Alveolar Epithelial Ion and Fluid Transport

Ca\(^{2+}\)-dependent stimulation of alveolar fluid clearance in near-term fetal guinea pigs

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Norlin, Andreas, and Hans G. Folkesson. Ca\(^{2+}\)-dependent stimulation of alveolar fluid clearance in near-term fetal guinea pigs. Am J Physiol Lung Cell Mol Physiol 282: L642–L649, 2002; 10.1152/ajplung.00417.2000.—We investigated the importance of changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) for amiloride-sensitive alveolar fluid clearance (AFC) in late-gestational guinea pigs. Fetal guinea pigs of 61, 68, and 69 days (term) gestation were investigated under normal conditions and after oxytocin-induced preterm labor. AFC or alveolar fluid secretion was measured using an impermeable tracer technique. At 61 days gestation there was net secretion of fluid into the lungs, and at birth the lungs cleared 49 ± 7% of the instilled fluid volume over 1 h. Induction of preterm labor with oxytocin induced AFC at 61 days gestation. When present, AFC was inhibited or reversed to net fluid secretion by amiloride (10\(^{-5}\) M). Inhibition of membrane Ca\(^{2+}\) channels by verapamil (10\(^{-4}\) M) or depletion of intracellular Ca\(^{2+}\) by thapsigargin (10\(^{-5}\) M) reduced AFC when net AFC was evident. Amiloride lacked an inhibitory effect on AFC when instilled with verapamil or thapsigargin. The results indicate that AFC via amiloride-sensitive pathways develops during late gestation, and that inducing preterm labor precociously may activate such pathways. Our results suggest that Ca\(^{2+}\) may act as a second messenger in mediating catecholamine-stimulated AFC.

amiloride; birth; sodium transport; oxytocin-induced preterm labor

IN UTERO, THE LUNGS SECRETE FLUID into the developing air spaces. This fluid secretion is necessary for development of future alveolar spaces (3, 24). At birth, the alveolar fluid must be removed to allow pulmonary gas exchange. Consequently, lung fluid secretion is reduced as full term approaches (2, 19), and at birth the alveolar fluid clearance (AFC) is rapid (4, 5, 19).

The increased AFC rate after spontaneous labor (4, 19) or oxytocin-induced preterm labor (19) is stimulated by labor-released epinephrine. Epinephrine stimulation of AFC is at least partly mediated by cAMP, which acts as an intracellular second messenger (5, 17, 30). It has also been demonstrated that intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is increased after terbutaline stimulation (14, 17, 28). In addition, a significant fraction of stimulated transepithelial Na\(^+\) transport may depend on increased [Ca\(^{2+}\)]\(_i\) (12, 13, 28).

AFC is secondary to Na\(^+\) absorption across the alveolar epithelium. Na\(^+\) enters through apical Na\(^+\) channels in the alveolar epithelial type II cells and is extruded basolaterally by the Na\(^+\)-K\(^+\)-ATPase (for reviews, see Refs. 15 and 16). A significant fraction of AFC is mediated through amiloride-sensitive pathways in fetal (19, 23) and newborn (5) animals. The epithelial Na\(^+\) channel (ENaC), which is amiloride sensitive, has been proposed as one pathway for Na\(^+\) absorption in near-term or newborn animals (1, 5, 32). Upregulation of the basolateral Na\(^+\)-K\(^+\)-ATPase may be involved in emptying the fetal air spaces of fluid (6).

