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Regulation of Cell Volume by β_2-adrenergic Stimulation in Rat Fetal Distal Lung Epithelial Cells

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Abstract. Cell-volume changes induced by terbutaline (a specific β_2-agonist) were studied morphometrically in rat fetal distal lung epithelium (FDLE) cells. Cell-volume changes qualitatively differed with the concentration of terbutaline. Terbutaline of 10^{-10}–10^{-8} M induced transient cell swelling. Terbutaline of 10^{-7} M induced transient cell swelling followed by slow cell shrinkage. Terbutaline of 10^{-6}–10^{-5} M induced rapid cell shrinkage followed by slow cell shrinkage. Terbutaline of 10^{-3} M induced transient cell shrinkage; then cell volume oscillated during stimulation. Benzamil of 10^{-6} M suppressed the cell swelling induced by 10^{-10}–10^{-8} M terbutaline and quinine of 10^{-3} M inhibited the cell shrinkage induced by 10^{-6}–10^{-5} M terbutaline. These results suggest that cell swelling would be induced by NaCl influx and the cell shrinkage is by KCl efflux. Dibutyryl cyclic AMP (DBcAMP) also induced similar cell-volume changes over a wide range of concentrations (10^{-9}–10^{-3} M): a low concentration induced transient cell swelling; a high concentration, rapid and slow cell shrinkage. Forskolin (10^{-4} M), like terbutaline (10^{-5} M), induced rapid cell shrinkage followed by slow cell shrinkage, and this decrease in the cell volume was enhanced by the presence of benzamil. On the other hand, cell shrinkage was induced by ionomycin (even low concentration; 3×10^{-10} M ionomycin), and after that cell volume remained at a plateau level. Removal of extracellular Ca^{2+} abolished the cell swelling caused by terbutaline of 10^{-10}–10^{-8} M. With removal of extracellular Ca^{2+}, the initial, rapid cell shrinkage induced by 10^{-5} M terbutaline became transient, but we still detected slow cell shrinkage similar to that in the presence of extracellular Ca^{2+}. Overall, at low concentrations (10^{-10}–10^{-8} M), terbutaline induced benzamil-sensitive cell swelling in FDLE cells, which was cAMP- and Ca^{2+}-dependent; high concentrations ($\geqslant 10^{-6}$) induced quinine-sensitive rapid cell shrinkage, which was Ca^{2+}-dependent; high concentrations ($\geqslant 10^{-7}$) induced slow cell shrinkage, which was cAMP-dependent. These findings suggest that terbutaline regulates cell volume in FDLE cells by cytosolic cAMP and Ca^{2+} through activation of Na^+ and K^+ channels.

Key words: Amiloride — Quinine — β-agonist — cAMP — Intracellular Ca^{2+} — Fluid absorption

Introduction

Fluid in the lumen of the fetal lung is absorbed immediately after birth, and the luminal space becomes filled with air for gas exchange. Lung fluid absorption begins during labor and the effects of labor can be mimicked by β-adrenergic stimulation (Enhorning et al., 1977; Olver, Schneeberger & Walters, 1981; Brown et al., 1983; Walters, Ramsden & Olver, 1990; O'Brodovich, 1991), whose effect can be blocked by amiloride (Olver et al., 1986). The effects of the β-agonists are mediated, at least in part, by cAMP (Barker et al., 1988; Walters et al., 1990) through β_2-adrenoreceptor stimulation. However, terbutaline has also been reported to increase the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) (Tohda et al., 1994). Fetal distal lung epithelium (FDLE) cells grown in primary culture have been prepared from rat (Post, Torday & Smith, 1984; Post & Smith, 1988) and used for Ussing chamber and patch clamp experiments to study lung epithelial Na^+ transport (O'Brodovich, Rafii & Post, 1990; Orser et al., 1991; Marunaka et al., 1992; Tohda et al., 1994; Marunaka & Tohda, 1995; Nakahari & Marunaka,

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1995*b*; Marunaka 1996). Studies of short-circuit current (O'Brodovich et al., 1990; Marunaka, Nakahari & Tohda, 1994) have demonstrated that the amiloride-blockable transepithelial currents are increased by terbutaline. Patch clamp studies (Orser et al., 1991; Marunaka et al., 1992; Tohda et al., 1994; Marunaka & Tohda, 1995; Nakahari & Marunaka, 1995*b*; Marunaka, 1996) using FDLE cells have revealed that amiloride-blockable Na^+ permeant channels (nonselective cation and Na^+ channels) which are present in the apical membrane are activated by cytosolic Ca^{2+}. These findings suggest that a rise in $[Ca^{2+}]_c$ may increase Na^+ entry into FDLE cells and thereby cause an *increase* in cell volume. However, other data suggest that agents which activate Na^+ permeant channels in FDLE cells may cause a *decrease* in cell volume. Terbutaline, which activates Na^+ permeant nonselective cation channels (Marunaka et al., 1994; Tohda et al., 1994), induces cell shrinkage and decreases the cytosolic Cl^- concentration ($[Cl^-]_c$) (Marunaka et al., 1994; Tohda et al., 1994; Marunaka, Doi & Nakahari, 1995). Indeed, the cell shrinkage induced by terbutaline has been proposed as a mechanism for the decrease in $[Cl^-]_c$ (Marunaka et al., 1994; Tohda et al., 1994). A recent study (MacLeod et al., 1994) has also demonstrated that 8-Br-cAMP and A23187 can decrease FDLE cell size. Cell shrinkage in secretory epithelia such as salivary gland and eccrine-secretory coil cells (Nauntofte & Poulsen, 1986; He et al., 1989; Foskett & Melvin, 1989; Nakahari, Murakami & Kataoka, 1989; Foskett, 1990; Nakahari et al., 1990; Nakahari et al., 1991; Steward et al., 1990; Suzuki et al., 1991; Takemura et al., 1991; Larcombe-McDouall, Seo & Steward, 1994) has already been reported to be evoked by the cholinergic or adrenergic stimulation and to be closely related to $[Ca^{2+}]_c$ or cytosolic cAMP concentration ($[cAMP]_c$) (He et al., 1989; Foskett & Melvin, 1989; Nakahari et al., 1991; Suzuki et al., 1991).

