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The Essential Role of Cytosolic Cl^- in Ca^{2+} Regulation of an Amiloride-Sensitive Channel in Fetal Rat Pneumocyte

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Abstract. An amiloride-sensitive, Ca^{2+}-activated nonselective cation (NSC) channel in the apical membrane of fetal rat alveolar epithelium plays an important role in stimulation of Na^+ transport by a beta adrenergic agonist (beta agonist). We studied whether Ca^{2+} has an essential role in the stimulation of the NSC channel by beta agonists. In cell-attached patches formed on the epithelium, terbutaline, a beta agonist, increased the open probability (P_o) of the NSC channel to 0.62 ± 0.07 from 0.03 ± 0.01 (mean ± SE; $n = 8$) 30 min after application of terbutaline in a solution containing 1 mM Ca^{2+}. The P_o of the terbutaline-stimulated NSC channel was diminished in the absence of extracellular Ca^{2+} to 0.26 ± 0.05 ($n = 8$). The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in the presence and absence of extracellular Ca^{2+} was, respectively, 100 ± 6 and 20 ± 2 nM ($n = 7$) 30 min after application of terbutaline. The cytosolic Cl^- concentration ($[Cl^-]_c$) in the presence and absence of extracellular Ca^{2+} was, respectively, 20 ± 1 and 40 ± 2 mM ($n = 7$) 30 min after application of terbutaline. The diminution of $[Ca^{2+}]_c$ from 100 to 20 nM itself had no significant effects on the P_o if the $[Cl^-]_c$ was reduced to 20 mM; the P_o was 0.58 ± 0.10 at 100 nM $[Ca^{2+}]_c$ and 0.55 ± 0.09 at 20 nM $[Ca^{2+}]_c$ ($n = 8$) with 20 mM $[Cl^-]_c$ in inside-out patches. On the other hand, the P_o (0.28 ± 0.10) at 20 nM $[Ca^{2+}]_c$ with 40 mM $[Cl^-]_c$ was significantly lower than that (0.58 ± 0.10; $P < 0.01$; $n = 8$) at 100 nM $[Ca^{2+}]_c$ with 20 mM $[Cl^-]_c$, suggesting that reduction of $[Cl^-]_c$ is an important factor stimulating the NSC channel. These observations indicate that the extracellular Ca^{2+} plays an important role in the stimulatory action of beta agonist on the NSC channel via reduction of $[Cl^-]_c$.

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Key words: Single channel current — Nonselective cation channel — β adrenergic agonist — Terbutaline — Amiloride — Alveolar type II cells

Introduction

The fetal lung epithelium is well recognized to secrete fluid into the lung's lumen throughout gestation [9], and the fetal lung fluid essentially plays an important role in development, differentiation and growth of the fetal lung. However, this fluid must be cleared from alveolar air spaces immediately after birth to allow normal gas exchange. Beta-adrenergic stimulation of the alveolar epithelium enhances fluid clearance at birth [1] by activating amiloride-sensitive Na^+ absorption [31–33] through amiloride-sensitive Na^+-permeable nonselective cation (NSC) channels [9, 14, 25, 37] which might be a member of the epithelial Na^+ channel (ENaC) family that has first been cloned from rat colon [2, 3, 6].

Fetal rat alveolar epithelium has two types of Ca^{2+}-activated, amiloride-blockable Na^+-permeable channels; one is an NSC channel with a single channel conductance of 28 pS, and the other is a Na^+ channel with a single channel conductance of 12 pS [10]. However, beta-adrenergic agonists stimulate only the NSC channel implying that the amiloride-sensitive Na^+ absorption occurs through activation of the NSC channel [11, 13, 23] which plays an essential role in cellular regulation (e.g., [8, 17, 19]). Our previous studies [20, 29, 37] indicate that: (i) cytosolic Ca^{2+} and a beta-adrenergic agonist (beta agonist) increase the open probability (P_o) of the NSC channel, and (ii) a beta agonist biphasically elevates the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$); i.e., a transient followed by a sustained increase. However, the relationship between the stimulation of the NSC channel

by beta agonists and Ca^{2+} is still unknown. The Ca^{2+} plays an important role in ion transport in fetal rat alveolar epithelium [14, 15] similar to other tissues (e.g., [7, 21, 36]). Therefore, the elevation of $[Ca^{2+}]_c$ is considered to be essentially important for the beta-agonist stimulation of the NSC channel that is activated by cytosolic Ca^{2+}. However, we have no direct evidence that the beta-agonist-induced sustained elevation of $[Ca^{2+}]_c$ is essentially required for the stimulatory action on the NSC channel. In the present study, we demonstrate that the beta agonist-induced sustained elevation of $[Ca^{2+}]_c$ is required for induction of a decrease in the cytosolic Cl^- concentration ($[Cl^-]_c$) and the stimulatory action of beta agonists on the NSC channel is mediated through this decrease in $[Cl^-]_c$.

