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FK506 inhibits Cl^- secretion in airway epithelium via calcineurin-independent mechanism

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Abstract

FK506 (tacrolimus)-binding protein (FKBP) is associated with intracellular Ca^{2+} release channel and modulates its function. To elucidate the effect of FK506 on Ca^{2+} dynamics and Ca^{2+}-mediated Cl^- secretion in airway epithelium, we studied intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration and Cl^--dependent short-circuit current (I_{sc}), in cultured bovine tracheal epithelial cells. Addition of ATP induced an increase in $[Ca^{2+}]_i$, and this response was dose dependently inhibited by FK506. Rapamycin, which binds FKBP with high affinity, likewise inhibited the $[Ca^{2+}]_i$ rise, but cyclosporin A, a specific calcineurin inhibitor, did not. In Cl^- secretion studies using Ussing chamber, ATP increased Ca^{2+}-mediated I_{sc} in amiloride-treated cells, an effect that was inhibited by FK506 and rapamycin but not by cyclosporin A. Therefore, FK506 inhibits Ca^{2+} mobilization in airway epithelium via FKBP but not calcineurin-dependent mechanism, which may result in the suppression of Ca^{2+}-activated Cl^- secretion.

Keywords: Airway epithelium; Adenosine 5′-triphosphate; Ion transport; FK506-binding protein

1. Introduction

In airway epithelium, intracellular Ca^{2+} is an important second messenger in the regulation of Cl^- transport and mucin secretion (Clancy et al., 1990; Mason et al., 1991; Kim and Lee, 1993). For example, several inflammatory mediators such as bradykinin and ATP stimulate their specific receptors on airway epithelium, activate phospholipase C, and produce inositol 1,4,5-trisphosphate (IP_3). Then the release of Ca^{2+} from endoplasmic reticulum occurs via IP_3 receptor, which results in the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Mason et al., 1991; Paradiso et al., 1991; Sakai et al., 1995). Consequently, elevated levels of $[Ca^{2+}]_i$ may affect transepithelial Cl^- secretion and, hence, water secretion toward the airway lumen (Clancy et al., 1990; Mason et al., 1991). Therefore, information may be useful about regulatory factors altering the function of airway epithelial Ca^{2+} release channels (CRC) such as IP_3 receptor for better understanding the mechanism of and therapeutic strategy to bronchial hypersecretions.

The 12-kDa FK506 (tacrolimus)-binding protein (FKBP12) is a member of immunophilins known as a target molecule of the macrolide immunosuppresant drug FK506. In addition to its crucial role in FK506-induced T-cell immunosuppression (Liu et al., 1991; Schreiber, 1991), FKBP12 associates physiologically with CRC including ryanodine receptor and IP_3 receptor (Jayaraman et al., 1992; Cameron et al., 1995), and functions as a chaperone-like protein (Liu, 1993; Brillantes et al., 1994). In the presence of FK506, the FKBP12 dissociates from the channel complex, which results in alteration of CRC conductance (Brillantes et al., 1994; Cameron et al., 1995). We have recently shown that FK506 interferes with intracellular Ca^{2+} oscillations in single airway epithelium (Kanoh et al., 1999). However, little is known of the effect of FK506 on the more common agonist-induced Ca^{2+} dynamics in population and Ca^{2+}-dependent function in airway epithelial cells. Therefore, we investigated the effect of FK506 on the ATP-induced increase in $[Ca^{2+}]_i$ and

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the related Ca^{2+}-activated Cl^- secretion in cultured bovine tracheal epithelial cells.

2. Materials and methods

2.1. Cell culture

Bovine tracheas were obtained from a slaughterhouse, and tracheal epithelial cells were isolated by protease as previously described (Kanoh et al., 1999). Briefly, strips of epithelium were pulled off the submucosa, washed four times with phosphate-buffered saline (PBS) containing 5 mM dithiothreitol, and rinsed twice with PBS. Epithelial tissues were digested with PBS containing 0.05% protease (type XIV; Sigma, St. Louis, MO, USA) at 4°C overnight. After neutralization of the protease with 5% fetal calf serum, cells were pelleted ($200 \times g$, 10 min) and suspended in 50% Dulbecco's modified Eagle's medium (DMEM) and 50% Ham's nutrient F12 that contained 5% fetal calf serum, non-essential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml). For the measurement of $[Ca^{2+}]_i$, the isolated cells were plated at a density of 2.0×10^5 cells/cm^2 on round coverslips (15-mm diameter; Matsunami, Tokyo, Japan) coated with human placental collagen (20 μg/cm^2; collagen Type IV, Sigma), and cultured for 4–5 days to confluent stage. For the measurement of bioelectric properties, the cells were seeded at a density of 2.5×10^5 cells/cm^2 onto collagen-coated polycarbonate inserts (porous filters of 24-mm diameter, 10-μm thickness and 0.4-μm pore size; Coster Transwell, Cambridge, MA, USA). The medium was changed every other day, and the cells were cultured under air–liquid interface condition at 37°C in a CO_2 incubator (95% air–5% CO_2) for 10 days (Kondo et al., 1997).