The recent development of video optics such as a video-enhanced optical microscopy and a laser confocal scanning microscopy has enabled us to directly visualize the changes in a living cell. In this report, video-enhanced optical microscopy was used to measure cell volume. FDLE cells changed in their volume with the stimulation of terbutaline over a wide range of concentrations, however, the qualitative aspect of volume changes were different for low and high concentrations of terbutaline. The present study evaluated these terbutaline-induced cell-volume changes and regulation by $[Ca^{2+}]_c$ and $[cAMP]_c$.

Materials and Methods

FDLE cells were isolated from the fetuses of pregnant Wistar rats whose gestational ages were 20 days (term, 22 days). FDLE cells were prepared according to the methods described in elsewhere (O'Brodovich, Hannam & Rafii, 1991). Briefly, the rats were anesthetized with inhalational ether, and the fetuses were removed from the uterus. Their lungs were removed immediately, washed in cold Hanks' solution (Mg^{2+}- and Ca^{2+}-free) to remove erythrocytes, and minced with scissors. The lung pieces were digested with trypsin (0.125%) and DNase (0.002%) for 20 min at 37°C. A cell suspension was filtered through a Nitex 100 filter. The cells were then incubated with collagenase (0.1%) and purified by a differential adhesion technique (Battenburg et al., 1988). The purity of FDLE cells as fetal alveolar type II cells has been previously established (Post, Torday & Smith, 1984; Post & Smith, 1988). The culture medium was MEM (GIBCO, Grand Island, NY) to which 10% fetal bovine serum (GIBCO) was added. For the cell-volume measurement, the cells were cultured at 37°C in a 95% air/5% CO_2 humidified incubator for 24–48 hr on a coverslip.

The solution used as control contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 glucose, and 10 HEPES, and had a pH 7.4. No $CaCl_2$ was added to the solution for Ca^{2+}-free experiments. DBcAMP and forskolin were purchased from Sigma Chemical (St. Louis, MO), thapsigargin from Calbiochem (La Jolla, CA) and ionomycin from Boehringer Mannheim (Mannheim, Germany); terbutaline sulfate was a gift from Astra Pharma (Canada), and benzamil from Merck Sharp & Dome Research laboratory (West point, PA). Benzamil, ionomycin, thapsigargin and forskolin were dissolved with dimethyl-sulfoxide (DMSO). The final concentration of DMSO in the medium never exceeded 0.1%. DMSO $\leqslant$ 0.1% had no effect on cell volume.

The coverslip was mounted on a thermal stage (37°C), which was set on the mechanical stage of an inverted microscope (Zeiss, Germany) connected to the video-imaging system. The perfusate was bubbled with 100% O_2; the perfusion rate was about 0.5 ml/min. The cavity of perfusion chamber was 0.5 ml. The cells were perfused with control solution for 10 min before the experiments. The images of the optical microscope were recorded continuously with a video-imaging system and a video-recorder. The images taken at every 10 or 20 sec were shown for volume measurements. During experiments we adjusted the focus frequently to obtain the maximal diameter of the cells.

To estimate the cell volume, the area of FDLE cells in the video image was measured. The averaged value of areas measured in the first 2 min was used as the control (A_O). The relative cell volume, $V/V_O = (A/A_O)^{1.5}$ was estimated, where V is the volume, A is the area, and subscript O is the value of the control. Cell-volume estimation was based on the assumption that cell-volume changes the same amount in all three dimensions. The method of cell-volume estimation and its reliability have been described in detail previously (Foskett & Melvin, 1989; Nakahari, Murakami & Kataoka, 1989; Nakahari et al., 1990; Nakahari et al., 1991; Suzuki et al., 1991; Takemura et al., 1991). Only spherical FDLE cells were selected for the volume measurements. When agonists altered cellular shape (for example causing budding, burst or intracellular bleb formations), these cells were discarded. We used only those cells that kept their spherical shape during the experiments for the volume measurements.

The values of V/V_O from 3–5 cells in 2–5 individual experiments were expressed as means ± SE.

Results

Cell-volume Changes Induced by Terbutaline

Figure 1 shows the cell-volume changes induced by terbutaline. Terbutaline of 10^{-10} M induced transient cell swelling. Cell volume reached a peak value (V/V_O = 1.2) about 2 min and then recovered to the control level at 4 min after infusion of terbutaline. After this transient

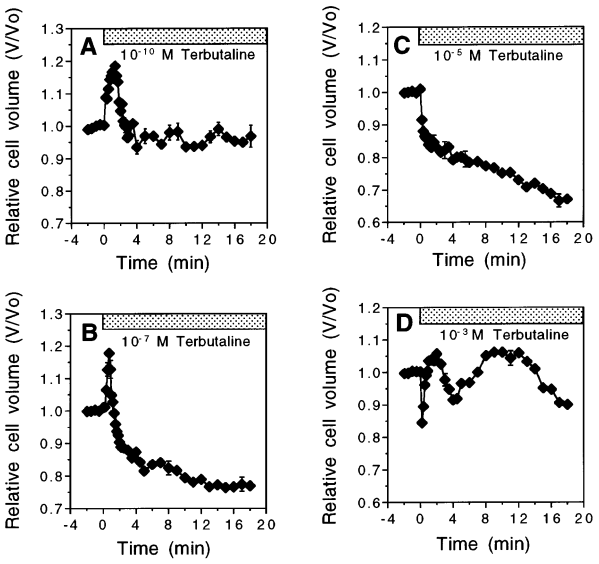

Fig. 1. Cell-volume changes induced by β-agonist (terbutaline) of various concentrations. The concentrations of terbutaline used for stimulation were *A*, 10^{-10} M; *B*, 10^{-7} M; *C*, 10^{-5} M; *D*, 10^{-3} M. Error bars show SEM. If error bars are not shown, the values of SEM are smaller than the symbols.

