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Short communication

Effects of Ca^{2+} channel blockers on amiloride-sensitive Na^+ permeable channels and Na^+ transport in fetal rat alveolar type II epithelium

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Abstract

A β-adrenergic agonist (β-agonist), terbutaline, stimulated amiloride-sensitive Na^+ absorption in fetal rat alveolar type II epithelium, contributing to the clearance of lung fluid. Cytosolic Ca^{2+} plays an important role in terbutaline-stimulated Na^+ absorption, since Ca^{2+}-activated, amiloride-sensitive Na^+-permeable channels are involved in transcellular Na^+ absorption and terbutaline stably elevates the cytosolic Ca^{2+} concentration by stimulating Ca^{2+} influx. Therefore, we studied whether Ca^{2+} channel blockers (Ni^{2+}, verapamil, and nifedipine) affect terbutaline-stimulated transcellular Na^+ absorption. Ni^{2+} partially blocked the channel responsible for the terbutaline-stimulated Na^+ absorption at the Na^+ entry pathway across the apical membrane of the epithelium, but did not diminish the terbutaline-stimulated transcellular Na^+ absorption. By measuring the capacity of the Na^+,K^+-pump activity, we determined that the rate-limiting step of the terbutaline-stimulated transcellular Na^+ absorption was the extrusion step across the basolateral membrane by the Na^+,K^+-pump. The other Ca^{2+} channel blockers, verapamil and nifedipine, had effects identical to those of Ni^{2+}. Based upon these observations, we conclude that, in the β-agonist-stimulated fetal rat alveolar type II epithelium, Ca^{2+} channel blockers diminish amiloride-sensitive channels, but do not affect transcellular Na^+ absorption, since under the β-agonist-stimulated condition the Na^+,K^+-pump is the rate-limitng step in Na^+ transport.

Keywords: Single channel current; Nonselective cation channel; β-Adrenergic agonist; Ni^{2+}; Verapamil; Nifedipine

1. Introduction

The fetal lung epithelium secretes fluid into the lung spaces throughout the fetal period [1]. This fluid stimulates the development, differentiation, and growth of the fetal lungs by generating positive pressure from the luminal space to the interstitium. Although this fluid plays an important role in the development of the lungs, it must be cleared from the alveolar spaces immediately after birth to allow normal gas exchange. β-Adrenergic stimulation of the alveolar epithelium enhances clearance of the lung fluid at birth [2] by activating amiloride-sensitive Na^+ absorption [3–5].

Two types of Ca^{2+}-activated, amiloride-sensitive Na^+-permeable channels have been reported in the apical membrane of fetal rat alveolar type II epithelium; one is a 28 pS NSC channel and the other is a 12 pS Na^+ channel [6,7]. However, the roles of these two types of channels and of Ca^{2+} in Na^+ absorption are still unclear. Therefore, in the present study, we investigated whether Ca^{2+} channel blockers (Ni^{2+}, verapamil, and nifedipine) can influence these Ca^{2+}-activated channels and transcellular Na^+ absorption in fetal rat alveolar type II epithelium under basal and β-adrenergic (β-agonist)-stimulated conditions.

2. Materials and methods

2.1. Solutions and chemicals

The vehicle solution used in the present study contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). All chemicals were purchased from the Sigma Chemical Co.

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Abbreviations: I_{sc}, short circuit current; I–V, current–voltage relation; NSC, nonselective cation; P_o, open probability; Vp, applied pipette potential.

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2.2. Cell culture

Rat alveolar type II epithelial cells were isolated from 20-day-old fetuses of Wistar rats (term, 22 days) [8,9]. These cells were seeded at a density of 1×10^6 cells/cm^2. Two or three days after seeding, the cultures were confluent and used for experiments.

2.3. Application of a β-agonist, terbutaline

Terbutaline (10 μM) was prepared in vehicle solution and applied to the basolateral side of the cell membrane. Single channel currents, the P_o of the channel, and the I_{sc} were recorded 30 min after the application of terbutaline.

2.4. Application of Ca^{2+} channel blockers

Apical and basolateral membrane blocking solutions were prepared by adding $NiCl_2$, verapamil, or nifedipine to the vehicle solution at a final concentration of 100 μM. Each blocker was added to the vehicle solution 10 min before the application of one of the β-agonists.