Because evidence is present that epinephrine stimulation of AFC may depend on changes in [Ca\(^{2+}\)]\(_i\) (14, 17, 28), we hypothesized that increased [Ca\(^{2+}\)]\(_i\) would lead to an increased AFC in near-term fetal guinea pigs. We therefore attempted to manipulate [Ca\(^{2+}\)]\(_i\) by blocking Ca\(^{2+}\) channels that could regulate [Ca\(^{2+}\)]\(_i\) by changing the exchange of Ca\(^{2+}\) with the external microenvironment. The first aim of this study was to investigate the role of extracellular Ca\(^{2+}\) influx for \(\beta\)-adrenergic stimulation of AFC by inhibiting membrane Ca\(^{2+}\) channels in normal fetuses and in age-matched fetuses.
after oxytocin-induced preterm labor. The second purpose was to investigate the effect of Ca\(^{2+}\)-channel inhibition on the amiloride-sensitive fraction of AFC during normal conditions and after oxytocin-induced preterm labor. The third goal was to investigate the effects of disrupting the intracellular Ca\(^{2+}\) balance by inhibiting Ca\(^{2+}\) reuptake by the sarcoplasmatic Ca\(^{2+}\)-ATPase (SERCA) using thapsigargin. This eventually results in a depletion of intracellular Ca\(^{2+}\) thus lowering the [Ca\(^{2+}\)]. We investigated thapsigargin inhibition during normal conditions and after oxytocin-induced preterm labor with and without amiloride inhibition of the AFC.

**MATERIALS AND METHODS**

**Animals and Oxytocin Pretreatment**

Fetuses obtained from timed-pregnant Dunkin-Hartley guinea pigs (Sahlin’s Forsöksdjursfarm, Malmö, Sweden) were used for the experiments (n = 165 animals from 52 litters). The timed-pregnant guinea pigs were maintained on a 12:12-h day-night rhythm and had free access to food (standard guinea pig chow; SDS, Witham, Essex, UK) and tap water. The Committee on Animal Experiments at Lund University approved this study.

Preterm labor was induced as we have done before (19) by subcutaneous injection of oxytocin (1 mg/kg body wt, which is equal to 1 IU/kg body wt; Ferring, Malmö, Sweden) every 15 min for 45 min. Fetuses were delivered by abdominal hysterectomy (see Surgical Preparations) after 45 min if normal vaginal delivery did not occur within that time. Timed-pregnant guinea pigs with 1 day of gestation remaining delivered their fetuses vaginally within 45 min after oxytocin administration, whereas fetuses of timed-pregnant guinea pigs with 8 days gestation remaining were delivered by abdominal hysterectomy.

**Solutions**

An isosmolar 5% albumin solution was prepared by dissolving 50 mg/ml bovine serum albumin (Sigma, St. Louis, MO) in 0.9% NaCl. In the studies of the role of extracellular Ca\(^{2+}\) influx, the L-type Ca\(^{2+}\)-channel inhibitor verapamil hydrochloride (10\(^{-4}\) M; Sigma) was used. In the studies of intracellular Ca\(^{2+}\) depletion, the intracellular Ca\(^{2+}\)-ATPase inhibitor thapsigargin (10 \(\mu\)M; Alomone Labs, Jerusalem, Israel) was used. Thapsigargin was dissolved in 0.1% DMSO. DMSO (0.3%) has been used directly in alveolar epithelial type II cell incubation medium without deleterious effects on cell integrity or surfactant phospholipid exocytosis (31). Therefore, we considered our DMSO concentration to be noninjurious in our experimental setup. In the studies of amiloride sensitivity, the Na\(^+\)-channel inhibitor amiloride hydrochloride (10\(^{-3}\) M; Sigma) was added to the instillate.

**Surgical Preparations**

The timed-pregnant guinea pigs were deeply anesthetized by injections of pentobarbital sodium (120 mg/kg body wt ip; Apoteksbolaget, Umeå, Sweden) and euthanized by intracardiac injections of 60 mg of pentobarbital sodium. A laparotomy was performed, and fetuses were carefully delivered. The fetal umbilical cord was ligated to prevent bleeding. The fetuses were immediately euthanized by 12 mg ip of pentobarbital sodium with 500 IU of heparin (Lövenas, Ballerup, Denmark). Newborn guinea pigs were euthanized by 18 mg ip of pentobarbital sodium with 500 IU of heparin.