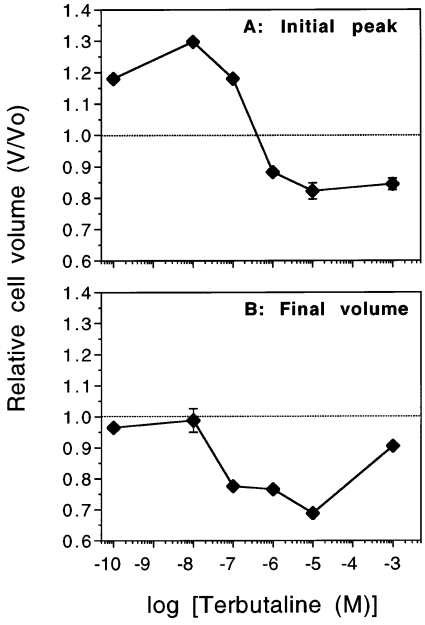

Fig. 2. Dose-response curve of cell-volume changes induced by terbutaline. (*A*) Peak values of initial cell swelling at 10^{-10}–10^{-7} and 10^{-3} M terbutaline, and plateau values of initial shrinkage within 3 min from start of stimulation at 10^{-5} M and 10^{-6} M were plotted. Low concentrations of terbutaline induced cell swelling; high concentrations induced cell shrinkage. (*B*) The final value at 16 min from the start of stimulation was plotted. The slow cell shrinkage was induced by high concentrations of terbutaline ($\geq 10^{-7}$ M).

cell swelling, the FDLE cells slightly shrank and reached a plateau level (V/V_O = 0.95 at 16 min; Fig. 1*A*). Terbutaline of 10^{-7} M induced rapid cell swelling (the peak value, V/V_O = 1.2 at 30 sec) followed by rapid (V/V_O = 0.9 at 2 min) and slow cell shrinkage (V/V_O = 0.8 at 16 min; Fig. 1*B*). Thus, the initial cell-volume change was biphasic during 10^{-7} M terbutaline stimulation. Terbutaline of 10^{-5} M induced rapid cell shrinkage (V/V_O = 0.85 at 30 sec). The cell volume stayed at this decreased level for 2–3 min, then began to decrease slowly again (V/V_O = 0.7 at 16 min; Fig. 1*C*). Terbutaline of 10^{-3} M induced transient cell shrinkage (peak value, V/V_O = 0.85 at 20 sec), then cell volume oscillated (Fig. 1*D*).

When the values of the initial peak and final cell volume were plotted against the terbutaline concentration (Fig. 2), the initial cell-volume change had two components: cell swelling induced by the low concentration of terbutaline ($\leq 10^{-7}$ M), and cell shrinkage induced by the high concentration of terbutaline ($\geq^{-6}$ M), as shown in Figure 2*A*. High concentrations of terbutaline ($\geq 10^{-7}$ M) induced slow cell shrinkage (Fig. 2*B*); however, terbutaline of 10^{-3} M induced cell-volume oscillation. Thus, cell-volume changes were dependent on the terbutaline concentrations. Terbutaline-induced cell-volume changes in FDLE consisted of three components: (i) cell swelling in the initial volume change, (ii) rapid cell shrinkage in the initial volume change, and (iii) slow cell shrinkage following to the rapid cell shrinkage.

To examine the ion conductive pathways responsible for cell volume changes, FDLE cells were treated with 10^{-6} M benzamil (Na^+ channel blocker) and 10^{-3} M quinine (K^+ channel blocker) (Fig. 3*A* and *B*, respectively). The cell swelling caused by terbutaline of 10^{-10} M was not observed in the presence of benzamil (10^{-6} M) (Fig. 3*A*) but was still observed in the presence of 10^{-3} M quinine (*data not shown*). On the other hand, 10^{-3} M quinine blocked the cell shrinkage caused by 10^{-5} M terbutaline (Fig. 3*B*), but 10^{-6} M benzamil could not block the cell shrinkage caused by 10^{-5} M terbutaline (*data not shown*).

Ca^{2+} Effects on Cell-volume Changes (10^{-10} M Terbutaline)

Removal of extracellular Ca^{2+}, which itself caused cell shrinkage (Fig. 4*A*), abolished the cell swelling evoked by 10^{-10} M terbutaline (Fig. 4*B*).

