknown.

The objective of this study was to examine the inhibitory effects of sodium nitroprusside, ouabain, and amiloride on transepithelial electrical resistance (RTE) of sheep pleura. Sodium nitroprusside is a donor of nitric oxide (NO), which inhibits amiloride-sensitive cation channels and Na+/K+ -ATPase and decreases vectorial Na+ transport. Ouabain inhibits Na+/K+ pump, whereas amiloride inhibits Na+ channels and Na+/H+ exchanger. Because of the anatomic differences between the visceral and parietal pleura (2, 3,
18), the measurements of the visceral pleura were compared with those of the parietal membrane.

METHODS

Intact sheets of visceral and parietal pleura were obtained from 30 adult sheep (males and females). The samples were collected from the slaughter house. The pleuras were kept in oxygenated Krebs-Ringer solution at 4°C and transferred to the laboratory within 30 min of the death of the animal. Care was taken to touch the surface as little as possible. Immediately after removal, the pleural tissue was placed in Krebs-Ringer bicarbonate (KRB) solution. The KRB solution was balanced at pH 7.4 and contained (in mM) 117.5 NaCl, 1.15 NaH₂PO₄, 24.99 NaHCO₃, 5.65 KCl, 1.18 MgSO₄, 2.52 CaCl₂, and 5.55 glucose. The KRB was bubbled with 95% O₂-5% CO₂. If experiments were not performed immediately, the pleura was then stored in KRB solution at 4°C along with a very small amount of silicone (Sylgard silicone-elastomer kit) in a recessed O rings, and a tight seal was obtained by applying a just-in-time method of mounting the tissue between the reservoirs was 1.43 cm². Because of this, the edge effect is minimized (16). Each piece of visceral pleura carefully stripped from underlying lung and examined for evidence of holes or adherent lung tissue by visual inspection. The pieces were mainly from the surface of the left and right caudal lobes as well as from middle and cranial lobes. Parietal pleura was stripped from the diagram and examined in a similar way. We studied the effect of stripping the tissue from the lung, the rib cage, and diaphragm. The results were the same using samples of the mediastinal pleura, which is free standing and requires no stripping for tissue bath studies.

The pleura was mounted as a planar sheet separating two reservoirs of fluid in acrylic Ussing-type chambers attached to glass reservoirs. The pleura was mounted between two recessed O rings, and a tight seal was obtained by applying a very small amount of silicone (Sylgard silicone-elastomer kit) along the rim of each O ring. This method of mounting the tissue has been shown to minimize the edge effect (16). Each chamber was conical in shape with a total volume (including the reservoir) of 20 ml. The cross-sectional area of the exposed tissue between the reservoirs was 1.43 cm². Because active transport of ions is influenced by temperature, measurements of transepithelial potential difference were done at 37°C.

The transepithelial potential difference across the visceral and parietal pleura was measured with 3 M KCl, 3% agar bridges placed 3 mm on either side of the membrane. They were connected on either side to Ag-AgCl electrodes, and the output was amplified (model DVC-3 with input impedance of 10¹² Ω; Word Precision Instruments). To determine the voltage response to an external current, direct current provided by a voltage-clamp apparatus (model DVC-1000, World Precision Instruments) was passed through the tissue via 3 M KCl agar bridges placed in the reservoirs connected to each hemichamber.

Visceral or parietal pleura was mounted in the chamber, bathed on both sides with KRB solution, and bubbled continuously with a 95% O₂-5% CO₂ gas mixture. The spontaneous potential difference across visceral (n = 6) and parietal (n = 6) pleura was measured in the absence of an external current source every 30 min for 5 h. A current of variable intensity (range 0–300 μA, −300–0 μA) was then applied, and the voltage response of the visceral (n = 10) and parietal (n = 9) pleura was measured. The $R_{TE}$ was calculated, using Ohm’s law, from the voltage deflections produced in response to constant current pulses across the tissue.

The experimental solution bathing the surface of the pleura that ordinarily faces the pleural liquid in vivo is referred to as the serosal solution, whereas the solution bathing the surface normally exposed to the blood supply is referred to as the mucosal solution. Similarly, the mesothelial cell membranes facing the pleural liquid or blood side is referred to as the basolateral or apical membrane, respectively.

In the initial set of experiments, electrical measurements were made on visceral and parietal pleura mounted in the chamber and bathed with KRB solution on both sides. After 30 min, spontaneous potential difference across the pleura and voltage response to applied current (range 0–300 μA) were measured. In some studies, the NO donor sodium nitroprusside (10⁻⁶ M) and sodium nitroprusside (10⁻⁵ M) plus N⁷-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, were added to the serosal solution of visceral (n = 6) and parietal (n = 6) pleura and to the mucosal solution of visceral (n = 6) and parietal (n = 6) pleura, and measurements were repeated after 10 min. In a number of cases, the Na⁺-transport blocker amiloride (10⁻⁵ M) or the Na⁺-K⁺ pump inhibitor ouabain (10⁻⁵ M) were added to the serosal solution of visceral (n = 6) and parietal (n = 6) pleura and to the mucosal solution of visceral (n = 6) and parietal (n = 6) pleura. Transepithelial potential difference and voltage response to applied current were measured after 10 min. These specific concentrations were chosen based on higher inhibitory effects of drugs. We obtained no effect on concentrations >10⁻⁸ M for all drugs. All solutions were freshly prepared before each experiment, heated to 37°C, and continuously bubbled with a 95% O₂-5% CO₂ in air. The results, which are presented in this paper, are the means of six different experiments.