After euthanasia, an endotracheal tube (PE-190; Clay Adams, Becton Dickinson, Parsippany, NJ) was inserted through a tracheotomy. The fetuses and newborn animals were immediately connected to a constant oxygen-flow device (oxygen fraction, 1.0; AGA, Lidingö, Sweden), and the lungs were expanded by adjusting the oxygen flow to provide a constant positive airway pressure (CPAP) of 5 cmH\(_2\)O. The entire surgical procedure after euthanasia required 5 min. Fetuses and newborn animals were placed between heating pads to maintain body temperature during the experiments. A rectal temperature probe measured body temperature, and heating was adjusted to maintain the temperature at 37–38°C. Airway pressure was continuously monitored by calibrated pressure transducers (UFI model 1050B or TSD104A, Biopac Systems, Goleta, CA) and analog-to-digital converters and amplifiers (UIM100 and MP100, Biopac Systems).

**Alveolar Fluid Clearance Experiments**

After surgery and connection to the CPAP circuit, the 5% albumin instillation solution (10 ml/kg body wt) was instilled into the lungs through the endotracheal tube: first, the fetuses and newborn animals were briefly disconnected from the CPAP circuit, and the lungs were deflated by gently aspirating residual air with the instillation syringe. The instillation solution was then instilled into the lungs and withdrawn again. This procedure was repeated four times to allow thorough and adequate mixing of instillate and preexisting fetal lung fluid, and then the fluid was finally instilled. The fetuses and newborn animals were reconnected to the CPAP circuit, and they remained on the CPAP device for the 1-h study period. A 0.1-ml sample of the instillation solution-lung fluid mixture (initial solution) remained in the syringe for protein measurement. After 1 h, the lungs and heart were carefully removed en bloc through a midline sternotomy, and a sample of remaining alveolar fluid was collected by gently advancing the PE-50 sampling tube to a wedged position and aspirating the fluid. Total protein concentrations in the instillates and in the initial and final solutions were determined spectrophotometrically (iEMS Reader MF, Labsystems, Helsinki, Finland) by the Lowry method (11) adapted for microtiter plates.

AFC or alveolar fluid secretion (AFS) was calculated from the change in alveolar protein concentration over 1 h. This was possible because the alveolar epithelium is relatively impermeable to large molecules such as albumin (mol wt 67,000). Therefore, water movement (absorption or secretion) results in a changed air-space protein concentration. Because the fetal lung is fluid filled in utero (3, 19, 24), we expected that a certain fraction of fluid would still be present in the lungs at the time of the experiments. This fluid is virtually protein free and will not add protein to the instilled albumin concentration. In contrast, it will dilute the protein concentration in the instillates and influence the calculations of AFC differently depending on the volume of fluid that is present in the lungs at the different developmental stages. To control for this volume, we instilled guinea pig fetuses with 10 ml/kg body wt of the 5% albumin instillation solution. The fluid was aspirated and reintroduced four times before a final 0.1-ml sample was taken and the rest was instilled, and the fetus was studied for 1 h. The entire procedure required ~1–2 min. During this time, it was unlikely that a significant quantity of protein either left or entered the air spaces or that significant volumes of fluid were reabsorbed from or secreted into the air spaces. Therefore, changes in protein concentra-
tion represent a dilution by preexisting fluid. The preexisting fluid volume (Vpre) calculated from Eqs. 1 and 2 was used to correct the instilled protein concentrations by the dilution of the instillate that would occur if fluid were already in the lung before the 5% albumin instillation. AFC or AFS was calculated from Eqs. 3 and 4

$$V_{\text{initial}} = \left( V_{\text{instilled}} \times C_{\text{instilled}} \right) / C_{\text{initial}}$$  \hspace{1cm} (1)

$$V_{\text{pre}} = V_{\text{initial}} - V_{\text{instilled}}$$  \hspace{1cm} (2)

$$V_{\text{final}} = \left( V_{\text{initial}} \times C_{\text{initial}} \right) / C_{\text{final}}$$  \hspace{1cm} (3)

$$\text{AFC or AFS} = \left[ (V_{\text{initial}} - V_{\text{final}}) / V_{\text{initial}} \right] \times 100$$  \hspace{1cm} (4)

where V_{\text{instilled}}, V_{\text{initial}}, and V_{\text{final}} are volumes of the instillate and the initial and final solutions, respectively, and C_{\text{instilled}}, C_{\text{initial}}, and C_{\text{final}} are the protein concentrations in the corresponding solutions.