Materials and Methods

Solutions and Chemicals

In the cell-attached configuration the bathing and pipette solutions used in the present study contained (in mM): 140 NaCl, 5KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (pH 7.4). In the excised inside-out configuration the pipette solution contained (in mM): 145 NaCl, 1 $MgCl_2$ and 10 HEPES (pH 7.4) with (Ca^{2+}-containing solution) or without (Ca^{2+}-free solution) 1 $CaCl_2$, and the bathing solution contained (in mM): 145 K^+, 1 $MgCl_2$ and 10 HEPES with various Ca^{2+} and Cl^- concentrations (pH 7.4). To change the Cl^- concentration ($[Cl^-]_c$), Cl^- was replaced with gluconate. The Ca^{2+} (free calcium) concentration was adjusted using known $CaCl_2$ and EGTA (10 mM as pure EGTA) concentrations, calculated using pK_d values of 10.86 for $EGTA^{4-}$ and 5.25 for $HEGTA^{3-}$, and we finally determined the free calcium concentration in the solution using the Ca^{2+}-sensitive fluorescent dye, fura 2 (Empix Imaging Incorporation, Mississauga, Ontario, Canada) with a computer program, Image-1 (Universal Imaging, West Chester, PA) [4]. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Cell Culture

We isolated fetal rat type II pneumocytes from the fetuses of pregnant Wistar rats whose gestational ages were 20 days (term, 22 days) [24, 25]. We completely anesthetized the rat with inhalational ether (overdose) for 15 min. Then we determined that there was no response to tactile stimulus, that respiration had ceased, and that there was no heartbeat. After confirming by these criteria that the rat had been successfully euthanized, we removed the fetuses from the uterus. Under these conditions, the fetuses were also euthanized; namely, no response to stimuli, no breathing, and no heartbeat were observed. We harvested distal lung epithelial cells from the fetuses, which were grown in primary culture according to the methods previously described [10, 15]. In brief, we minced fetal rat excised lungs into 1 mm^3 pieces, incubated the lung fragments at 37°C with 0.125% trypsin and 0.002% DNase. Dissociated cells were then passed through a Nitex 100 mesh filter (B. and S.H. Thompson, Scarborough, Ontario, Canada). We then incubated the cells with 0.1% collagenase and purified the cells using differential adhesion techniques. The majority of these cells are known to have morphologic and biochemical characteristics of type II alveolar epithelial cells [34, 35]. We immediately seeded the harvested epithelial cells (1×10^6 cells/cm^2) onto translucent porous Nunc filter inserts (Nunc Tissue Culture Inserts, Nunc, Roskilde, Denmark). All cells were grown in MEM with 10% fetal bovine serum (Gibco, Grand Island, NY) and penicillin-streptomycin at 37°C in a humidified 95% air/5% CO_2 environment. We subsequently used these epithelia 2 or 3 days after seeding under confluent conditions for experiments.

Application of Terbutaline

We applied 10 μM terbutaline from the basolateral side. In experiments using the cell-attached configuration, we recorded channel activity (the open probability) from the same patch before and 30 min after application of terbutaline while maintaining the patch. In the excised inside-out patch configuration for single channel current recording from the terbutaline-stimulated cell, we applied the following protocol; (i) first we made cell-attached patches, (ii) we then applied 10 μM terbutaline to cells while keeping cell-attached patches, and (iii) 30 min after application of terbutaline, we made inside-out patches.

Single Channel Recordings and Data Analysis

We performed single channel recordings and data analysis using the same methods as we have previously reported [16, 25, 27, 37]. We measured single channel currents at 28–30°C from cell-attached or inside-out patches, digitized current signals at a sampling rate of 5,000 Hz, and analyzed the data with a 2,000-Hz low-pass Gaussian filter. To present the actual traces, we used a 500-Hz low-pass filter with a software Gaussian filter.

Open Probability (P_o) of a Single Channel

Channel activity is expressed as open probability (P_o) as previously reported [10, 12].

$$P_o = \frac{1}{N} \frac{\sum_{i=1}^{N} (i \cdot T_i)}{T_t} \quad (1)$$

where N is the maximum number of the channels being simultaneously open observed in a patch, i is the number of the channels being simultaneously open, T_i is the time of just i channels being simultaneously open and T_t is the total recording time. Also, as previously reported [10], the channels studied in the present report were activated by cytosolic Ca^{2+}. Even if the P_o of an unstimulated patch was small, we could estimate the number of the channels per patch by making an inside-out patch with the cytosolic surface of the channel exposed to a high Ca^{2+} concentration (10 mM) leading to high P_o; and then either a low Ca^{2+} concentration (1 nM) or a high Cl^- concentration (140 mM) to produce a very low P_o. Such treatments allowed us to observe both events with all channels open and all channels closed, so we could accurately determine the actual number of channels in a patch. Further, our estimate of the number of channels per patch membrane could be statistically substantiated at the 95% confidence level using methods we have previously described [12, 18].

The Relationship Between the Open Probability and Cytosolic Ca^{2+} Concentration ($[Ca^{2+}]_c$)

As we previously reported, the NSC channel has one Ca^{2+}-binding site [14].

$$P_o = \frac{P_o^{max}}{1 + \frac{K_{[Ca]_{1/2}}}{[Ca^{2+}]_c}} \quad (2)$$

where P_o^{max} is the maximal value of P_o and $K_{[Ca]_{1/2}}$ is the $[Ca^{2+}]_c$ required for a half of P_o^{max}. Therefore, we obtained the relationship between the open probability (P_o) and $[Ca^{2+}]_c$ by fitting the data to the following equation using a nonlinear least squares fitting algorithm.

Measurement of Cytosolic Ca^{2+} and Cl^- Concentrations

As previously described [37], the cytosolic Ca^{2+} and Cl^- concentrations were measured by a cell imaging system (Empix Imaging, Mississauga, Ontario, Canada) using the Ca^{2+}-sensitive dye, fura 2, and the Cl^--sensitive dye, 6-methoxy-*N*-(3-sulfopropyl)quinolium (SPQ), respectively.