2.2. Measurement of $[Ca^{2+}]_i$

The coverslip on which the cells were grown was washed with Hanks' balanced salt solution (HBSS) which contained 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.4 and loaded with 2 μM acetoxymethyl ester of fura-2 (fura-2/AM) for 20 min at 37°C. The coverslip was then washed again and held with a rigid holder in a continuously stirred cuvette containing HEPES-buffered HBSS maintained at 37°C, and the fluorescence intensity was measured with a spectrophotometer (CAF-110; Japan Spectroscopic, Tokyo, Japan) (Kondo et al., 1998). For excitation of fura-2 fluorescence, UV lights of 340- and 380-nm wave length were automatically exchanged at a rate of 50 Hz, the emitted light from cells (F340 and F380) was detected with a photomultiplier tube through a 510 ± 10-nm band-pass filter, and the fluorescence intensity ratio, F340/F380, was automatically calculated. Maximal and minimal values for the ratio were determined in the presence of 10 μM ionomycin and 5 mM ethyleneglyco-*bis*-(β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA), respectively, and $[Ca^{2+}]_i$ was calculated using the external calibration standards and the formula (Grynkiewicz et al., 1985).

2.3. Measurement of bioelectric properties

The short-circuit technique for measuring electrical properties of cultured airway epithelium had been described previously (Tamaoki et al., 1992). Briefly, the porous filter on which tracheal epithelial cells were grown was mounted between Ussing chambers (0.5-cm^2 surface area) and bathed with Krebs Ringer bicarbonate solution of the following composition (in mM): 115 NaCl, 25 $NaHCO_3$, 2.4 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, equilibrated with 95% O_2–5% CO_2 and warmed to 37°C. Transepithelial potential difference was measured with two polyethylene bridges containing 3% agar in saline, positioned within 1 mm from each side of the epithelial surface and connected to calomel electrodes (model 2080A-06T, Horiba, Tokyo, Japan) and a high-impedance voltmeter (model CEZ-9100, Nihon Kohden, Tokyo). Another pair of polyethylene bridges (3% agar in saline), positioned 10 mm from the orifice and connected to Ag/AgCl wires, was used to pass sufficient current through both the chamber and cells to bring the potential difference to zero. This short-circuit current (I_{sc}) was automatically corrected for solution resistance between the potential difference-detecting bridges, and recorded continuously on a pen recorder (model SR6335, Graphtec, Tokyo). The cells were allowed to equilibrate for 20 min, and amiloride (100 μM) was added to the mucosal side of the solution to eliminate a component of Na^+ movement (Al-Bazzaz and Zevin, 1984). When the response of I_{sc} to amiloride became a stability that did not vary by more than 0.2 μA/cm^2 in any 5-min intervals thereafter, ATP (100 μM) was added to the mucosal solution.

DMEM, Ham's F-12, non-essential amino acids, and 1,2-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-AM were purchased from Gibco BRL (Tokyo, Japan). Fura-2/AM was obtained from Dojindo Lab. (Kumamoto, Japan). All other chemicals and the recombinant human FKBP12 were obtained from Sigma. FK506 was a gift from Fujisawa Pharmaceutical (Osaka, Japan). FK506, repamycin and cyclosporin A were dissolved in ethanol and used at final ethanol concentration of less than 0.1%.

2.4. Statistics

All data are expressed as means ± S.E. Statistical analysis was performed by two-tailed paired Student's *t*-test or one-way analysis of variance using Fisher's Protected Least Significant Difference (PLSD) test, and a P value of less than 0.05 was considered significant.