2.5. Single channel recordings and data analysis

Single channel recordings at 37° and data analysis were performed using the same methods mentioned in our previous reports [8,10–13]. Single channel currents were recorded from cell-attached patches, and digitized current signals were sampled at a rate of 5000 Hz. The data were analyzed with a 2000-Hz low-pass Gaussian filter. To present the actual traces, a 500-Hz low-pass filter was used with a software Gaussian filter.

2.6. P_o of a single channel

Channel activity is expressed as P_o, as shown below.

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

where N is the maximum number of simultaneously open channels observed in a patch, i is the number of the channels simultaneously open, T_i is the time when just i channels are simultaneously open, and T_t is the total recording time. As previously reported [6], the channels studied in the present report were activated by cytosolic Ca^{2+}. Even though the P_o of an unstimulated patch was small, we could estimate the number of channels per patch by forming an inside-out patch with the cytosolic surface of the channel exposed to a high Ca^{2+} concentration (10 mM) leading to a high P_o; a low Ca^{2+} concentration (1 nM) with a high Cl^- concentration (140 mM) produced a very low P_o. Such treatments allowed us to observe both events with all channels open and all channels closed. Therefore, we could accurately determine the actual number of channels in a patch. Moreover, our estimate of the number of channels per patch of membrane could be statistically substantiated at the 95% confidence level using methods previously described [14,15].

2.7. Measurement of I_{sc}

I_{sc} was measured at 37° as previously reported [16]. Amiloride (10 μM)-sensitive I_{sc} was used as a measure of transcellular macroscopic Na^+ absorption, and ouabain (1 mM)-sensitive I_{sc} as a measure of the capacity of the Na^+,K^+-pump-generated I_{sc} following permeabilization of the apical membrane to Na^+ by 50 μM nystatin [16].

2.8. 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 (The Hospital for Sick Children Research Institute).

2.9. Statistical analyses

Results are presented as means ± SD. Statistical significance was measured by Student's *t*-test or ANOVA, as appropriate. A P value of <0.05 was considered significant.

3. Results and discussion

Fig. 1 shows single channel currents (Fig. 1, A-a, b and B-a, b) and I–V relations (Fig. 1, A-c and B-c) of the Na^+ and NSC channels obtained from cell-attached patches under basal and terbutaline-stimulated conditions. The Na^+ channel had an inward-rectifying I–V relation, with no outward currents being observed at the Vp between −60 and 60 mV (Fig. 1A-c). On the other hand, the NSC channel had a linear I–V relation, with some outward currents being observed at −Vp of 40 and 60 mV (Fig. 1B-c). Based upon these characteristics of the Na^+ and NSC channels, we were able to differentiate between Na^+ and NSC channels in a cell-attached patch from the I–V relationship.

Fig. 2A shows the effect of a β-agonist, terbutaline (10 μM), on the P_o of the Na^+ and NSC channels. Under basal conditions, the P_o of the Na^+ channel was much larger than that of the NSC channel, suggesting that the Na^+ channel is the main contributor to basal Na^+ absorption in the cell. Terbutaline activated the NSC channel, but not the Na^+ channel, and under the terbutaline-stimulated condition the P_o of the NSC channel was larger than that of the Na^+ channel (Fig. 2A). Further, terbutaline increased the number of the NSC channels by about 5-fold, but did not affect the number of the Na^+ channels. The NSC channel had an almost identical permeability to Na^+ and K^+ (i.e. $P_{Na}/P_K = 1.1$) as reported previously [6]; therefore, the equilibrium potential of the permeable ions