Statistics. Statistical analysis was performed with SPSS for Windows. All data are expressed as means ± SD. Statistical comparison was determined by paired t-test. We accepted a P value of <0.05 as being significantly different.

RESULTS

The spontaneous electrical potential difference across parietal pleura was 0.5 ± 0.1 mV, whereas that across visceral pleura was 0.4 ± 0.1 mV. Neither value was significantly different from zero. Measurements of potential difference every 30 min for 5 h did not show any development of transepithelial potential difference (Fig. 1).

As shown in Fig. 2, the voltage-current relationship of both the visceral and parietal pleura was linear, indicating their behavior as an ohmic resistor. Electrical resistance of both pleura was very low: 22.02 ± 0.3 MΩ cm² for visceral pleura and 22.02 ± 3.5 MΩ cm² for parietal pleura.

The inhibitory effects of ouabain, amiloride, and sodium nitroprusside on the basolateral and apical membrane of parietal pleura are shown in Fig. 3. On the basolateral membrane, an increase in $R_{TE}$ was observed with the addition of 10⁻³ M ouabain and 10⁻⁵ M amiloride (Fig. 3A). These effects were reversed by washing the chamber with KRB solution (data not shown). Addition of 10⁻⁵ M sodium nitroprusside to the serosal bathing solution increased $R_{TE}$ (Fig. 3A). In another set of experiments, we used sodium nitroprusside plus L-NAME, an inhibitor of NO synthase. With 10⁻⁴ M L-NAME, we showed no increase in $R_{TE}$. Ouabain also increased the $R_{TE}$ when it was added to the apical surface of parietal pleura (Fig. 3B).
On the basolateral surface of visceral pleura, an increase in $R_{TE}$ was observed only when sodium nitroprusside ($10^{-5}$ M) was added to this side of the membrane (Fig. 4A). Addition of sodium nitroprusside plus L-NAME elicited no change in $R_{TE}$. Addition of $10^{-3}$ M ouabain to the mucosal bathing solution of visceral pleura resulted in an increase of the $R_{TE}$ (Fig. 4B). It was reversible, with a return to control levels after the chamber was washed with KRB solution (data not shown). An increase in $R_{TE}$ was also observed when sodium nitroprusside was added to the mucosal surface of visceral pleura. It was returned to control levels by addition of sodium nitroprusside plus L-NAME ($10^{-4}$ M) to the mucosal bathing solution.

**DISCUSSION**

Our data show that there is no measurable spontaneous potential difference across the visceral and parietal sheep pleura. We further demonstrated an increase in the $R_{TE}$ when sodium nitroprusside, ouabain, and amiloride were added to the pleura.

Engelberg and Radin (8) found a vascular-pleural potential that was about $2.5$ mV. In the isolated lung, this electric potential was abolished when a solution with cyanide or ouabain was applied to the lung surface, suggesting that it was generated by an active transport. This potential was also abolished when sodium was substituted by choline, or chloride by sulfate, on both sides, suggesting that both Na$^+$ and Cl$^-$ are necessary for the vascular-pleural potential (8). We found no measurable potential difference across the sheep visceral and parietal pleura. This finding is the same as that reported for canine visceral and parietal pleura.

More specifically, this increase was observed when 1) ouabain was added to the mucosal surface of visceral pleura and to the serosal or mucosal surface of parietal pleura; 2) amiloride was added to the serosal bathing solution in parietal pleura; and 3) sodium nitroprusside was added to the serosal surface of parietal pleura and to the serosal or mucosal bathing solutions of visceral pleura.

Engelberg and Radin (8) found a vascular-pleural potential that was about $-5$ mV. In the isolated lung, this electric potential was abolished when a solution with cyanide or ouabain was applied to the lung surface, suggesting that it was generated by an active transport. This potential was also abolished when sodium was substituted by choline, or chloride by sulfate, on both sides, suggesting that both Na$^+$ and Cl$^-$ are necessary for the vascular-pleural potential (8). We found no measurable potential difference across the sheep visceral and parietal pleura. This finding is the same as that reported for canine visceral and parietal pleura.

**Fig. 1.** Transepithelial potential difference did not change significantly over 5 h in parietal and visceral sheep pleura. Bathing solution was Krebs-Ringer bicarbonate bubbled with 95% O$_2$-5% CO$_2$. Values are means ± SD.

**Fig. 2.** Voltage-current relationship for sheep visceral and parietal pleura. Tissue resistance for these specimens was 22.02 Ω·cm$^2$.