Ca^{2+} Effects on Cell-volume Changes (10^{-5} M Terbutaline)

When the FDLE cells were stimulated by 10^{-5} M terbutaline with Ca^{2+}-free perfusate, cell volume rapidly decreased, but recovered to the control level during stimulation; then cell volume slowly decreased (Fig. 5*A*). The final cell volume (V/V_O at 16 min) was about 0.8. Thus,

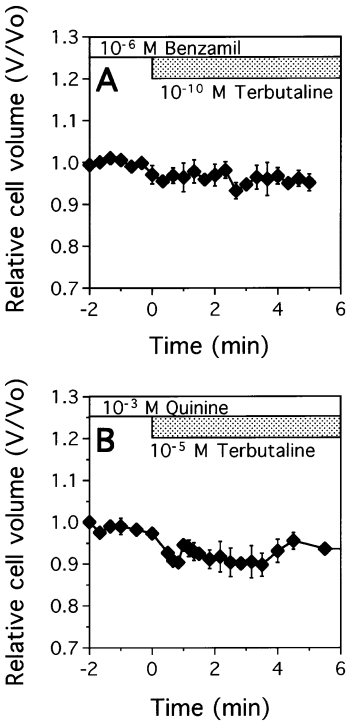

Fig. 3. The effects of benzamil (10^{-6} M) and quinine (10^{-3} M) on terbutaline evoked cell volume changes. (*A*) The FDLE cells were treated with 10^{-6} M benzamil. The cells were stimulated by 10^{-10} M terbutaline. The cell swelling was abolished by benzamil. (*B*) The FDLE cells were treated with quinine (10^{-3} M). The cell was stimulated with 10^{-5} M terbutaline. The cell shrinkage was abolished by quinine.

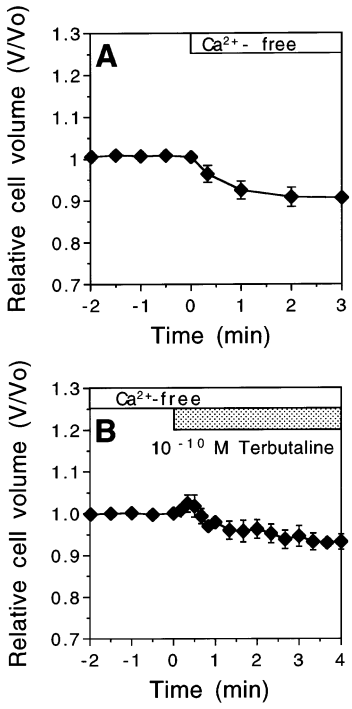

Fig. 4. Volume changes in FDLE cells induced by Ca^{2+} free perfusate and terbutaline (10^{-10} M). (*A*) The FDLE cells were perfused with a control solution (10^{-3} M Ca^{2+}) and then Ca^{2+} was removed from the perfusate. Cell shrinkage was induced by removal of extracellular Ca^{2+}. (*B*) The FDLE cells were perfused with Ca^{2+}-free solution. Cell swelling induced by terbutaline (10^{-10} M) was completely suppressed.

the initial cell shrinkage induced by terbutaline (10^{-5} M) was not sustained (i.e., transient) when Ca^{2+} was removed from the perfusate. When we added $NiCl_2$ (10^{-3} M, a Ca^{2+} channel blocker) into the perfusate, FDLE cells shrank in their volume similar to removal of extracellular Ca^{2+} (Figs. 4*A* and 5*B*). In the presence of Ni^{2+}, 10^{-5} M terbutaline caused cell shrinkage (Fig. 5*B*) similar to that observed in the Ca^{2+}-free solution (Fig. 5*A*). These observations suggest that Ca^{2+} entry into the cell from extracellular space would contribute to maintain the basal cell volume and the sustained phase of 10^{-5} M terbutaline-induced rapid cell shrinkage. Even under extracellular Ca^{2+}-free conditions, however, 10^{-5} M terbutaline still induced rapid cell shrinkage, though it was transient. If Ca^{2+} is also supplied from the endogenous store, the depletion or decrease of the endogenous Ca^{2+} store should inhibit or decrease initial shrinkage. Therefore, FDLE cells were stimulated twice in a short interval without extracellular Ca^{2+} (Fig. 6). First the FDLE cells were stimulated with terbutaline (10^{-5} M) for 30 sec. The cell volume rapidly decreased and then recovered to the control level when terbutaline was removed from the perfusate. The cells were then stimulated again with 10^{-5} M terbutaline over a short interval (1.5 min). Rapid, transient cell shrinkage was induced again by the second stimulation, but to a much smaller than that by the first. After this smaller transient shrinkage, cell volume recovered to the control level during stimulation, and then the cell began shrinking slowly as before. FDLE cells were treated with thapsigargin (2×10^{-6} M, a specific inhibitor of nonmitochondrial microsomal Ca^{2+}-ATPase) in Ca^{2+}-free perfusate (Fig. 6*B*). Thapsigargin itself evoked transient cell shrinkage, which might be caused by an increase in $[Ca^{2+}]_c$ due to the suppression of Ca^{2+}-ATPase. These cells were stimulated with 10^{-5} M terbutaline twice over a short interval (1.5 min). The first stimulation, whose period was 30 sec, evoked cell shrinkage; then cell volume recovered to the prestimulation level. The second stimulation evoked only slow cell shrinkage without rapid cell shrinkage (Fig. 6*B*). These observations suggest that the terbutaline-induced rapid cell shrinkage would be triggered by elevation of $[Ca^{2+}]_c$ supplied from endogenous stores and maintained by Ca^{2+} supplied from extracellular fluid.

Effects of Ionomycin

We used ionomycin to elevate $[Ca^{2+}]_c$. Ionomycin (10^{-7} M) induced rapid cell shrinkage, but slower cell shrink-

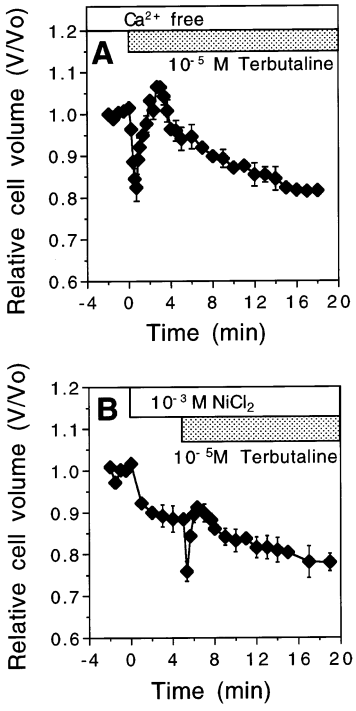

Fig. 5. The effects of removal of extracellular Ca^{2+} and Ni^{2+} (10^{-3} M) on cell volume changes induced by terbutaline of 10^{-5} M. (*A*) The FDLE cells were stimulated by 10^{-5} M terbutaline with Ca^{2+}-free perfusate. Terbutaline of 10^{-5} M evoked transient cell shrinkage followed by slow shrinkage (*B*) Addition of Ni^{2+} caused cell shrinkage similar to that caused by removal of extracellular Ca^{2+}. Terbutaline of 10^{-5} M evoked transient followed by slow cell shrinkage similar to that in Ca^{2+}-free solution.