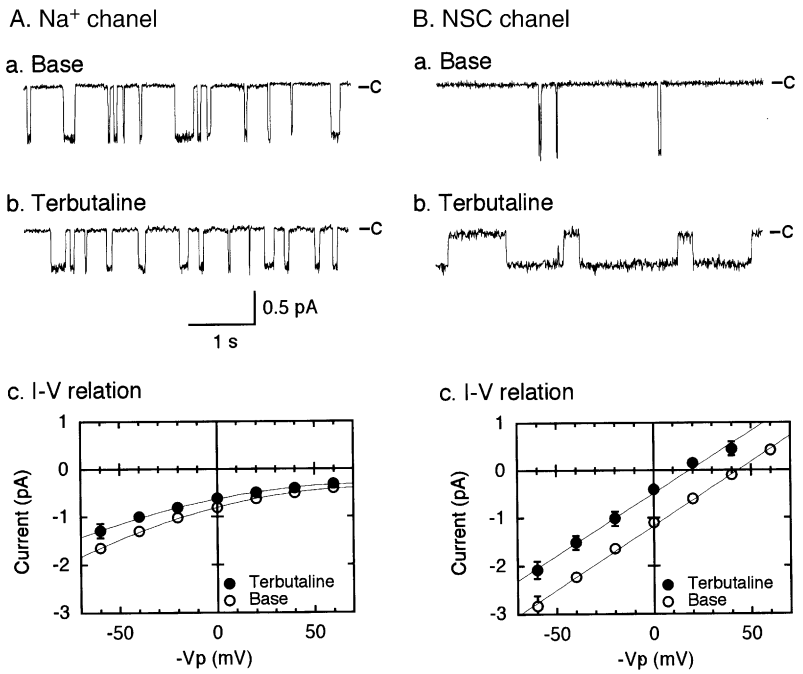

Fig. 1. Effect of terbutaline on the Na^+ channel (A) and the NSC channel (B). The single channel currents and I–V relations shown were recorded from cell-attached patches. The single channel currents were recorded without an applied pipette potential. The closed level of the channel current is indicated by the horizontal line at 0 in "c." The downward direction of the single channel current trace shows an inward current across the patch membrane from the pipette into the cytosolic space. Vp is the magnitude of the applied pipette potential; therefore, −Vp is displacement of the membrane potential from the resting membrane potential, i.e. −Vp of 60 mV means that the membrane potential of the patch membrane is 60 mV more positive than the resting membrane potential. (A) The Na^+ channel was not responsive to terbutaline application. The I–V relation showed an inward rectification irrespective of terbutaline application. No outward currents were observed within the range of the applied pipette potentials. (B) The NSC channel increased its P_o as it responded to terbutaline application. The I–V relation was linear irrespective of terbutaline application. Values in Fig. 1A–c and B-c are means ± SD, N = 9.

in this NSC channel would be 2.4 mV. From this information and the reversal potential of the channel observed in the cell-attached patches (Fig. 1B-c), the apical membrane potentials were estimated to be about −40 and −14 mV under basal and terbutaline-stimulated conditions, respectively. The values for the apical membrane potential and the inward current in the absence of an applied pipette potential, as shown in Fig. 1B-a and -b, indicate that the dominant charge carrier through the NSC channel is Na^+, and not K^+. Terbutaline also increased the amiloride-sensitive I_{sc} (Fig. 2B). These observations suggest that terbutaline increases transcellular Na^+ absorption by activating the NSC channel, but not the Na^+ channel.

Under basal conditions, Ni^{2+} (100 μM; [17]), a Ca^{2+} channel blocker, diminished the P_o of the Na^+ channel; however, the NSC channel was not affected by Ni^{2+}

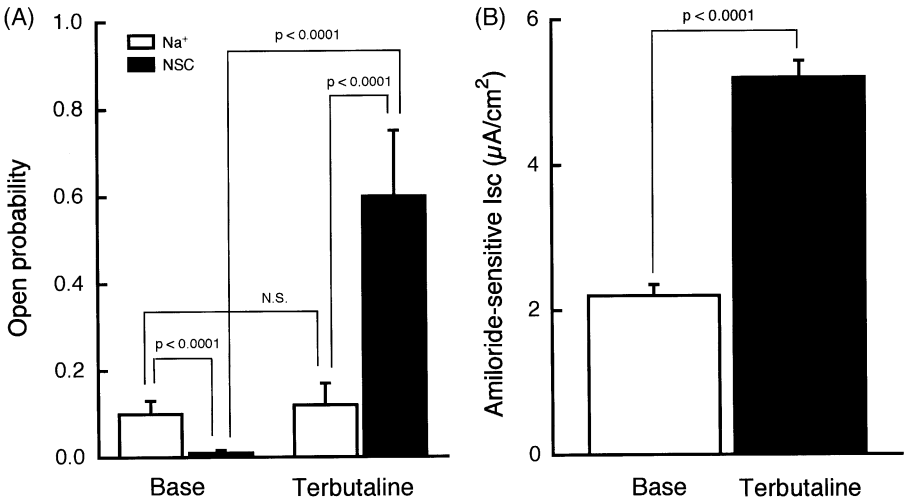

Fig. 2. Effect of terbutaline on the P_o of the Na^+ and NSC channels (A) and the amiloride-sensitive I_{sc} (B). Values are means ± SD, N = 9. N.S. = not significant.