3. Results

3.1. Effect of FK506 on ATP-induced $[Ca^{2+}]_i$ rise

The baseline $[Ca^{2+}]_i$ in the bovine tracheal epithelium was 126 ± 5 nM ($n = 48$). Exposure to 100 μM ATP caused a rapid increase in F340/F380 (Fig. 1A). This $[Ca^{2+}]_i$ response was biphasic, consisting of an initial transient rise that peaked within 15 s and a following sustained response. The peak value of $[Ca^{2+}]_i$ was 513 ± 32 nM, indicating an increase in $[Ca^{2+}]_i$ by 387 ± 32 nM ($n = 48$, $P < 0.001$). We then examined the effect of FK506 on ATP-induced $[Ca^{2+}]_i$ rise. The cells were incubated for various periods (1–60 min) with 1 μM FK506, and 100 μM ATP was added. As shown in Fig. 2, FK506 caused a significant reduction in ATP-induced $[Ca^{2+}]_i$ rise 5 min after the incubation, and this effect was similar at any other subsequent time points within 60 min. Thus, to avoid a possible effect of FK506 on various transcription factors or protein syntheses, the period of 10 min was selected as a incubation time in this study. Pretreatment of cells with FK506 at 1 and 10 μM did not alter the baseline $[Ca^{2+}]_i$ but reduced ATP-induced increase in $[Ca^{2+}]_i$ (Fig. 1B, C). As shown in Fig. 3, the effect of FK506 was concentration-dependent, the maximal inhibition of $[Ca^{2+}]_i$ response being $91 \pm 2\%$ ($P < 0.001$). This inhibition was prevented when recombinant human FKBP12 was added to extracellular milieu prior to FK506 (Fig. 3). Pretreatment with rapamycin, another immunosuppresant drug that binds FKBP with high affinity, inhibited ATP-induced $[Ca^{2+}]_i$ response as did FK506, whereas pretreatment with cyclosporin A, a specific calcineurin inhibitor that does not bind FKBP, had no effect (Fig. 4).

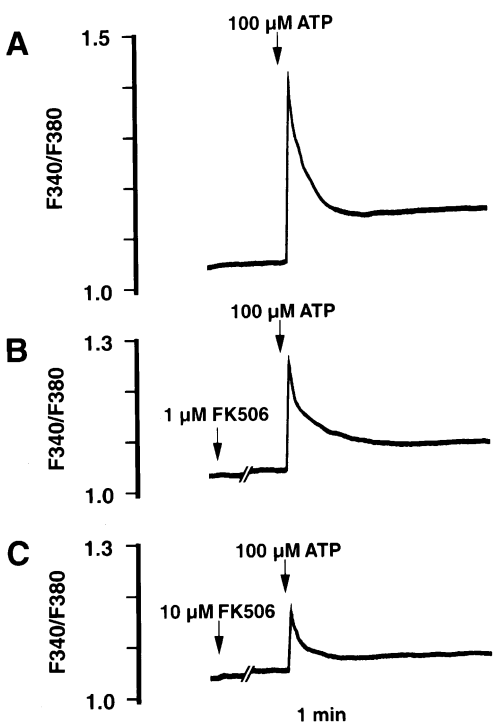

Fig. 1. Representative recordings of ATP-induced Ca^{2+} responses in fura 2-loaded bovine tracheal epithelial cells treated with and without FK506. (A) ATP (100 μM) induced a transient rapid increase in $[Ca^{2+}]_i$, followed by a sustained response. (B, C) After treatment with FK506 (1 or 10 μM) for 10 min, ATP (100 μM) was added. FK506 reduced ATP-induced increase in $[Ca^{2+}]_i$.

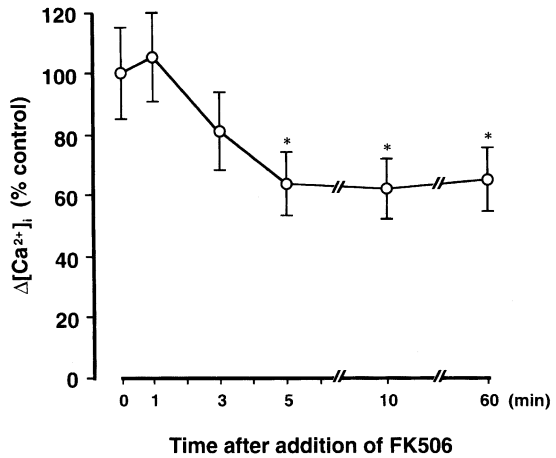

Fig. 2. Time-dependent effect of FK506 on ATP-induced Ca^{2+} responses. The cells were incubated with FK506 (1 μM) for 1–60 min, and the response of $[Ca^{2+}]_i$ to ATP (100 μM) was determined. Values are expressed as percentage of the control response (ATP alone). Data are means ± S.E.; $n = 4$ for each point. $^{*}P < 0.05$, significantly different from the control value.