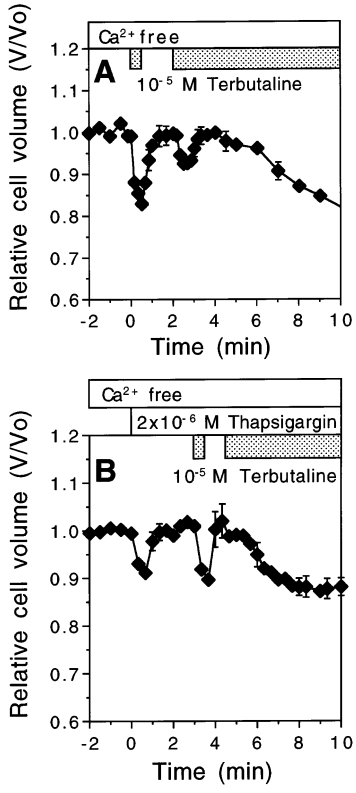

Fig. 6. The FDLE cells were stimulated twice with terbutaline (10^{-5} M) over a short interval in Ca^{2+}-free perfusate. The period of the first stimulation was 30 sec. After a short interval (1.5 min) the cells were stimulated again. (*A*) The first stimulation induced a rapid decrease in cell volume, which recovered to the control level after removal of terbutaline. The second stimulation also induced transient cell shrinkage, which was smaller than that by the first stimulation. Cell volume gradually decreased after the transient cell shrinkage. (*B*) Thapsigargin (2×10^{-6} M; a nonmitochondrial Ca^{2+}-ATPase inhibitor) was added into the Ca^{2+}-free perfusate. Thapsigargin itself induced transient cell shrinkage. The first stimulation induced rapid shrinkage and the cell volume recovered to the control level after removal of terbutaline. The second stimulation only induced slow cell shrinkage without the transient cell shrinkage.

age did not occur: cell volume reached a plateau level within 2 min (Fig. 7*A*). FDLE cells lost their volume with ionomycin treatment over a wide range of concentrations ($3 \times 10^{-10} - 3 \times 10^{-6}$ M), as shown in Fig. 7*B*. Ionomycin of 3×10^{-10} M induced a small amount of cell shrinkage ($V/V_O = 0.9$) as shown in Fig. 7*B*. On the other hand, ionomycin of 3×10^{-5} M induced cell swelling after rapid transient shrinkage (Fig. 7*B*). These findings suggest that an excessive increase in $[Ca^{2+}]_c$ induces cell swelling. The dose-response curve of ionomycin is summarized in Fig. 8, in which the V/V_O at 2 min after addition of ionomycin is plotted. These findings suggest that the elevation of $[Ca^{2+}]_c$ induces cell shrinkage. On the other hand, the terbutaline-induced slow shrinkage, which occurred even without extracellular Ca^{2+} and with Ni^{2+}, is unlikely to be regulated by changes in $[Ca^{2+}]_c$.

Effects of Forskolin

Forskolin (10^{-4} M), which is known to increase $[cAMP]_c$ by activating the adenylate cyclase, induced rapid cell shrinkage followed by slow cell shrinkage (Fig. 9*A*). The cell volume change induced by forskolin of 10^{-4} M was similar to that induced by terbutaline of 10^{-5} M. The effects of forskolin were also examined in benzamil-treated FDLE cells. Benzamil (10^{-6} M) itself induced cell shrinkage. Forskolin (10^{-4} M) also evoked rapid followed by slow cell shrinkage in benzamil-treated FDLE cells (Fig. 9*B*); the final cell volume (V/V_O) was about 0.5 The decreasing rate of cell volume at the phase of the slow cell shrinkage in the presence of benzamil (Fig. 9*B*) was larger than that in the absence of benzamil (Fig. 9*A*).

Effects of Dibutyryl cAMP

DBcAMP, a membrane-permeable analogue of cAMP, induced cell-volume changes (Fig. 10). Cells were tran-

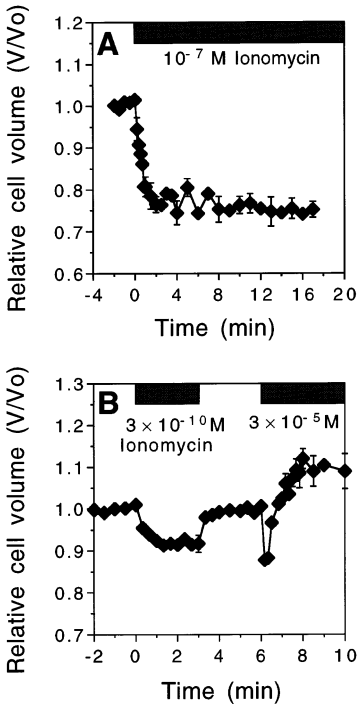

Fig. 7. The effects of ionomycin on cell volume. (*A*) Cell shrinkage induced by ionomycin (10^{-7} M). Rapid cell shrinkage occurred, but the slow decrease in cell volume did not. Cell volume stayed at a plateau level. (*B*) Cell-volume changes induced by 3×10^{-10} and 3×10^{-5} M ionomycin. Ionomycin of 3×10^{-10} M induced cell shrinkage. Ionomycin of 3×10^{-5} M induced cell swelling after rapid shrinkage.