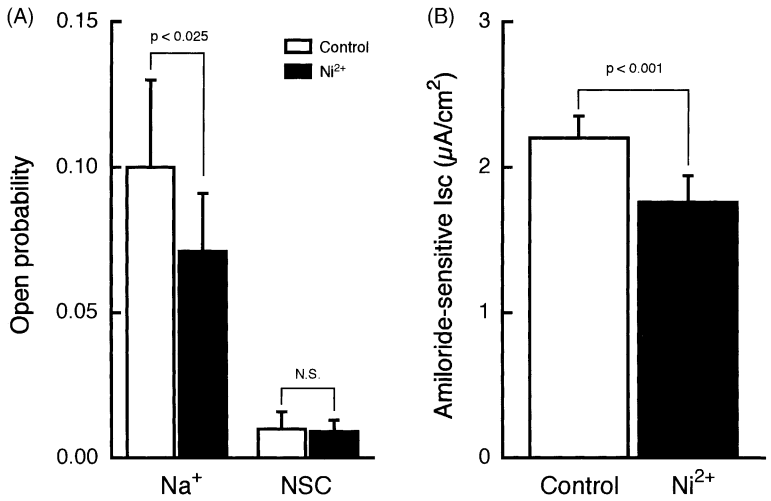

Fig. 3. Effect of Ni^{2+} on the P_o of the Na^+ and NSC channels (A) and the amiloride-sensitive I_{sc} (B) under basal conditions. Values are means ± SD, N = 9. N.S. = not significant.

(Fig. 3A). The amiloride-sensitive I_{sc} was also decreased by Ni^{2+} compared to the control (Fig. 3B). These observations suggest that Ni^{2+} diminishes basal transcellular Na^+ absorption by partially inhibiting the Na^+ channel.

Next, we studied the effect of Ni^{2+} on the P_o of the channels and the amiloride-sensitive I_{sc} under terbutaline-stimulated conditions. Ni^{2+} decreased the P_o of both the Na^+ and NSC channels (Fig. 4A). The inhibitory action of Ni^{2+} on the Na^+ channel was identical under basal and terbutaline-stimulated conditions (compare Figs. 3A and 4A). On the other hand, Ni^{2+} inhibited the terbutaline-stimulated NSC channel (Fig. 4A), but not the NSC channel under basal conditions (Fig. 3A). These observations indicate that the inhibitory action of Ni^{2+} on the channels under terbutaline-stimulated conditions was greater than under basal conditions. Therefore, we anticipated that the inhibitory action of Ni^{2+} on the amiloride-sensitive I_{sc} would be larger under terbutaline-stimulated conditions than under basal conditions. Contrary to our expectation, however, the amiloride-sensitive I_{sc} was not affected by Ni^{2+} under terbutaline-stimulated conditions (Fig. 4B).

To clarify this unexpected result, we examined transcellular Na^+ absorption. Transcellular Na^+ absorption is mediated through two steps: the Na^+ entry step and the Na^+ extrusion step. The rate-limiting step of transcellular Na^+ absorption is generally considered to be the Na^+ entry across the apical membrane (i.e. channel). If so, the Ni^{2+}-induced diminution of the P_o of the Na^+ and NSC channels should cause a decrease in the amiloride-sensitive I_{sc} under terbutaline-stimulated conditions. However, we observed no inhibitory action of Ni^{2+} on the terbutaline-stimulated I_{sc}. Therefore, we considered another possibility, i.e. that under terbutaline stimulation the rate-limiting step of transcellular Na^+ absorption is not Na^+ entry, but Na^+ extrusion. Since the Na^+ extrusion step

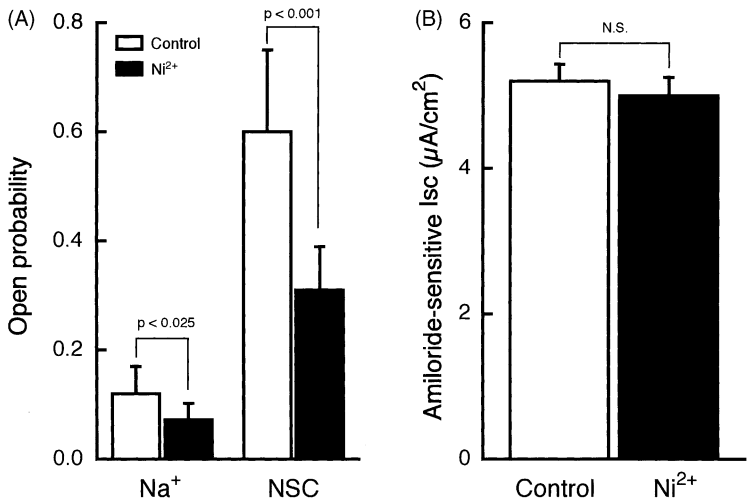

Fig. 4. Effect of Ni^{2+} on the P_o of the Na^+ and NSC channels (A) and the amiloride-sensitive I_{sc} (B) under terbutaline-stimulated conditions. Values are means ± SD, N = 9. N.S. = not significant.

is mediated through the Na^+,K^+-pump, we examined Na^+,K^+-pump activity.