Specific Protocols

Guinea pig fetuses of 61 and 68 days postconception gestational age and newborn animals (69 days; term) were studied. Day of conception was set as the day when the timed-pregnant guinea pigs gave birth to their earlier litter, because guinea pigs enter estrus immediately after birth. All groups contain fetuses from at least two litters. All fetuses were studied for 1 h after which the lungs were removed and a sample of remaining alveolar fluid was collected. Protein concentrations were measured and AFC was calculated.

Control studies. Preterm guinea pig fetuses of 61 (n = 6) and 68 (n = 6) days gestation and newborn guinea pigs (69 days gestation, term; n = 6) were surgically delivered from timed-pregnant guinea pigs, prepared as described (see Surgical Preparations), and instilled with the 5% albumin solution.

Oxytocin studies. Guinea pig fetuses from oxytocin-injected timed-pregnant guinea pigs of 61 (n = 6) and 68 (n = 6) days gestation were surgically prepared as described and instilled with the 5% albumin solution.

Amiloride studies. Guinea pig fetuses of 61 (n = 6) and 68 (n = 6) days gestation and newborn guinea pigs (69 days gestation, term; n = 6) were delivered from timed-pregnant guinea pigs, surgically prepared as described, and instilled with the 5% albumin solution containing 10^{-3} M amiloride. Another set of guinea pig fetuses from oxytocin-injected timed-pregnant guinea pigs of 61 (n = 5) and 68 (n = 4) days gestation was also prepared and instilled with the 5% albumin solution containing 10^{-3} M amiloride. The 10^{-3} M concentration of amiloride was used because a large fraction of amiloride becomes protein bound, and a significant fraction rapidly leaves the air spaces due to its low molecular weight (21, 33); therefore, the effective alveolar concentration was probably lower. Also, the same amiloride concentration has been used in other studies of AFC in both developing and adult animals (5, 8, 18).

Extracellular Ca^{2+} influx (verapamil) studies. Guinea pig fetuses of 61 (n = 6) and 68 (n = 6) days gestation and newborn guinea pigs (69 days gestation, term; n = 5) were delivered from timed-pregnant guinea pigs, surgically prepared as described, and instilled with the 5% albumin solution containing 10^{-4} M of the L-type Ca^{2+} channel inhibitor verapamil. Another set of guinea pig fetuses from oxytocin-injected timed-pregnant guinea pigs of 61 (n = 7) and 68 (n = 6) days gestation was also prepared and instilled with the 5% albumin solution containing 10^{-4} M verapamil.

Extracellular Ca^{2+} influx and Na^{+} channel (verapamil plus amiloride) studies. Guinea pig fetuses of 61 (n = 4) and 68 (n = 6) days gestation and newborn guinea pigs (69 days gestation, term; n = 5) were delivered from timed-pregnant guinea pigs, surgically prepared as described, and instilled with the 5% albumin solution containing 10^{-4} M verapamil and 10^{-3} M amiloride. Another set of guinea pig fetuses from oxytocin-injected timed-pregnant guinea pigs of 61 (n = 4) and 68 (n = 6) days gestation was also prepared and instilled with the 5% albumin solution containing 10^{-4} M verapamil and 10^{-3} M amiloride.

Intracellular Ca^{2+} mobilization (thapsigargin) studies. Guinea pig fetuses of 61 (n = 6) and 68 (n = 6) days gestation and newborn guinea pigs (69 days gestation, term; n = 5) were delivered from timed-pregnant guinea pigs, surgically prepared as described, and instilled with the 5% albumin solution containing 10^{-5} M of the intracellular Ca^{2+}-ATPase inhibitor thapsigargin. Another set of guinea pig fetuses from oxytocin-injected timed-pregnant guinea pigs of 61 (n = 7) and 68 (n = 6) days gestation was also prepared and instilled with the 5% albumin solution containing 10^{-5} M thapsigargin.