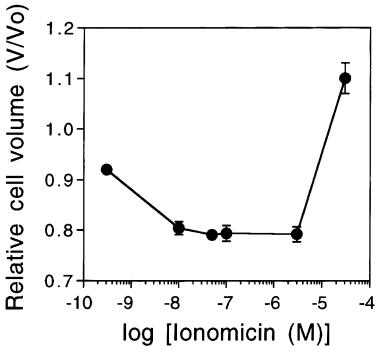

Fig. 8. Dose-response curve of cell-volume change induced by ionomycin. Cell shrinkage depends on ionomycin concentration. The plateau levels induced over a wide range of concentrations of ionomycin (10^{-8}–3×10^{-6} M) was identical ($V/V_O = 0.8$). The values at 2 min from the start of stimulation were plotted.

siently swelled by 10^{-9} or 10^{-8} M DBcAMP and stayed at a plateau level ($V/V_O \approx 1.0$; Fig. 10*A* and *B*). DBcAMP of 10^{-5} M decreased the cell volume, which reached a plateau level (about 0.88) about 2–3 min after stimulation (Fig. 10*C*). DBcAMP of 10^{-3} M induced rapid cell shrinkage within 30 sec followed by a gradual decrease; cell volume reached a plateau level ($V/V_O \approx 0.8$) about 4 min after stimulation (Fig. 10*D*). Figure 11 summarizes

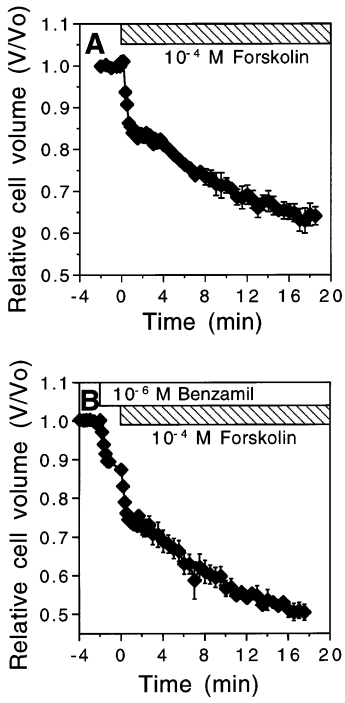

Fig. 9. The effects of forskolin on cell volume. (*A*) Cell shrinkage induced by forskolin (10^{-4} M). This cell-volume change was similar to that induced by terbutaline (10^{-5} M). (*B*) FDLE cells were treated with benzamil (10^{-6} M). Benzamil induced the cell shrinkage. Further addition of forskolin (10^{-4} M) induced the rapid followed by slow cell shrinkage.

these results: the initial volume change at 20 sec and the final cell volume at 16 min.

Discussion

FDLE cells in primary culture have been demonstrated to have intracellular filaments, and lectin binding characteristics of alveolar type II epithelial cells (Post, Torday & Smith, 1984; Post & Smith, 1988; O'Brodovich et al., 1990). Since FDLE monolayer in primary culture forms tight junction and shows terbutaline-stimulated amiloride-sensitive short-circuit currents (O'Brodovich et al., 1990), these cells have been widely used for the studies of short circuit currents (O'Brodovich et al., 1990; Hagiwara et al., 1992) and patch clamp (Orser et al., 1991; Marunaka et al., 1992; Tohda et al., 1994; Marunaka & Tohda, 1995; Nakahari & Marunaka, 1995*b*; Marunaka, 1996) as a model of the fetal type II cell.

In the present study, cell volume was measured by a morphometric method whose reliability has been described in detail previously (Foskett & Melvin, 1989; Foskett, 1990; Nakahari et al., 1991; Suzuki et al., 1991). The values of cell volume changes in salivary gland detected at single cell and whole gland levels by the morphometric (Foskett & Melvin, 1989; Nakahari, Mu-

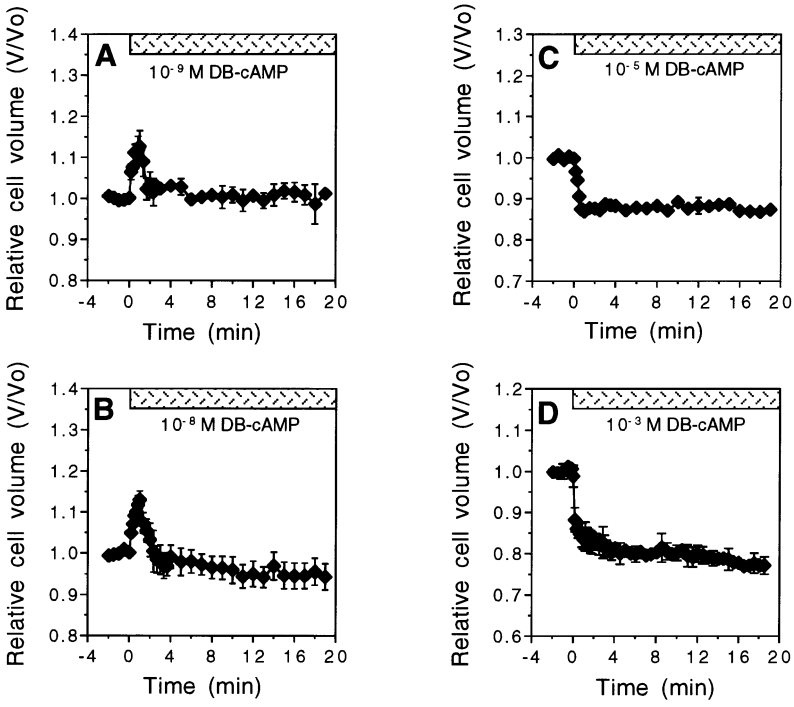

Fig. 10. Cell-volume changes induced by various concentrations of DBcAMP. The concentrations of DBcAMP used for stimulations were *A*, 10^{-9} M; *B*, 10^{-8} M; *C*, 10^{-5} M; *D*, 10^{-3} M.