Ethical Approval of Animal Care

The present study complied with the principles and guidelines of the Canadian Council on Animal Care and it had institutional ethical approval (Hospital for Sick Children Research Institute).

Statistical Analyses

Results are presented as mean ± SE. Statistical significance was tested with Student's *t*-test or ANOVA as appropriate. A P value of <0.05 was considered significant.

Results

Single Channel Currents of Nonselective Cation (NSC) Channel Before and After Application of Terbutaline in the Presence and Absence of Extracellular Ca^{2+}

The apical membrane of fetal rat alveolar pneumocytes has an NSC channel with a single channel conductance of 28 pS [14]. Figure 1 shows traces of single channel currents through the NSC channel obtained from cell-attached patches at no applied potential before (Fig. 1*A*; 120 sec-traces in A-a, b and 4 sec-traces in A-a′, b′) and 30 min after (Fig. 1*B*; 120 sec-traces in B-a, b and 4 sec-traces in B-a′, b′) application of 10 μM terbutaline while the patch was maintained in the presence and absence of 1 mM extracellular Ca^{2+}. Under the basal condition, the open probability (P_o) of the NSC channel was very low (Figs. 1*A* and 2). In a Ca^{2+}-free solution, the P_o of the NSC channel under the basal condition was small and identical to that in a Ca^{2+}-containing solution (Ca^{2+} (+)/(−) in Fig. 1*A*). This means that extracellular Ca^{2+} has no significant effects on the P_o of the NSC channel under the basal condition. Terbutaline, a beta agonist, activated the NSC channel, irrespective of the presence of extracellular Ca^{2+} (*see* Figs. 1*A*, *B* and 2). Unlike the channel activity under the basal condition, the P_o of the terbutaline-stimulated channel was diminished by absence of extracellular Ca^{2+} (Figs. 1*B* and 2). The amplitude of the single channel current in the presence of extracellular Ca^{2+} (Ca^{2+} (+) in Fig. 1*B*) was smaller than that in the absence of extracellular Ca^{2+} (Ca^{2+} (−) in Fig. 1*B*). The activity (P_o) of the NSC channel was higher in the presence of extracellular Ca^{2+} than that in the absence of extracellular Ca^{2+} (Fig. 2). This higher activity of the channel would cause more depolarization of the apical membrane in the presence of extracellular Ca^{2+} than that in the absence of extracellular Ca^{2+}. The larger depolarization of the apical membrane would induce a smaller driving force for the inward current through the NSC channel in the presence of extracellular Ca^{2+} than that in the absence of extracellular Ca^{2+}. The smaller driving force would be a reason why the amplitude of the inward current through the NSC channel was smaller in the presence of extracellular Ca^{2+} than that in the absence of extracellular Ca^{2+}. These observations suggest that; (i) extracellular Ca^{2+} has no effects on the P_o of the unstimulated NSC channel, and (ii) absence of extracellular Ca^{2+} diminished the stimulatory action of terbutaline on the NSC channel.

Roles of Extracellular Ca^{2+} in Regulation of the Cytosolic Ca^{2+} Concentration ($[Ca^{2+}]_c$)

The NSC channel is activated by cytosolic Ca^{2+} [14]. Therefore, we studied roles of extracellular Ca^{2+} in regulation of the $[Ca^{2+}]_c$ to investigate whether diminution of terbutaline action by absence of extracellular Ca^{2+} is mediated through a decrease in $[Ca^{2+}]_c$. First, we studied the effect of absence of extracellular Ca^{2+} on the $[Ca^{2+}]_c$. In the presence of extracellular Ca^{2+}, terbutaline elevated a transient increase in $[Ca^{2+}]_c$ followed by a sustained one (Ca^{2+} (+) in Fig. 3*A*). In a Ca^{2+}-free solution, terbutaline only elicited a transient increase in $[Ca^{2+}]_c$ without a sustained one (Ca^{2+} (−) in Fig. 3*A*). Figure 3*B* shows the statistical value of $[Ca^{2+}]_c$ before (Base) and 30 min (Terbutaline) after application of terbutaline in the presence (open bars in Fig. 3*B*) and absence (filled bars in Fig. 3*B*) of extracellular Ca^{2+}. Absence of extracellular Ca^{2+} lowered the sustained level of $[Ca^{2+}]_c$ in terbutaline-stimulated cells ($P < 0.001$; *see* open and filled bars in Terbutaline in Fig. 3*B*).

Roles of Extracellular Ca^{2+} in Regulation of the Cytosolic Cl^- Concentration ($[Cl^-]_c$)

As reported in our previous studies [14, 37], terbutaline decreases $[Cl^-]_c$, and this reduction in $[Cl^-]_c$ is one of the factors activating the NSC channel. Therefore, we stud-