Intracellular Ca^{2+} mobilization and Na^{+} channel (thapsigargin plus amiloride) studies. Guinea pig fetuses of 61 (n = 4) and 68 (n = 6) days gestation and newborn guinea pigs (69 days gestation, term; n = 5) were delivered from timed-pregnant guinea pigs, surgically prepared as described, and instilled with the 5% albumin solution containing 10^{-5} M thapsigargin and 10^{-3} M amiloride. Another set of guinea pig fetuses from oxytocin-injected timed-pregnant guinea pigs of 61 (n = 4) and 68 (n = 4) days gestation was also prepared and instilled with the 5% albumin solution containing 10^{-5} M thapsigargin and 10^{-3} M amiloride.

Statistics

Values are presented as means ± SD. Statistical analysis was carried out with one-way ANOVA with Tukey’s test post hoc. Differences were considered statistically significant when P < 0.05.

RESULTS

We instilled an isosmolar 5% albumin solution into the lungs of fetal guinea pigs and calculated AFC or AFS from the change in protein concentration over 1 h. Calculations were always corrected for the initial dilution of the instilled protein solution by endogenous fetal lung fluid.

AFC or AFS

At 61 days gestation, fetal guinea pig lungs secreted fluid into the lung lumen (Fig. 1). When 1 day remained until delivery (at 68 days gestation), the lungs had begun to absorb fluid; during the last gestational day, the fluid absorption rate rapidly increased and reached very high rates at birth. When preterm labor was induced by repeated oxytocin injections (four 1 mg/kg injections over 45 min), the fluid secretion observed in the 61-day gestation control fetuses was converted to fluid absorption. Also, oxytocin-induced preterm labor tended to increase the AFC 1 day before birth (at 68 days gestation), but this increase did not reach significance.

Effect of Amiloride on AFC or AFS

AFC depends on water absorption secondary to Na^{+} absorption through amiloride-sensitive and -insensi-
To investigate the fraction of AFC that was mediated through amiloride-sensitive Na\(^+\)/H\(^+\) absorption, we used the Na\(^+\)/H\(^+\) channel inhibitor amiloride (10\(^{-3}\) M). Amiloride instillation had no inhibitory effect in 61-day gestation (normal) control fetuses (Fig. 2), but amiloride inhibited AFC completely after oxytocin-induced preterm labor at this age (Fig. 3). In 68-day gestation fetuses, amiloride instillation completely inhibited AFC both in control fetuses (Fig. 4) and in oxytocin-induced preterm labor fetuses (Fig. 5). In newborn animals, amiloride inhibited AFC by 88 ± 29% (Fig. 6).

**Effect of Extracellular Ca\(^{2+}\) Influx (Verapamil) on AFC and AFS**

In these studies, verapamil (10\(^{-4}\) M), which is an inhibitor of L-type voltage-gated Ca\(^{2+}\) channels, was added to the instillate. Verapamil instillation did not affect secretion of fluid in control animals at 61 days gestation (see Fig. 2). After oxytocin-induced preterm labor at 61 days gestation, verapamil instillation reversed the induced AFC to net fluid secretion (see Fig. 3). At 68 days gestation, when the lungs had begun to absorb fluid, verapamil instillation reversed the absorption to secretion (see Fig. 4). Also, at 68 days gestation, verapamil inhibited AFC after oxytocin induction of preterm labor (see Fig. 5). In newborn animals, verapamil instillation inhibited AFC by 86 ± 8% (Fig. 6). We also added verapamil and amiloride simultaneously to the instillate. At all gestational ages investigated, irrespective of treatment (control and oxytocin), the combination of the drugs resulted in an attenuation of the amiloride sensitivity (see Figs. 2–6).

