

Soichiro Kanoh, Mitsuko Kondo, Jun Tamaoki, Hideki Shirakawa, Kazutetsu Aoshiba, Shunichi Miyazaki, Hideo Kobayashi, Naokazu Nagata and Atsushi Nagai
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The Conserved Sites for the FK506-binding Proteins in Ryanodine Receptors and Inositol 1,4,5-Trisphosphate Receptors Are Structurally and Functionally Different

G. Bultynck, D. Rossi, G. Callewaert, L. Missiaen, V. Sorrentino, J. B. Parys and H. De Smedt

J. Biol. Chem., December 7, 2001; 276 (50): 47715-47724.

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Differential Regulations between Adenosine Triphosphate (ATP)- and Uridine Triphosphate-Induced Cl⁻ Secretion in Bovine Tracheal Epithelium . Direct Stimulation of P1-like Receptor by ATP

S. Kanoh, M. Kondo, J. Tamaoki, H. Kobayashi, K. Motoyoshi and A. Nagai

Am. J. Respir. Cell Mol. Biol., September 1, 2001; 25 (3): 370-376.

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Effect of FK506 on ATP-induced intracellular calcium oscillations in cow tracheal epithelium

SOICHIRO KANO^H,¹ MITSUKO KONDO,² JUN TAMAOKI,² HIDEKI SHIRAKAWA,³ KAZUTETSU AOSHIBA,² SHUNICHI MIYAZAKI,³ HIDEO KOBAYASHI,¹ NAOKAZU NAGATA,¹ AND ATSUSHI NAGAI²

¹Third Department of Medicine, National Defense Medical College, Saitama 359; and ²First Department of Medicine and ³Department of Physiology, Tokyo Women's Medical College, Tokyo 162, Japan

Kanoh, Soichiro, Mitsuko Kondo, Jun Tamaoki, Hideki Shirakawa, Kazutetsu Aoshiba, Shunichi Miyazaki, Hideo Kobayashi, Naokazu Nagata, and Atsushi Nagai. Effect of FK506 on ATP-induced intracellular calcium oscillations in cow tracheal epithelium. *Am. J. Physiol.* 276 (*Lung Cell. Mol. Physiol.* 20): L891–L899, 1999.—To elucidate the effect of FK506 on Ca²⁺ oscillations in airway epithelium, we investigated cultured cow tracheal epithelial cells with a Ca²⁺ image-analysis system. ATP (1 μM) induced long-lasting Ca²⁺ oscillations, having nearly constant peak values (300–400 nM) and intervals (20–40 s) in subconfluent cells but not in confluent cells. These responses were gradually attenuated and abolished by the addition of FK506. Rapamycin, which binds the FK506-binding protein (FKBP), likewise inhibited Ca²⁺ oscillations, whereas cyclosporin A, a calcineurin inhibitor, did not. Treatment of cells with FK506 decreased Ca²⁺ content in thapsigargin-sensitive stores, suggesting that the partial depletion of the stores causes the inhibition of Ca²⁺ oscillations. Immunocytochemistry revealed the existence of cytoplasmic FKBP-like immunoreactivities. The expression of a 12-kDa FKBP was greater in subconfluent cells than in confluent cells as determined by Western blotting, suggesting that the 12-kDa FKBP may be one of the factors that regulates Ca²⁺ oscillations. Therefore, FK506 possesses an inhibitory action on the Ca²⁺ response via intracellular FKBP but not via calcineurin, which may result in modification of airway epithelial functions.

airway epithelium; adenosine 5'-triphosphate; FK506-binding protein; rapamycin; cyclosporin

IN AIRWAY EPITHELIUM, several inflammatory mediators such as bradykinin and ATP induce an increase in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) (19, 22, 24), which mediates various cell functions including ion transport (8, 19) and mucus secretion (14). Repetitive spikes of [Ca²⁺]_i (Ca²⁺ oscillations) are observed during agonist stimulation in a wide variety of electrically nonexcitable and excitable cells and are considered as physiologically significant Ca²⁺ kinetics that regulate cellular functions (2, 11, 20). Airway epithelial cells have been reported to show Ca²⁺ oscillations in response to mechanical stimulation (4), acetylcholine (25), and neutrophil elastase (18). Although the mechanism of Ca²⁺ oscillations is not fully understood, the intracellular Ca²⁺ release channel (CRC) of the endoplasmic reticulum (ER) such as the inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃R) plays a central role (2, 4, 11, 20).