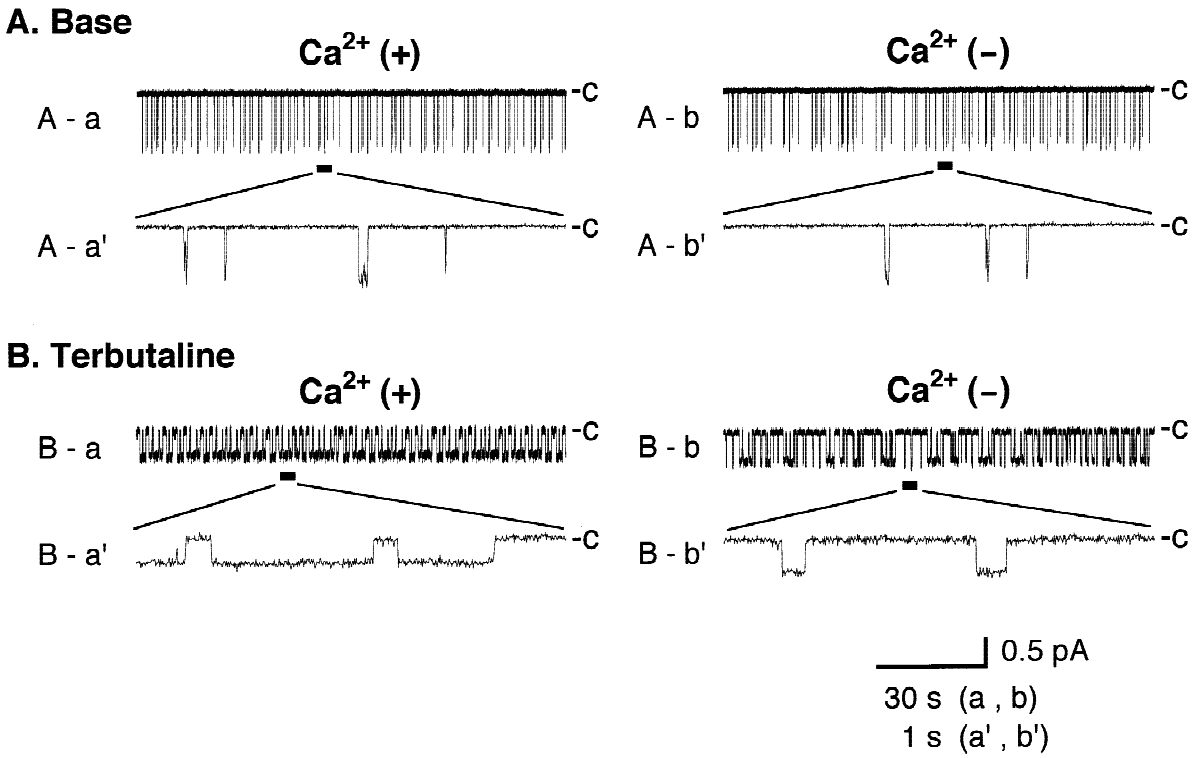

Fig. 1. Actual traces of single channel currents obtained from cell-attached patches formed on the apical membrane of fetal rat lung alveolar epithelium cultured on a permeable support before (*A*, Base) and 30 min after (*B*, Terbutaline) basolateral application of 10 μM terbutaline, a beta-adrenergic agonist, at no applied potential in 1 mM Ca^{2+}-containing [Ca^{2+} (+)] and Ca^{2+}-free [Ca^{2+} (−)] solutions. The current level with no channels open in a patch is marked with a horizontal dash and "C" next to each trace. Downward deflections are inward currents across the patch membrane (current from pipette to cell). The time scale bar indicates 30 sec in the traces of single channel currents in A-a, A-b, B-a and B-b, and a part of each trace marked with a horizontal bar under each trace (A-a, A-b, B-a and B-b) is expanded in time with the time scale bar of 1 sec in A-a′, A-b′, B-a′ and B-b′.

ied roles of extracellular Ca^{2+} in regulation of $[Cl^-]_c$. Figure 4*A* shows a typical time course of terbutaline action on $[Cl^-]_c$ in the presence and absence of extracellular Ca^{2+}. In a Ca^{2+}-containing solution, terbutaline decreased the $[Cl^-]_c$ (open circles in Fig. 4*A*). On the other hand, in a Ca^{2+}-free solution, terbutaline elicited a biphasic decrease in $[Cl^-]_c$; i.e., a transient decrease followed by a sustained one in $[Cl^-]_c$ (closed squares in Fig. 4*A*). Although terbutaline induced a sustained decrease in $[Cl^-]_c$ 30 min after its application in Ca^{2+}-containing and -free solutions, the sustained level in a Ca^{2+}-containing solution was lower than that in a Ca^{2+}-free solution (Fig. 4*A*). The statistical results of the $[Cl^-]_c$ are shown in Fig. 4*B*. The basal $[Cl^-]_c$ was not significantly affected by the absence of extracellular Ca^{2+}, while in terbutaline-stimulated cells the $[Cl^-]_c$ was kept at a higher level in the absence of extracellular Ca^{2+} (Terbutaline in Fig. 4*B*).

Which Change in $[Ca^{2+}]_c$ or $[Cl^-]_c$ Diminishes the Terbutaline Action on P_o?