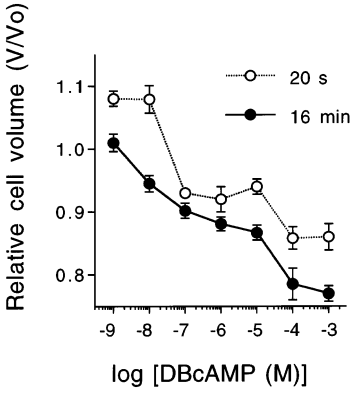

Fig. 11. The dose-response curve of cell-volume changes induced by DBcAMP. Open circles show the value at 20 sec after DBcAMP stimulation. The low concentrations of DBcAMP (10^{-9} and 10^{-8} M) induced cell swelling; DBcAMP of 10^{-7}–10^{-3} M induced cell shrinkage (V/V_O = 0.9–0.85). Closed circles show the final values at 16 min from start of stimulation. The slow cell shrinkage also depended on DBcAMP concentration.

rakami & Kataoka, 1989; Nakahari et al., 1990; Nakahari et al., 1991), impedance (Nakahari et al., 1990) and NMR (Steward et al., 1990; Larcombe-McDouall, Seo & Steward, 1994) methods, are identical. A study using a coulter counter (MacLeod et al., 1994) has indicated that 8-Br-cAMP and A23187 induce cell shrinkage in FDLE cells. This cell shrinkage induced by 8-Br-cAMP or A23187 is similar to that induced by DBcAMP or ionomycin in the present study. These reports support the usefulness and reliability of the morphometric method used in this report.

Our preliminary observations indicate that removal of extracellular Ca^{2+} or application of quinine (10^{-3} M) and benzamil (10^{-6} M) abolished the effects of 3×10^{-10} M ionomycin on cell volume. On the other hand, using Fura-2 (Tohda et al., 1994) we preliminarily observed that 3×10^{-10} M ionomycin increased $[Ca^{2+}]_c$ to 50 nM from the basal level of 20–30 nM. Taken together, these observations suggest that the action of 3×10^{-10} M ionomycin on cell volume would occur by activating ion channels through an increase in $[Ca^{2+}]_c$, but would not be a nonspecific effect.

We previously reported that in FDLE cell 10^{-5} M terbutaline induced an increase in $[Ca^{2+}]_c$ (Tohda et al., 1994), which activated K^+ currents (Nakahari & Marunaka, 1995*b*), and that a decrease in $[Cl^-]_c$ was caused by 10^{-5} M terbutaline (Tohda et al., 1994). In the present study, we show the terbutaline-induced rapid cell shrinkage was blocked by quinine (10^{-3} M), a blocker of Ca^{2+}-activated K^+ channel (the rapid cell shrinkage in Fig. 3*B*). These findings suggest that the cell shrinkage in FDLE cells is due to KCl loss from cell.

The observations shown in the present study suggest that the slow cell shrinkage induced by 10^{-5} M terbutaline would be dependent on cAMP but not on Ca^{2+}. The cell shrinkage have already been demonstrated by cAMP in a rat salivary cell line (RSMT-A5) (He et al., 1989) and FDLE cells (MacLeod et al., 1994) through activa-

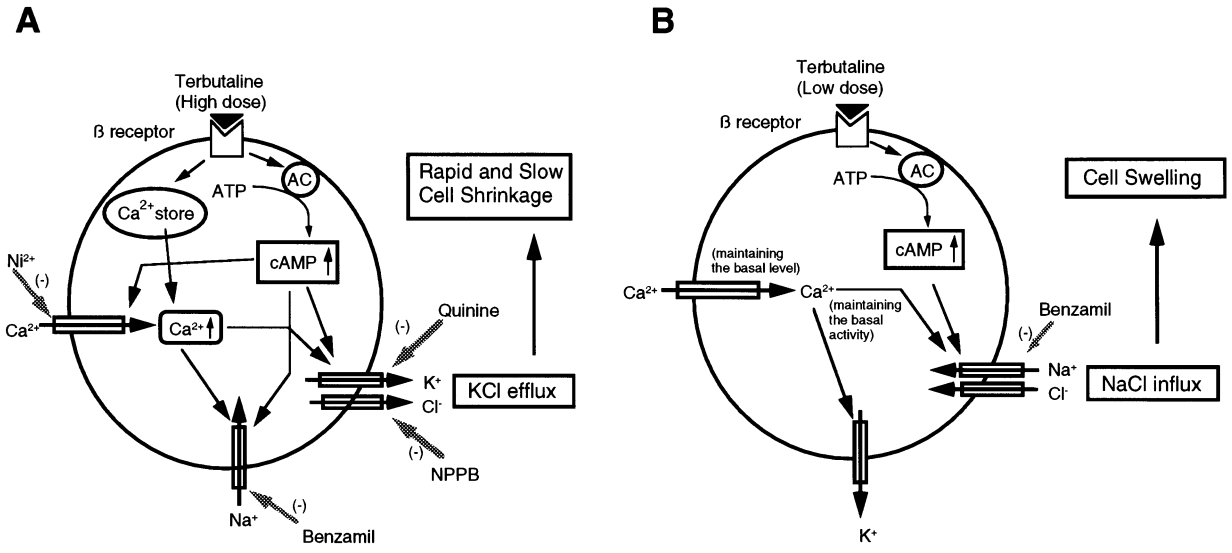

Fig. 12. The possible mechanism of cell volume changes induced by terbutaline. (*A*) High concentration of terbutaline. (*B*) Low concentration of terbutaline.