**Fig. 3.** Effect of ouabain ($10^{-3}$ M), amiloride ($10^{-5}$ M), sodium nitroprusside (SNP; $10^{-6}$ M), and combined effect of SNP ($10^{-5}$ M) plus N$^o$-nitro-L-arginine methyl ester (L-NAME; $10^{-4}$ M) on transepithelial electrical resistance ($R_{TE}$) to the basolateral (A) and apical (B) membrane of parietal pleura. Ouabain resulted in an increase in $R_{TE}$ when it was added to either the basolateral or apical membrane, whereas amiloride and SNP increased the $R_{TE}$ only when they were added to the basolateral membrane of the pleura. Values are means ± SD.
pleura and sheep visceral pleura (13, 19), as well as for another mesothelial tissue, toad peritoneum (9).

The ohmic resistance of the pleura was very low, lying between the values reported for “leaky” epithelial tissues such as renal proximal tubule and rabbit gallbladder (10). The tissue resistance values for both visceral and parietal pleura were 22.02 Ω·cm² and were found to be similar to those reported for sheep visceral pleura (27.1 Ω·cm²) (13) and canine visceral and parietal pleura (20.2 and 22.2 Ω·cm², respectively) (19). These properties indicate that the pleura is extremely leaky to ionic currents but do not by themselves rule out the possibility that active transport occurs across the pleura (6). It remains possible, however, that active ion transport takes place across the pleura in such a way that no electrical potential difference can be observed.

Most of the experimental evidence is that pleural fluid is produced via a Starling mechanism through the capillaries of the parietal pleura and is reabsorbed via pleural lymphatics. The visceral pleura does not contribute substantially to passive fluid and solute exchanges in physiological conditions. Recently, indirect evidence was provided to support an active electrolyte transport by mesothelial cells (22, 23). The corresponding solute-coupled water transport remains so far undetermined, although results from the isolated specimens of parietal and visceral pleura tend to exclude any significant role (13, 19). The permeability to electrolytes of stripped specimens of either visceral or parietal pleura is high (13, 19), but it could be smaller under physiological conditions. Evidence for a small, active transport of Na⁺ from the serosal to the interstitial side of the dog parietal pleura in vitro was found by D’Angelo et al. (5).

The results reported above show the occurrence of an electrolyte transport across the pleural mesothelium. The increase of the $R_{TE}$ when ouabain was added to the mucosal solution of visceral pleura suggests the occurrence of a Na⁺-K⁺ pump on the apical membrane transporting Na⁺ out of the cells and thus out of the pleural space. Addition of ouabain to the serosal surface of parietal pleura leads to the increase in $R_{TE}$ and suggests the existence of a Na⁺-K⁺ pump on the basolateral side of the mesothelium transporting K⁺ into the cells.

Ten minutes after the addition of amiloride to the serosal solution of parietal pleura, the $R_{TE}$ was increased. Because amiloride inhibits the Na⁺ channels and the Na⁺/H⁺ exchanger, this finding suggests the occurrence of Na⁺ channels or of Na⁺/H⁺ double exchange in the basolateral side of parietal pleura. The absence of ouabain in the serosal surface of parietal pleura and to the apical side of the parietal pleura could be explained by the hypothesis that the amiloride exerts its action on the endothelium of the lymphatic stomata, which are on the basolateral side of the parietal pleura (21).

If this interpretation of the above findings is correct, we suggest the occurrence of two kinds of cells in the pleural mesothelium. Those with the double exchange mechanism or Na⁺ channels on the serosal side are likely provided with the Na⁺-K⁺ pump on the mucosal side. These cells should transport Na⁺ (and hence liquid) out of the pleural space. The second kind of cells is likely provided with the Na⁺-K⁺ pump on the serosal side and could be involved in recycling K⁺ and perhaps in other functions. It should be kept in mind that these mechanisms are probably involved in maintaining an adequate volume and composition, not only of the pleural liquid, but also of the mesothelial cells. Evidence suggesting a Na⁺-K⁺ pump on the opposite side of two kinds of cells has been provided in the pleural mesothelium (22) and in the alveolar epithelium (4). Morphologically, two kinds of cells have been described in the pleural mesothelium, the flat cells, which are the most numerous and have well-developed tight junctions, and the cuboidal cells, which are less in number and have less tight junctions than the typical flat cells (18). NO was found to inhibit both apical amiloride-sensitive cation channels and the basolateral Na⁺-K⁺-ATPase and decrease vectorial Na⁺ transport across cultured alveolar type II monolayers (12, 17), as well as across cultured distal lung epithelial cells (7). The
increase in the $R_{TE}$ when a NO donor, sodium nitroprusside, was added to the serosal bathing solution of parietal pleura suggests the existence of the inhibition of one or more of the above mechanisms. This increase in the $R_{TE}$ returned to control levels by the addition of l-NAME, an inhibitor of NO synthesis. The occurrence of the electrolyte transport could be either to the pleural mesothelium, or to endothelium of lymphatic stomata, or both.

In conclusion, our results show that the values of the spontaneous potential difference across all visceral and parietal pleura tested were not significantly different from zero. We have also found evidence for active transport across both visceral and parietal pleura. Thus the sheep pleural mesothelium appears to play a role in the exchange of electrolytes between the pleural capillaries and the pleural space.

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REFERENCES