Absence of extracellular Ca^{2+} diminished the stimulatory action of terbutaline on the NSC channel. As shown above (Fig. 4), extracellular Ca^{2+} affected the $[Ca^{2+}]_c$ and $[Cl^-]_c$ in terbutaline-stimulated cells: (i) the $[Ca^{2+}]_c$ was 100 and 20 nM in the presence and absence of extracellular Ca^{2+}, respectively (*see* Fig. 3*B*); (ii) the $[Cl^-]_c$ was 20 and 40 mM in the presence and absence of extracellular Ca^{2+}, respectively (*see* Fig. 4*B*). Namely, absence of extracellular Ca^{2+} caused the low $[Ca^{2+}]_c$ and high $[Cl^-]_c$ in terbutaline-stimulated cells. Both low $[Ca^{2+}]_c$ and high $[Cl^-]_c$ have the possibility to diminish the P_o of the terbutaline-stimulated NSC channel [14, 37]. Therefore, it was still unclear which change in $[Ca^{2+}]_c$ or $[Cl^-]_c$ diminishes the terbutaline action on P_o. To clarify this point, we measured the P_o of the channel in inside-out patches in the bathing (cytosolic) solutions containing various concentrations of Ca^{2+} and Cl^-. The P_o of the terbutaline-stimulated channel in inside-out patches with 20 or 40 mM $[Cl^-]_c$ is plotted against $[Ca^{2+}]_c$ (Fig. 5*A*). The P_o in 20 mM $[Cl^-]_c$ was larger than that in 40 mM $[Cl^-]_c$ at all tested $[Ca^{2+}]_c$ (Fig. 5*A*). This means that the channel is more sensitive to cytosolic Ca^{2+} in its activity at a lower $[Cl^-]_c$. To study which change in $[Ca^{2+}]_c$ or $[Cl^-]_c$ essentially diminishes the action of terbutaline on P_o, we compared the P_o at 20 nM $[Ca^{2+}]_c$ with that at 100 nM $[Ca^{2+}]_c$ when the $[Cl^-]_c$ was kept 20 mM.

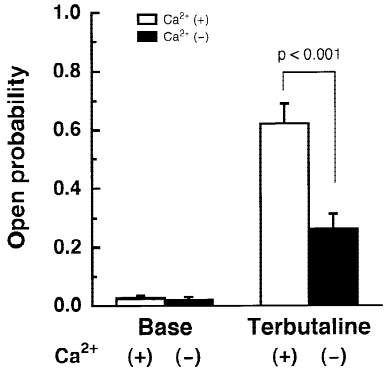

Fig. 2. The open probability (P_o) of the basal and terbutaline-stimulated NSC channels in 1 mM Ca^{2+}-containing [Ca^{2+} (+)] and Ca^{2+}-free [Ca^{2+} (−)] solutions. Under the basal condition, absence of extracellular Ca^{2+} had no significant effects on the P_o. Terbutaline increased the P_o of the NSC channel that was measured 30 min after basolateral application of 10 μM terbutaline. The P_o of the terbutaline-stimulated channel was diminished by absence of extracellular Ca^{2+}. $n = 8$.

Namely, this condition means a case that the absence of extracellular Ca^{2+} caused just a decrease in $[Ca^{2+}]_c$ without any effects on the decrease in $[Cl^-]_c$. Lowering $[Ca^{2+}]_c$ from 100 to 20 nM had no significant effects on the P_o when the $[Cl^-]_c$ was kept at 20 mM (*see* the open bars at 100 and 20 nM $[Ca^{2+}]_c$ in Fig. 5*B*). On the other hand, even if the $[Ca^{2+}]_c$ was kept high (100 nM), the P_o was diminished as the $[Cl^-]_c$ was kept high (i.e., 40 mM; *see* the open and filled bars at 100 nM $[Ca^{2+}]_c$ in Fig. 5*B*). In intact cells, this high level of $[Cl^-]_c$ was observed when the extracellular Ca^{2+} was absent (*see* Fig. 4). Further, the diminution of P_o caused by keeping $[Cl^-]_c$ high was more obviously observed at 20 nM $[Ca^{2+}]_c$ (*see* the open and closed bars at 20 nM $[Ca^{2+}]_c$ in Fig. 5*B*). These observations suggest that the absence of extracellular Ca^{2+} diminishes the NSC channel activity by keeping the $[Cl^-]_c$ at a higher level, but not directly by decreasing the $[Ca^{2+}]_c$ in the terbutaline-stimulated cell.

Discussion

In the present study, we report that the role of extracellular Ca^{2+} in the beta agonist-stimulated NSC channel in fetal rat alveolar cells is to decrease the $[Cl^-]_c$ which has inhibitory action on the NSC channel.

Mechanisms of Elevation of $[Ca^{2+}]_c$ by Terbutaline and the Role of Extracellular Ca^{2+} in Elevation of $[Ca^{2+}]_c$

Terbutaline induced a biphasic increase in the $[Ca^{2+}]_c$; i.e., a transient increase followed by a sustained one (Fig. 3). The sustained one was abolished by the absence of extracellular $[Ca^{2+}]_c$. As we previously reported [29], the sustained one is due to a Ca^{2+} influx from the extracellular space in a cAMP-dependent pathway. On the other hand, the transient one is due to a release of Ca^{2+} from cytosolic store sites in a cAMP-independent pathway [29]. Detail mechanisms of the cAMP-independent pathway are still unknown.

Mechanisms of Changes in $[Cl^-]_c$ by Terbutaline and the Role of Extracellular Ca^{2+} in the Change of $[Cl^-]_c$

Terbutaline induced a decrease in the $[Cl^-]_c$ (Fig. 4). This decrease is due to the terbutaline-induced cell shrinkage as we previously reported [13, 22, 23]; i.e., (i) terbutaline increases the $[Ca^{2+}]_c$; (ii) the elevation of $[Ca^{2+}]_c$ stimulates a K^+ efflux by activating a quinine-sensitive K^+