**Effect of Intracellular Ca\(^{2+}\) Mobilization (Thapsigargin) on AFC and AFS**

The intracellular Ca\(^{2+}\)-ATPase inhibitor thapsigargin (10\(^{-5}\) M) was added to the instillate to deplete the intracellular Ca\(^{2+}\) stores. Thapsigargin instillation did not affect net alveolar fluid movement in 61-day gestation (see Fig. 2) and 68-day gestation (see Fig. 4) preterm fetuses. After oxytocin-induced preterm labor, thapsigargin instillation inhibited the induced AFC at 61 days gestation (see Fig. 3). Also, at 68 days gestation, thapsigargin instillation inhibited the AFC (see Fig. 5). Thapsigargin instillation significantly inhibited AFC (81 ± 17% inhibition) in newborn guinea pigs (see Fig. 6). When given together, thapsigargin and amiloride attenuated the amiloride sensitivity in all age groups studied both in control and oxytocin-treated animals (see Figs. 2–6).

**DISCUSSION**

Previous studies have shown that the fetal lung fluid volume decreases before delivery (2, 19) and that absorption of fluid starts during or before labor (4, 5, 10, 19). It has been demonstrated that labor releases fetal epinephrine that can stimulate AFC (4, 5, 19). Catecholamines (e.g., epinephrine) stimulate AFC at least partly via cAMP as an intracellular second messenger in fetal, newborn, and adult animals (5, 16, 18). There...
is also evidence that catecholamines may act through alternative or complementary intracellular second-messenger systems; terbutaline has been demonstrated to increase \([\text{Ca}^{2+}]_i\) and to increase \([\text{Ca}^{2+}]-\text{sensitive Na}^{+}\) transport in rat fetal distal lung epithelial (FDLE) cells (13, 28). Because AFC depends on trans-epithelial Na\(^+\) transport (16) and this Na\(^+\) transport can be stimulated by the \(\beta\)-adrenergic system (16), we studied basal and epinephrine-stimulated alveolar fluid transport after inhibition of \([\text{Ca}^{2+}]_i\) flux in developing guinea pig lungs.

AFC was measured in situ using a method whereby the fetal guinea pig lungs were kept expanded with a CPAP. It has previously been demonstrated that although the animals do not have pulmonary circulation and are not ventilated, the calculated AFC does not differ significantly from the results obtained in experiments where the animals have been ventilated (9, 19, 26). Therefore, we believe that the results we have obtained using this method are reliable and are not influenced by the fact that the study is carried out on postmortem lungs.

At 61 days gestation (i.e., 8 days before normal birth), the fetal guinea pig lung displayed a net secretion of fluid, but when 1 day remained to birth (at 68 days gestation), there was a small but significant net fluid absorption. In newborn animals, AFC was stimulated and \(~50\%\) of the instilled solution was absorbed over 1 h (see Fig. 1). These findings were well in accordance with our previous studies (5, 19), which showed that lung fluid production ceases 3–5 days before birth and the lung slowly begins to absorb fetal lung fluid. Our results also confirm our earlier study (19) where we demonstrated that induction of preterm labor by oxytocin stimulated AFC at 61 days gestation (when there normally was net fluid secretion). The earlier study demonstrated that oxytocin stimulated AFC by release of fetal epinephrine, which in turn stimulated AFC through \(\beta\)-adrenergic stimulation (19).

Studies of several animal species have demonstrated that a significant fraction of AFC (5, 16, 19, 23) or Na\(^+\) transport at a cellular level (12, 14, 28) can be inhibited...
by amiloride. We observed in this study that amiloride inhibited net fluid absorption once AFC became stimulated, which is similar to what we have demonstrated before (19). These findings suggest that amiloride-sensitive pathways are major pathways for AFC in preterm guinea pigs. At birth, amiloride inhibits a large fraction of AFC, and within a few days after birth, the amiloride-inhibited fraction of AFC has decreased to 40–50% (5), which is similar to the adult inhibition (18). This developmental pattern of amiloride sensitivity largely conforms to the development suggested for ENaC in rats (20, 32) and guinea pigs (1).