3.2. ATP-induced Cl^- secretion and its inhibition by FK506

The effect of exogenous ATP on Cl^- secretion in bovine tracheal epithelium was assessed by measuring the

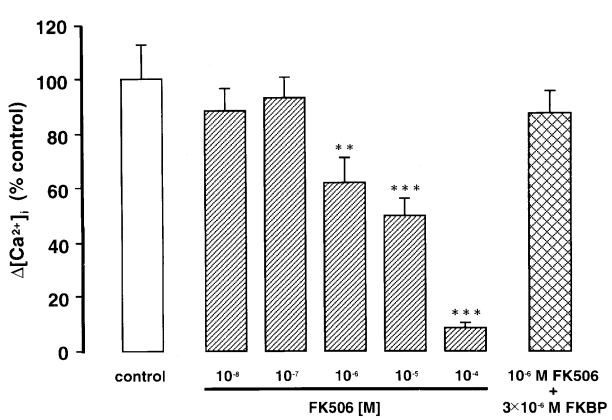

Fig. 3. Concentration-dependent effect of FK506 on ATP-induced increase in $[Ca^{2+}]_i$ and its inhibition by FKBP. After incubation with FK506 for 10 min, the response of $[Ca^{2+}]_i$ to ATP (100 μM) was determined. Values are expressed as percentage of the control response (ATP alone). FK506 reduced the ATP-induced Ca^{2+} responses, and excess recombinant human FKBP12 (rhFKBP12) prevented the inhibitory effect of FK506. Data are means ± S.E.; $n = 12$ for each column. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from the control value.

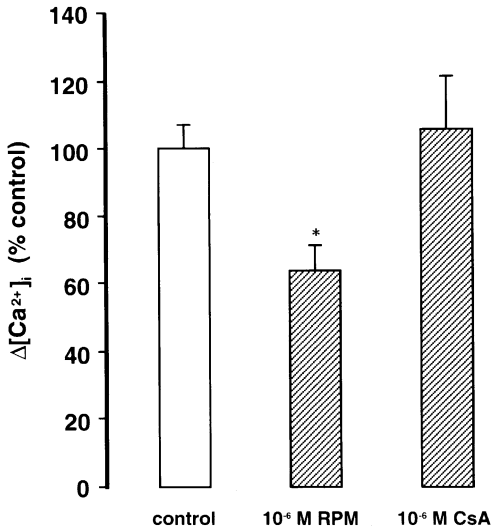

Fig. 4. Effect of rapamycin and cyclosporin A on ATP-induced increase in $[Ca^{2+}]_i$. After incubation with rapamycin (RPM, 1 μM) or cyclosporin A (CsA, 1 μM) for 10 min, the response of $[Ca^{2+}]_i$ to ATP (100 μM) was determined. Values are expressed as percentage of the control response (ATP alone). Data are means ± S.E.; $n = 6$ for each column. $^{*}P < 0.05$, significantly different from the control value.

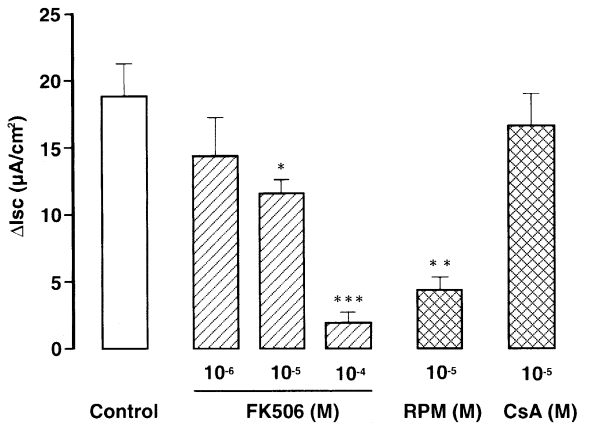

Fig. 6. Effects of FK506, rapamycin and cyclosporin A on ATP-induced increase in I_{sc}. After incubation without (*control*) and with FK506 (1–100 μM), rapamycin (RPM, 10 μM) or cyclosporin A (CsA, 10 μM) for 10 min, the response of I_{sc} to ATP (100 μM) was determined. Data are means ± S.E.; $n = 5$ for each column. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from the control value.