To measure the capacity of the Na^+,K^+-pump activity, we applied nystatin to the apical solution to increase the cytosolic Na^+ concentration. To confirm whether the apical application of nystatin was sufficient to measure the Na^+,K^+-pump current, we also applied amiloride (10 μM) to the apical membrane of the cells (in conjunction with the application of nystatin) in the presence and absence of terbutaline. Even if the apical application of nystatin was not sufficient in making the membrane more porous for Na^+ entry and in elevating the concentration of cytosolic Na^+, amiloride should still diminish the I_{sc}. However, amiloride (10 μM) had no effects on the I_{sc} obtained from nystatin-treated cells (data not shown), indicating that the apical application of nystatin increased the porosity of the apical membrane for Na^+ entry. We also measured transepithelial conductance. The transepithelial conductance of the cells treated with terbutaline was increased significantly from 435 ± 150 to 769 ± 243 μS/cm^2 by the apical application of nystatin (the nystatin-induced conductance $= 335 \pm 143$ μS/cm^2; $81 \pm 41\%$ increase; $N = 9$, $P < 0.001$), suggesting that nystatin has some effect on apical membrane conductance and that the cytosolic Na^+ concentration increases at least to some extent. The nystatin-induced conductance (335 ± 143 μS/cm^2, $N = 9$) was much larger than the amiloride-sensitive conductance in the terbutaline-treated cells (95 ± 51 μS/cm^2, $N = 9$; $P < 0.001$), indicating that nystatin significantly increases Na^+ entry into the cytosolic space across the apical membrane. These observations suggest that: (a) Na^+-permeable pores at the apical membrane are generated by nystatin, and (b) cytosolic Na^+ concentration increases to some extent after the apical application of nystatin. Furthermore, most of the I_{sc} was diminished by K^+ channel blockers, such as Ba^{2+} (2 mM) and quinine (0.1 mM), in all cases with/without apical application of nystatin under both basal and terbutaline-stimulated conditions. This observation suggests that although the NSC channel and nystatin provide pathways for the release of K^+ across the apical membrane to the extracellular space, the major K^+-releasing pathway is located at the basolateral membrane. This pathway serves as a recycling route for K^+, which is taken up into the intracellular space by the Na^+,K^+-pump. These observations indicate that the majority of the I_{sc} would be due to the transcellular movement of Na^+ via two steps: (a) the entry step through the apical NSC channel or nystatin-generated pores at the apical membrane, and (b) the extrusion step by the Na^+,K^+-pump across the basolateral membrane.

Under steady-state conditions, the amount of Na^+ entry through the apical channel is, in general, equal to the amiloride-sensitive I_{sc}. However, the time period (30 min) after the application of terbutaline would not be long enough for the cell to reach its steady state. Namely, under the terbutaline-stimulated conditions of the present study, the amount of Na^+ entry through the apical channel would be much larger than the capacity of Na^+ extrusion by the Na^+,K^+-ATPase, since nystatin did not increase the I_{sc} (i.e. the ouabain-sensitive I_{sc} of nystatin-treated cells was not larger than the amiloride-sensitive I_{sc}; see Fig. 5). Further, when Ni^{2+} decreased the P_o of the NSC channel in cells treated with terbutaline (Fig. 4A), the reversal potential of the NSC channel in cell-attached patches with terbutaline treatment moved from −14 mV to −27 mV. This indicates that the driving force for Na^+ entry was increased by Ni^{2+}. These observations suggest that the Ni^{2+}-induced decrease in the amount of Na^+ entry across the apical membrane would be smaller than that expected from the Ni^{2+}-induced decrease in P_o of the NSC channel. Nevertheless, the amount of Na^+ entry across the apical membrane would be decreased by Ni^{2+}.