Several in vitro studies (14, 28) have suggested that Ca²⁺ may act as a second messenger for terbutaline (a β-adrenergic agonist)-stimulated Na⁺ transport in the lung, which consequently suggests that stimulated AFC may depend on changes in [Ca²⁺]i. [Ca²⁺]i can be increased by mobilization from two sources: intracellular stores (endoplasmic reticulum) and/or the extracellular spaces (29). In this study, we investigated the role of extracellular Ca²⁺ influx for stimulation of AFC by inhibiting L-type voltage-gated Ca²⁺ channels with verapamil. Generally, this drug is used to inhibit Ca²⁺ channels in cardiac or smooth muscles, but there is strong evidence that this type of channel is also located in the alveolar epithelium, because verapamil and the verapamil analog nifedipine can block phosphatidylincholine secretion from rat alveolar type II pneumocytes (27, 31). In all animals where AFC was stimulated (i.e., in newborn guinea pigs at 68 days gestation and after oxytocin-induced preterm labor), verapamil very effectively inhibited AFC (see Figs. 3–6). We know from our earlier studies that endogenous plasma epinephrine is significantly elevated at these gestational ages (19). These findings thus support the hypothesis that β-adrenergic stimulation of AFC may be associated with changes in [Ca²⁺]i. When we had established that verapamil could inhibit epinephrine-stimulated AFC, and because AFC is at least partially mediated through amiloride-sensitive pathways, we investigated whether the amiloride-sensitive fraction was affected by Ca²⁺ channel inhibition by verapamil. The combined inhibitory effect of amiloride and verapamil never differed

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**Fig. 5.** AFC over 1 h in developing guinea pigs at 68 days gestation after oxytocin-induced preterm labor. AFC was measured with (open bars) or without (solid bars) amiloride (10⁻³ M) in controls or in combination with either verapamil (10⁻⁴ M; A) or thapsigargin (10⁻⁵ M; B) added to the instillate. *P < 0.05 compared with control.

**Fig. 6.** AFC over 1 h in newborn guinea pigs. AFC was measured with (open bars) or without (solid bars) amiloride (10⁻³ M) in controls or in combination with either verapamil (10⁻⁴ M; A) or thapsigargin (10⁻⁵ M; B) added to the instillate. *P < 0.05 compared with control.
from that when any of the drugs were given alone. This indicates that verapamil affects the same fraction of AFC as amiloride; i.e., stimulation of amiloride-sensitive pathways for AFC may be associated with influx of Ca$^{2+}$ through verapamil-sensitive Ca$^{2+}$ channels in the plasma membrane of the alveolar epithelial cells. Marunaka and co-workers (14) reported that a nonspecific cation channel (NSC) can be stimulated by increased [Ca$^{2+}$]. Several reports suggest that ENaC is expressed in the lung epithelium near birth (1, 5, 20, 32), but no reports are present on its dependence or relation to changes in [Ca$^{2+}$] in fetal lungs. However, because the amiloride sensitivity was completely abolished after verapamil administration, it appears as if β-adrenergic stimulation of ENaC depends on Ca$^{2+}$ from extracellular spaces in the in vivo situation. There is also evidence that ENaC in fact might be stimulated at low [Ca$^{2+}$], (25), and as such our result may suggest that the observed fluid clearance in the fetal guinea pig may be mediated by the NSC-type channels, as these results also do not exclude involvement of the NSC channels because these channels probably are amiloride sensitive at the concentrations of amiloride used in here.

Would changes in [Ca$^{2+}$], from intracellular stores yield the same result? Thapsigargin, an inhibitor of Ca$^{2+}$-ATPase (which is responsible for Ca$^{2+}$ reuptake into the endoplasmic reticulum and restoration of a low resting [Ca$^{2+}$]), produced similar results as when verapamil was added to the instillate (see Figs. 3–6). This suggests that stimulated amiloride-sensitive AFC in fetal or newborn guinea pigs depends on changes in [Ca$^{2+}$] independently of its origin. A model for the role of Ca$^{2+}$ for activation of NSC in rat FDLE cells has been suggested (21) where mobilization of Ca$^{2+}$ occurs in two steps. First, terbutaline or dibutylryl cAMP produces a transient [Ca$^{2+}$], elevation caused by Ca$^{2+}$ release from the endoplasmic reticulum. Second, the continuous stimulation is caused by a subsequent influx of Ca$^{2+}$ through membrane Ca$^{2+}$ channels. Consequently, by blocking either of the pathways for altering [Ca$^{2+}$], we may expect a reduced AFC rate.