tion of Cl^- channels. This is supported by our preliminary observation that NPPB (5-Nitro-2-(3-phenylpropylamino)-benzoic acid, 2×10^{-5} M, a generous gift from Prof. R. Greger) treatment also abolished the cell volume changes induced by terbutaline, but did not affect the cell volume under the unstimulated condition. Thus, the elevation of cAMP might induce the slow cell shrinkage by activating Cl^- channels resulting in an increase in KCl efflux. However, it was still unclear whether terbutaline of high concentrations also activates Na^+ entry pathway. Forskolin (10^{-4} M) induced huge shrinkage of benzamil-treated FDLE cells compared with benzamil-untreated cells. This observation suggests that Na^+ entry pathways are also activated by forskolin of 10^{-4} M. Furthermore, the studies of short circuit currents have demonstrated that terbutaline (10^{-5} M) increases amiloride-sensitive currents (O'Brodovich et al., 1990), and the patch clamp studies have also demonstrated activation of amiloride-blockable Na^+-permeant nonselective cation channels by 10^{-5} M terbutaline (Tohda et al., 1994; Nakahari & Marunaka, 1995*b*). If 10^{-5} M terbutaline activates Na channels, 10^{-5} M terbutaline is expected to increase the cell volume in the presence of quinine which blocks K^+ loss. Our observation shown in Fig. 3*B* indicates that 10^{-5} M terbutaline could not increase cell volume in the presence of quinine (10^{-3} M). This phenomenon can be explained by either of the following speculations: i.e., (i) quinine of 10^{-3} M could not completely block terbutaline-activated K^+ channels, or (ii) terbutaline could activate quinine-insensitive K^+ channels. Our preliminary observation indicated that terbutaline of 10^{-5} M could not increase the cell volume even in the presence of quinine of higher concentration such as 5×10^{-3} M, suggesting that terbutaline might also activate quinine-insensitive K^+ channels. To clarify this point further studies will be required. Based on the observations shown in the present study, we propose a possible model of cell volume regulation by terbutaline of high concentration such as 10^{-5} M (Fig. 12*A*).

The transient cell swelling induced by terbutaline of 10^{-10} M was suppressed by removal of extracellular Ca^{2+} or by benzamil. Our preliminary observations suggest that terbutaline of 10^{-10} M does not significantly increase $[Ca^{2+}]_c$ (the basal $[Ca^{2+}]_c$ = 20–30 nM) and that removal of extracellular Ca^{2+} reduced $[Ca^{2+}]_c$ to <1 nM. The transient cell swelling was also induced by low concentration of DBcAMP ($\leqslant 10^{-8}$ M) but not induced by ionomycin alone even with a wide range of concentrations (3×10^{-10}–3×10^{-5} M). Amiloride-blockable, Ca^{2+}-activated Na^+-conductive channels (nonselective cation channel and Na^+ channel) have already been reported to exist in the apical membrane of FDLE cells (Orser et al., 1991; Marunaka et al., 1992; Tohda et al., 1994; Marunaka, 1996). These findings suggest that the cell swelling is due to the cAMP-induced activation of Na^+ entry which requires the basal level of $[Ca^{2+}]_c$, however only an increase in $[Ca^{2+}]_c$ without any elevation of $[cAMP]_c$ is not sufficient to induce the cell swelling. Based on the observations shown in the present study, we propose a possible model of cell volume regulation by terbutaline of low concentration such as 10^{-10} M. Unlike high concentrations of terbutaline, low concentrations of terbutaline could not activate K^+ channels (Fig. 12*B*).

We observed the extreme decrease in cell volume induced by the combination action of forskolin and benzamil (Fig. 9*B*). Many cells have a nonosmotic volume of about 34% (Drewnowska & Baumgarten, 1991) due to insoluble cell proteins. Our previous report (Toh-

da et al., 1994) indicates that $[Cl^-]_c$ of FDLE cells is about 50 mM. The total salts of the control solution used in the present study was approximately 150 mM, which would be equivalent to that in the osmotic space of the cell. These mean that the decrease in cell volume due to loss of KCl should be less than one-third (50/150) of the osmotic volume (66%); i.e., 22%. This suggests that the cell volume after KCl loss caused by benzamil and forskolin could be as large as 78%. The observation of the extreme decrease in cell volume (50%) by benzamil and forskolin could be due to exit of cellular osmolyte such as surfactant in addition to KCl loss (Post, Torday & Smith, 1984; Post & Smith, 1988).

Recently, $[Cl^-]_c$ has been reported to regulate cell functions, including the K^+ channel in cardiac atrial cells (Nakajima, Sugimoto & Kurachi, 1992), respiratory burst in neutrophils (Grinstein, Furuya & Downey, 1992) and cation conductance in A6 cells (Nakahari & Marunaka, 1995*a*). A decrease in $[Cl^-]_c$ has also been reported to activate a nonselective cation channel in the apical membrane of FDLE cells (Tohda et al., 1994). Cell shrinkage is a cellular mechanism that decreases $[Cl^-]_c$ and maintains it at a lowered level (Foskett, 1990), which plays a physiologically important role in activation of Na^+ absorption that causes water clearance from lung air space. Therefore, cell shrinkage is likely to be related to some cellular functions. The physiological meanings of the stimulatory action of terbutaline of high concentration on K^+ channels would be acceleration of the Na^+ channel's activation, which induces water clearance from lung air space, by decreasing $[Cl^-]_c$.

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