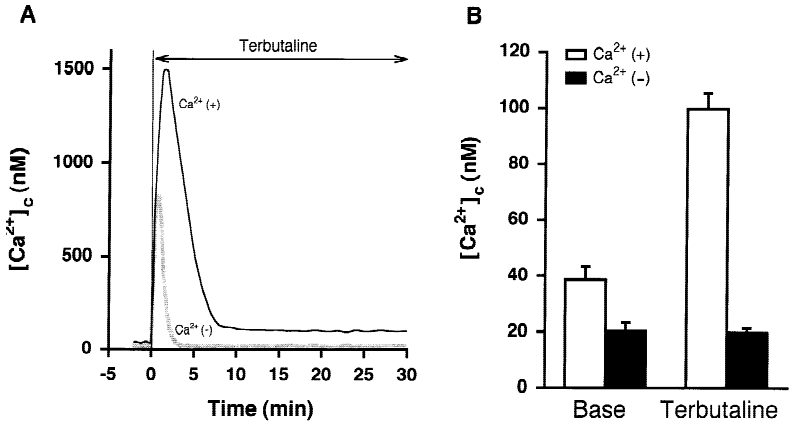

Fig. 3. Effects of terbutaline on $[Ca^{2+}]_c$ and the role of extracellular Ca^{2+} in the action of terbutaline. (*A*) Typical time courses of changes in $[Ca^{2+}]_c$ caused by terbutaline in the presence and absence of extracellular Ca^{2+}. Terbutaline induced a biphasic increase in $[Ca^{2+}]_c$; a transient followed by a sustained increase in the presence of 1 mM extracellular Ca^{2+} [Ca^{2+} (+)]. On the other hand, terbutaline only induced a transient increase in $[Ca^{2+}]_c$ without a sustained one in the absence of extracellular Ca^{2+} [Ca^{2+} (−)]. (*B*) Statistical results of the $[Ca^{2+}]_c$ under the basal and terbutaline-stimulated conditions in the presence and absence of 1 mM extracellular Ca^{2+}. The $[Ca^{2+}]_c$ under the terbutaline-stimulated condition was measured at 30 min after basolateral application of 10 μM terbutaline. Under the basal condition, absence of extracellular Ca^{2+} decreased the $[Ca^{2+}]_c$ (*see* the open and filled bars in Base; $P < 0.005$; $n = 7$). Terbutaline increased the $[Ca^{2+}]_c$ in the presence of extracellular Ca^{2+} (*see* the open bars in Base and Terbutaline; $P < 0.001$; $n = 7$), while in the absence of extracellular Ca^{2+} terbutaline did not affect the $[Ca^{2+}]_c$ (*see* the filled bars in Base and Terbutaline; not significant; $n = 7$).

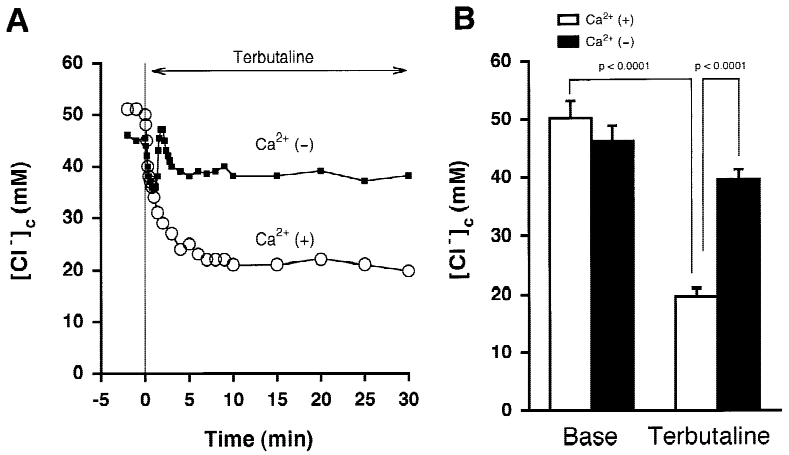

Fig. 4. Effects of terbutaline on $[Cl^-]_c$ and the role of extracellular Ca^{2+} in the action of terbutaline. (*A*) Typical time courses of changes in $[Cl^-]_c$ caused by terbutaline in the presence (open circles) and absence (closed squares) of extracellular Ca^{2+}. Terbutaline decreased the $[Cl^-]_c$ in the presence of 1 mM extracellular Ca^{2+} [Ca^{2+} (+)]. On the other hand, terbutaline induced a transient decrease in $[Cl^-]_c$ with a sustained but smaller one in the absence of extracellular Ca^{2+} [Ca^{2+} (−)] than that in the presence of 1 mM extracellular Ca^{2+} [Ca^{2+} (+)]. (*B*) Statistical results of the $[Cl^-]_c$ under the basal and terbutaline-stimulated conditions in the presence and absence of 1 mM extracellular Ca^{2+}. The $[Cl^-]_c$ under the terbutaline-stimulated condition was measured at 30 min after basolateral application of 10 μM terbutaline. Extracellular Ca^{2+} had no significant effects on the $[Cl^-]_c$ under the basal condition (*see* the open and filled bars in Base; not significant; $n = 7$). Terbutaline decreased the $[Cl^-]_c$ in the presence of extracellular Ca^{2+} (*see* the open bars in Base and Terbutaline; $P < 0.0001$; $n = 7$). However, the magnitude of the terbutaline-induced decrease in $[Cl^-]_c$ was significantly diminished by the absence of extracellular Ca^{2+} (*see* the open and filled bars in Terbutaline; $P < 0.0001$; $n = 7$).