Thapsigargin per se might be expected to stimulate AFC, because the immediate result of thapsigargin administration would be increased [Ca$^{2+}$], owing to a constant leak of Ca$^{2+}$ from the endoplasmic reticulum (6). However, this was not the case in our studies. This deviation from the expected result can be explained in several possible ways. First, the thapsigargin concentrations used together with the length of the experiment would more likely cause an overall reduction in [Ca$^{2+}$], because virtually all cytosolic Ca$^{2+}$ buffering would be lost; i.e., there would be no Ca$^{2+}$ release from the endoplasmic reticulum. Second, because terbutaline also increases intracellular cAMP concentration in addition to increasing [Ca$^{2+}$], it may be that Ca$^{2+}$ cannot act without a concomitant activation of cAMP. Third, several studies have demonstrated that continuous (long-term) conditions where [Ca$^{2+}$], is mediating a signal to an effector protein occur through [Ca$^{2+}$] oscillations (33). It is possible that we inhibited the Ca$^{2+}$ oscillations in our study. Fourth, thapsigargin may result in a new [Ca$^{2+}$] level, which the cell must readjust to maintain normal cell functions. The ability to adjust to altered [Ca$^{2+}$], depends on cell type, and if a readjustment is not possible, transport systems in the cell that depend on [Ca$^{2+}$] may cease functioning. Accordingly, transepithelial Na$^{+}$ transport would be expected to decrease, and as a consequence, AFC would decrease.

The effect from verapamil may be compromised by the fact that verapamil also can inhibit Ca$^{2+}$-gated K$^{+}$ channels (7). The effect from this may indirectly lead to an inhibited AFC (22), because when K$^{+}$ channels are inhibited, less K$^{+}$ will leave the cell. The normal continuous flux of K$^{+}$ out of the cell is connected to a decreased intracellular Cl$^{-}$ concentration ([Cl$^{-}$]). A decreased [Cl$^{-}$], increases the sensitivity of NSC to Ca$^{2+}$ (28). Consequently, if or when K$^{+}$ channels are inhibited, the [Cl$^{-}$], will remain relatively high or even increase, which may prevent the NSC from responding to the increased Ca$^{2+}$. However, the hypothesis that verapamil acts directly on the L-type Ca$^{2+}$ channels is more likely because it also has been shown to affect surfactant release (31), but a limited effect of verapamil on K$^{+}$ channels cannot be excluded.

In summary, AFC is rapidly increased during the last day of gestation and can be stimulated 8 days before birth after oxytocin-induced preterm labor. Preterm AFC (either control or oxytocin induced) was always amiloride sensitive. Verapamil instillation as well as thapsigargin instillation inhibited AFC when stimulated by preterm or near-term labor induction and in newborn animals. These results, together with the findings that terbutaline increases the open probability of ENaC (15) and amiloride-sensitive NSC channels (14, 28), support the hypothesis that [Ca$^{2+}$], may be involved in stimulation of amiloride-sensitive AFC in preterm animals. Stimulation of AFC depends on Ca$^{2+}$ mobilization from both extracellular compartments (via membrane Ca$^{2+}$ channels) and intracellular compartments (such as the endoplasmic reticulum). The exact mechanism for Ca$^{2+}$ stimulation of AFC was not investigated in this study, but we speculate that intracellular Ca$^{2+}$ may act as a second messenger, probably by increasing the open probability of amiloride-sensitive Na$^{+}$ channels in the alveolar epithelium.

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REFERENCES