As shown in Fig. 5, under basal conditions, the amplitude of the ouabain-sensitive I_{sc} in the nystatin-treated cells was larger than the amiloride-sensitive I_{sc} in cells without nystatin treatment, suggesting that the rate-limiting step is the Na^+ entry step mediated through the amiloride-sensitive apical Na^+-permeable channel. On the other hand, under the terbutaline-stimulated conditions, the amplitude of the ouabain-sensitive I_{sc} in the cells treated with nystatin, which increases Na^+ entry across the apical membrane, did not exceed the amiloride-sensitive I_{sc} in the cells without nystatin treatment (Fig. 5), suggesting that the rate-limiting step is the Na^+ extrusion step mediated through the Na^+,K^+-pump. Although Ni^{2+} decreased Na^+ entry across the apical membrane, Ni^{2+} had no effects on the ouabain-sensitive I_{sc} in the nystatin-treated cells (data not

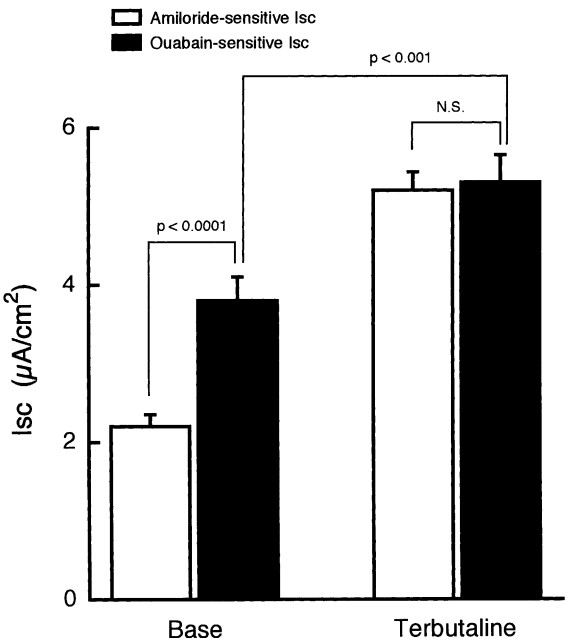

Fig. 5. Relation of the amiloride-sensitive I_{sc} in the untreated cells (open columns) and the ouabain-sensitive I_{sc} in the nystatin-treated cells (closed columns) under basal and terbutaline-stimulated conditions. Values are means ± SD, $N = 9$. N.S. = not significant.

shown). Taken together, these observations suggest that even if the amount of Na^+ entry through the apical channel is decreased by a Ca^{2+} channel blocker (Ni^{2+}), the amount of Na^+ entry through the apical channel would not yet be less than the capacity of the Na^+ extrusion by Na^+,K^+-ATPase, since the Na^+ extrusion step by Na^+,K^+-ATPase is the rate-limiting step of transepithelial Na^+ transport.

We also studied the effects of two other Ca^{2+} channel blockers, verapamil (100 μM) and nifedipine (100 μM) [18], on the P_o of the channels and the I_{sc}. We found that the effect of verapamil and of nifedipine was identical to that of Ni^{2+} (data not shown).

We applied the Ca^{2+} channel blockers Ni^{2+}, verapamil, and nifedipine to the cells. If these compounds act on the I_{sc} by affecting the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), it should be mediated through a decrease in Ca^{2+} influx. As we reported previously [19], terbutaline elevates $[Ca^{2+}]_c$ biphasically, i.e. a transient elevation followed by a sustained one. Most of the transient elevation (around 5 min after terbutaline application) is caused by Ca^{2+} release from intracellular Ca^{2+} store sites, and the sustained elevation is due to Ca^{2+} influx from the extracellular space [19]. When we recorded the P_o and I_{sc} after terbutaline application in the present study (i.e. 30 min after terbutaline application), the sustained elevation of $[Ca^{2+}]_c$ was maintained by Ca^{2+} influx from the extracellular space. Therefore, the Ca^{2+} channel blockers used in the present study would diminish $[Ca^{2+}]_c$ when recording the P_o and I_{sc} in the terbutaline-stimulated cells. We also tried to measure the I_{sc} in a Ca^{2+}-free solution. Unfortunately we were unable to do so because the monolayer (tight junction) of the cells became very leaky due to Ca^{2+} removal.

Based upon these observations, we conclude that under terbutaline-stimulated conditions, the rate-limiting step of transcellular Na^+ absorption is the Na^+ extrusion step driven by the Na^+,K^+-pump. The inhibitory effect of the Ca^{2+} channel blocker on Na^+ entry through the channels is not large enough to diminish the amount of the Na^+ entry to a level below the capacity of the Na^+,K^+-pump activity.

Acknowledgments

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