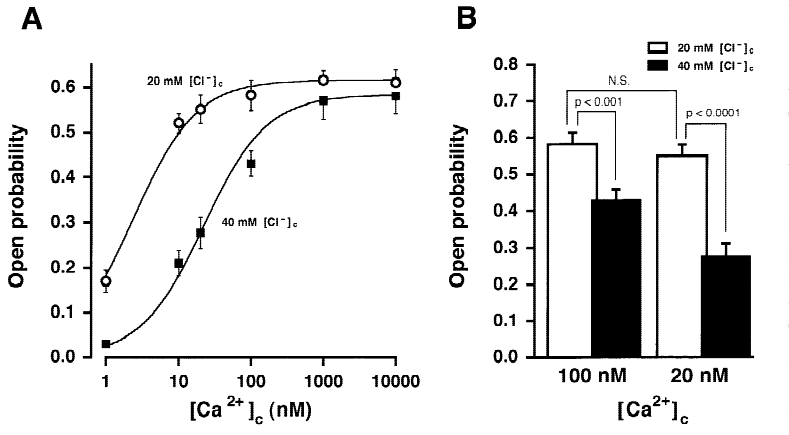

Fig. 5. Ca^{2+}-dependency of open probability (P_o) at 20 and 40 mM $[Cl^-]_c$ in inside-out patches obtained from cell-attached patches that had been maintained with application of 10 μM terbutaline for 30 min. (*A*) The Ca^{2+}-dependency of P_o of the terbutaline-stimulated NSC channel in inside-out patches with bathing solutions containing either 20 mM (open circles) and 40 mM (closed squares) Cl^-. The solid line is obtained by fitting the data to Eq. (2) using a nonlinear least squares fitting algorithm. The relationship between the P_o and $[Ca^{2+}]_c$ was shifted toward left by lowering $[Cl^-]_c$ to 20 from 40 mM. (*B*) The P_o of the terbutaline-stimulated NSC channel in inside-out patches with bathing solutions containing 100 and 20 nM Ca^{2+} with either 20 mM (open bars) and 40 mM (closed bars) Cl^-. When the $[Cl^-]_c$ was reduced to 20 mM, the reduction of $[Ca^{2+}]_c$ had no significant effects on the P_o (*see* the open bars at 100 and 20 nM $[Ca^{2+}]_c$). This observation suggests that the reduction of $[Ca^{2+}]_c$ itself does not diminish the action of terbutaline on the P_o if the terbutaline-induced reduction of $[Cl^-]_c$ is large enough (i.e., $[Cl^-]_c = 20$ mM). On the other hand, when the $[Cl^-]_c$ was kept higher (40 mM), the higher $[Cl^-]_c$ itself diminished the terbutaline action on the P_o (compare the filled bar with the open bar in each case of 100 or 20 nM $[Ca^{2+}]_c$). In other words, if the terbutaline-induced reduction of $[Cl^-]_c$ is not large enough (i.e., $[Cl^-]_c = 40$ mM), the higher level of $[Cl^-]_c$ itself diminishes the terbutaline action on the P_o irrespective of $[Ca^{2+}]_c$ (100 or 20 nM). $n = 8$.

channel; (iii) to keep the electroneutrality, cytosolic Cl^- is released following the K^+ efflux; (iv) the KCl efflux produces an osmotic gradient followed by water loss; (v) the water loss with KCl release (isosmotic movement) causes cell shrinkage; (vi) even though cell shrinkage occurs isosmotically, the $[Cl^-]_c$ decreases due to the presence of fixed (membrane impermeable) anion in the cytosolic space (*see* a review [13]; Fig. 6). However, it is still unknown what kind of Cl^- channel contributes to terbutaline-induced Cl^- release. We only know that the Cl^- release induced by terbutaline is blocked by 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB, a Cl^- channel blocker) [5, 26, 28, 30, 38], suggesting that an NPPB-sensitive Cl^- channel would contribute to the terbutaline-induced Cl^- release.

Relationship Between Action of Cytosolic Ca^{2+} and Cl^- on the NSC Channel Activity

As shown in Fig. 5*A*, the change in $[Cl^-]_c$ seems to affect the sensitivity of the NSC channel to cytosolic Ca^{2+}. The sensitivity has been studied in detail in our previous report [14]. Namely, cytosolic Cl^- does not influence the maximum P_o of the NSC channel in stimulation by cytosolic Ca^{2+}, which appears in $[Ca^{2+}]_c$ exceeding the physiological concentration, but the affinity of the channel to the cytosolic Ca^{2+} within the range of the physiological concentration is increased by lowering $[Cl^-]_c$ [14]. This observation suggests that the inhibitory action of cytosolic Cl^- is apparently competitive with the stimulatory action of cytosolic Ca^{2+} on the NSC channel.

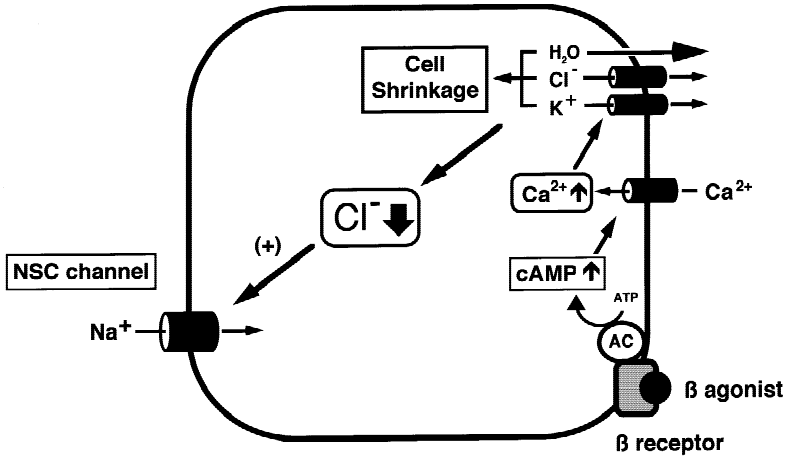

Fig. 6. A scheme of the role of the extracellular Ca^{2+} in the stimulatory action of terbutaline on the NSC channel. AC indicates adenylate cyclase. (+) means activation.