bioelectric properties. Application of 100 μM ATP to mucosal side of amiloride-treated cells produced a rapid increase in I_{sc} consisting of an initial transient spike that peaked within 20 s and a following sustained response (Fig. 5A). The change in I_{sc} from the baseline level to the peak value was 19.6 ± 2.1 μA/cm^2 ($n = 12$). Pretreatment of cells with 100 μM niflumic acid, a Ca^{2+}-activited Cl^- channel blocker (White and Alwyn, 1990), or replacement of medium on both sides with Cl^--free solution, in which Cl^- was substituted with iodide that cannot be transported across the airway epithelium, abolished the ATP-induced increase in I_{sc} (Fig. 5B, C). Furthermore, incubation of cells with 100 μM BAPTA-AM, a chelating agent of intracellular storage of Ca^{2+} (Yamaya et al., 1993), abolished the ATP-induced initial peak in I_{sc} (Fig. 5D). In addition, the I_{sc} response induced by mucosal addition of 10 μM ionomycin mimicked the initial peak response to ATP (Fig. 5E). FK506 per se had no effect on the baseline I_{sc}, but pretreatment with 10 μM FK506 for 10 min reduced the response of I_{sc} to ATP (Fig. 5F). This effect was concentration-dependent, and rapamycin likewise inhibited the ATP-induced increase in I_{sc}, but cyclosporin A did not (Fig. 6).

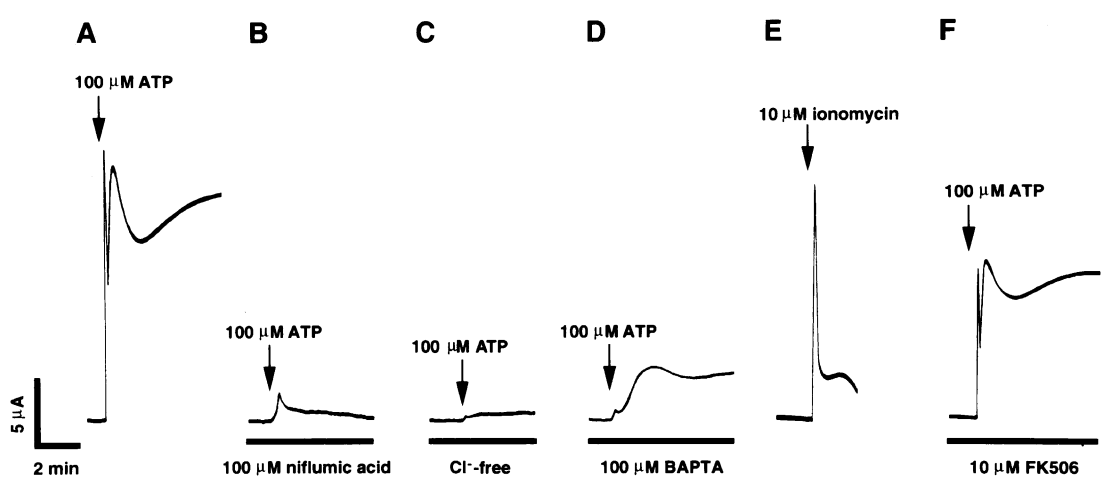

Fig. 5. Representative tracings of short-circuit current (I_{sc}) in bovine cultured tracheal epithelium in the presence of amiloride (100 μM). (A) Mucosal ATP (100 μM) induced an initial transient increase in I_{sc}, followed by a biphasic response. (B, C, D) Mucosal niflumic acid (100 μM, B), substitution of Cl^- in the bathing medium with iodide (Cl^--free, C), or pretreatment with BAPTA-AM (100 μM, D) inhibited the ATP-induced increase in I_{sc}. (E) Mucosal ionomycin (10 μM) caused a rapid transient increase in I_{sc}. (F) Pretreatment with FK506 (10 μM) for 10 min reduced the ATP-induced initial peak and the following response in I_{sc}.