The Action of Reduced $[Cl^-]_c$ on the Apical Cl^- Channels

As shown in Fig. 4, terbutaline induced reduction of $[Cl^-]_c$, which may affect Cl^- efflux through the apical Cl^- channel. Namely, the reduction of $[Cl^-]_c$ decreases the chemical potential for Cl^- efflux (secretion) through the apical Cl^- channel. Further, the depolarization of the apical membrane [14] due to the terbutaline-induced increase in P_o of the NSC channel also decreases the electrical potential for Cl^- efflux (secretion) through the apical Cl^- channel. Therefore, the terbutaline-caused reduction of $[Cl^-]_c$ and depolarization of the apical membrane would be one of the factors accelerating the clearance of lung fluid.

Normal $[Cl^-]_c$ in Fetal Rat Pneumocyte

The $[Cl^-]_c$ was measured to be about 50 mM (Fig. 4). On the other hand, our previous report [14] and preliminary observations (*unpublished data* by Y. Marunaka and N. Niisato) on apical Cl^- channels in fetal rat pneumocyte indicate that the apical membrane potential is about −40 mV and the reversal potential of the apical Cl^- channel is more positive by 10 mV than the apical membrane potential. These observations suggest that the equilibrium potential for Cl^- would be −30 mV. Based upon these data, the $[Cl^-]_c$ is estimated to be 45 mM, which is very closed to the value of $[Cl^-]_c$ measured by a Cl^--sensitive dye in the present study.

Time Course of Terbutaline-Induced Activation of the NSC Channel

The time course of activation of the NSC channel by terbutaline was variable. In some cases the channel activity reached to the maximum within 5–10 min after application of terbutaline, and this time course is very similar to that for the change in $[Cl^-]_c$. In other cases, however, we observed slower activation of the NSC channel by terbutaline than that for the change in $[Cl^-]_c$; even in these cases we observed the full activation of the NSC channel within 20 min after application of terbutaline. Therefore, although we could not conclude that the time courses of the channel activation and the change in $[Cl^-]_c$ are absolutely the same, we may suggest that they are similar within our experimental variation.

Effect of Depletion of Cell Stores of Ca^{2+} on Terbutaline Action

Even when Ca^{2+} store was depleted and extracellular Ca^{2+} was absent, the activation of NSC channels by terbutaline was observed (*unpublished data* by Y. Marunaka and N. Niisato). However, the time course and stimulatory effect were, respectively, much slower and smaller than those under the normal condition. The terbutaline-induced reduction of $[Cl^-]_c$ was also observed under the condition that Ca^{2+} store was depleted and extracellular Ca^{2+} was absent, but the time course and magnitude of the change were, respectively, much slower and smaller than those under the normal condition. cAMP, an intracellular second messenger of terbutaline, would directly activate Cl^- channels (*unpublished data* by T. Nakahari and Y. Marunaka), resulting in reduction of cell volume and $[Cl^-]_c$ [13].

Conclusion

The Extracellular Ca^{2+} Plays an Important Role in the Stimulatory Action of Terbutaline on the NSC Channel by Reducing the $[Cl^-]_c$

The present study showed absence of extracellular Ca^{2+} diminished the stimulatory action of terbutaline on the

NSC channel. The NSC channel in fetal rat alveolar epithelium is activated by the cytosolic Ca^{2+} [10], therefore the absence of extracellular Ca^{2+} would be considered to diminish the stimulatory action of terbutaline on the NSC channel directly via a decrease in the $[Ca^{2+}]_c$. Terbutaline biphasically elevated the $[Ca^{2+}]_c$ in the presence of extracellular Ca^{2+}; a transient followed by a sustained elevation of $[Ca^{2+}]_c$. However, in the absence of extracellular Ca^{2+} a transient but not a sustained elevation of $[Ca^{2+}]_c$ was observed at stimulation of terbutaline. As described above, the elevation of $[Ca^{2+}]_c$ was required for the terbutaline-caused reduction of $[Cl^-]_c$. The reduction of $[Cl^-]_c$ was essentially important for terbutaline action on the NSC channel. The present study shows that a decrease in the $[Ca^{2+}]_c$ did not directly diminish the stimulatory action of terbutaline on the NSC channel, but it acted on the NSC channel by keeping the $[Cl^-]_c$ higher compared with that in the presence of normal extracellular Ca^{2+}. As a conclusion, the present study indicates that: (i) the extracellular Ca^{2+} is required for the terbutaline-induced decrease in $[Cl^-]_c$ via the sustained elevation in $[Ca^{2+}]_c$; (ii) the terbutaline-induced transient elevation in $[Ca^{2+}]_c$ itself is insufficient for the terbutaline-induced decrease in $[Cl^-]_c$; (iii) the decrease in $[Cl^-]_c$ caused by the terbutaline-induced sustained increase in $[Ca^{2+}]_c$ essentially plays in the activation of the NSC channel; (iv) the role of the extracellular Ca^{2+} in the stimulatory action on the NSC channel is to decrease the $[Cl^-]_c$ which has an inhibitory effect on the NSC channel (Fig. 6).

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