4. Discussion

Our present study demonstrated that FK506 inhibited ATP-stimulated Ca^{2+} response and the concomitant Ca^{2+}-activited Cl^- secretion in bovine tracheal epithelial cells. It is well known that FK506 binds FKBP, and FK506–FKBP complex inhibits the Ca^{2+}-dependent phosphate calcineurin, which results in the inhibition of calcineurin-dependent responses such as interleukin-2 transcription and T-cell proliferation (Liu et al., 1991; Schreiber, 1991). We therefore assessed the effects of rapamycin and cyclosporin A on airway epithelial $[Ca^{2+}]_i$ and I_{sc} to determine the role of FKBP and calcineurin, respectively. Pretreatment of cells with rapamycin, which binds FKBP with high affinity, attenuated the ATP-induced increases in $[Ca^{2+}]_i$ and I_{sc} as did FK506, whereas cyclosporin A, a calcineurin inhibitor that does not bind FKBP, was without effect. These results suggest that FK506 may have exerted its effect only by binding FKBP and that the involvement of the subsequent interference with calcineurin-dependent effect seems unlikely. Furthermore, prior addition of excess FKBP12 prevented the inhibitory effect of FK506 on ATP-induced $[Ca^{2+}]_i$ rise, suggesting that extracellular FKBP12 may have trapped FK506 outside the cells, and FK506 was thus unable to penetrate the cells and bind intracellular FKBP. Therefore, intracellular FKBP appears to be crucial for maintenance of Ca^{2+} response. It has been proposed that the physiological role of FKBP is to stabilize CRC function of IP_3 receptor as well as ryanodine receptor by enhancing the cooperativity among four subunits resulting in full conductance channels, and that dissociation of FKBP12 from IP_3 receptor by FK506 or rapamycin causes leakiness in the channel's gating properties (Marks, 1996). The leakiness of CRC causes partial depletion of Ca^{2+} in ryanodine receptor-gated or IP_3 receptor-gated stores (Timerman et al., 1993, 1995; Brillantes et al., 1994; Cameron et al., 1995). We have recently shown that the cells employed in this study have cytoplasmic FKBP12, which plays an important role in the regulation of Ca^{2+} contents in endoplasmic reticulum, and that FK506 significantly decreases the Ca^{2+} contents (Kanoh et al., 1999). Taken together, the inhibitory effect of FK506 on ATP-induced increase in $[Ca^{2+}]_i$ may be due to the decreased intracellular Ca^{2+} stores.

Airway epithelium regulates eletrolyte and fluid transport across the mucosa and, hence, the output and composition of the respiratory tract secretions (Nadel et al., 1985). The epithelial cells grown under air–liquid interface condition have been found to possess active ion transport function with a level similar to the native tissue (Kondo et al., 1993), in which Cl^- channels including Ca^{2+}-activated Cl^- channel localized to the apical membrane play a key role (Anderson et al., 1992). Accordingly, we assessed the effect of FK506 on Cl^- transport property in our cells to address whether FK506 affects Ca^{2+}-dependent cellular function. In the present study, The ATP-induced I_{sc} rise in amiloride-treated cells was abolished by mucosal niflumic acid, a Ca^{2+}-activated Cl^- channel blocker, or replacement of medium with Cl^--free solution, suggesting that Cl^- transport through Ca^{2+}-dependent Cl^- channel may principally be involved in the response to ATP. This notion was further supported by the findings that the intracellular Ca^{2+} chelator BAPTA-AM inhibited the ATP-induced initial peak response of I_{sc} and that ionomycin caused a transient Cl^- current as ATP did. These results confirmed the existence of Ca^{2+}-activated Cl^- channel in our cultured epithelium. Then, pretreatment with FK506 inhibited the ATP-induced increase in I_{sc} in a concentration-dependent fashion, which was similar to the experiment with $[Ca^{2+}]_i$. Moreover, the effects of rapamycin and cyclosporin A on I_{sc} were consistent with those observed in $[Ca^{2+}]_i$ studies, indicating that the inhibitory effect of FK506 on Cl^- secretion may be attributable to the inhibition of Ca^{2+} dynamics by binding FKBP but not by inactivation of calcineurin.

FK506 is a potent immunosuppressant drug that prevents graft and autoimmune disorders. Recently, FK506 and its derivatives have been found to possess unique biological activities other than their immunosuppressive properties (Dhar et al., 1996; Rokaw et al., 1996; Gold et al., 1997). Our present data add novel calcineurin-independent pharmacologic action of FK506 that inhibits the Ca^{2+} response, which may result in the prevention of Ca^{2+}-mediated inflammatory responses such as Cl^- secretion in airway epithelium.

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