FK506, a macrolide immunosuppressant drug, binds the FK506-binding protein (FKBP), and the FK506-

FKBP complex inhibits the Ca²⁺-dependent phosphatase calcineurin, thereby preventing calcineurin-dependent interleukin-2 transcription and T-cell proliferation (17, 26). The 12-kDa (FKBP12) and 12.6-kDa FKBP are associated with the ryanodine receptor (RyR) of the sarcoplasmic reticulum in skeletal (12) and cardiac muscle (29) cells, respectively. FK506 causes dissociation of FKBP from RyR and alters its CRC function (3). Likewise, recent evidence suggests that FKBP12 is also associated with the IP₃R and that disrupting this complex by FK506 results in alternation of CRC conductance (6). However, the effect of FK506 on Ca²⁺ oscillations remains unknown. In the present study, we demonstrate Ca²⁺ oscillations induced by exogenous ATP and its inhibition by FK506 in fura 2-loaded cow tracheal epithelial cells.

MATERIALS AND METHODS

Cell culture. Cow tracheae were obtained from a slaughterhouse, and tracheal epithelial cells were isolated by protease as previously described (15). Briefly, strips of epithelium were pulled off the submucosa, washed four times with phosphate-buffered saline (PBS) containing 5 mM dithiothreitol, and rinsed two times with PBS. Epithelial tissues were digested with PBS containing 0.05% protease at 4°C overnight. After neutralization of the protease with 5% fetal calf serum (FCS), the cells were pelleted (200 g for 10 min) and suspended in 50% Dulbecco's modified Eagle's medium (DMEM) and 50% Ham's F-12 nutrient mixture that contained 5% FCS, 1% nonessential amino acids, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 50 μg/ml of gentamicin. The isolated cells were plated at a density of 1.0 × 10⁵ cells/cm² on a glass-bottomed petri dish (MatTek, Ashland, MA) coated with human placental collagen. The medium was changed every 2 days. The cells were cultured for 3–5 or 7–8 days to a subconfluent or confluent stage, respectively, to observe the difference in ATP-induced Ca²⁺ responses between these different culture conditions.

Measurement of [Ca²⁺]_i in single cells. The dish on which the cells were grown was washed with Hanks' balanced salt solution (HBSS) that contained 10 mM HEPES, pH 7.4, and was loaded with 10 μM fura 2-AM for 1 h at 37°C. The dish was then washed several times with HEPES-buffered HBSS and mounted on the stage of an inverted microscope (Diaphot 300, Nikon, Tokyo, Japan). The temperature was kept at 37°C by a ring heater surrounding the dish. For excitation of fura 2 fluorescence, ultraviolet (UV) light of 340- or 380-nm wavelength was produced by a xenon lamp and narrow band-pass filters and applied to the cells through a ×40 objective lens (Fluor 40, Nikon). Emission fluorescence (F) was led to a silicon-intensifier target camera through a 510 ± 10-nm band-pass filter. Ca²⁺ images of the cells were obtained at 3- or 4-s intervals unless otherwise indicated by alternately

applying 340- and 380-nm UV light for 0.125 s (four video frames) for each. Data sets were stored on the hard disk of the computer as eight-bit digital images (256×256 pixels) and processed to calculate the ratio of 340- to 380-nm fluorescence later. The averaged values of the ratios in individual cells were obtained in an optical field in which ~ 40 cells were sampled simultaneously. A calibration curve between the ratio and $[Ca^{2+}]_i$ was obtained by measuring the ratios of Ca^{2+} -*N*-(2-hydroxyethyl)EDTA buffer solutions. All these procedures were performed with an image processor (Argus-50/CA system, Hamamatsu Photonics, Hamamatsu, Japan) (10).

Electron microscopy. To examine morphological differences between subconfluent and confluent cells, the cells on glass-bottomed petri dishes were fixed in 2.5% glutaraldehyde for 2 h and postfixed in 1% osmium tetroxide for 1 h. After dehydration in a graded series of alcohols, the cells were embedded in Epon. Thin sections (80 nm), which were cut perpendicular to the plane of the cell sheet, were mounted on copper grids, stained with lead citrate and uranyl acetate, and examined with a transmission electron microscope (H-7000, Hitachi, Tokyo, Japan).

Immunocytochemistry for FKBP12. Indirect immunofluorescence was used to detect FKBP12 in the cultured tracheal epithelial cells that had been fixed in 3% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were incubated with a 1:10 dilution of monoclonal anti-FKBP12 antibody (2C1) for 30 min at room temperature. After the antibody was removed, the cells were washed three times for 10 min each with PBS. Then the cells were incubated with a 1:100 dilution of the FITC-conjugated anti-mouse IgG goat antibody for 1 h at room temperature, washed with PBS, and observed under a fluorescence microscope. As negative controls, PBS was used as the first antibody instead of the anti-FKBP12 antibody to evaluate nonspecific staining.

Immunoblotting for FKBP12. Western blot analysis was used to assess a possible difference in the amount of FKBP12 between different culture conditions. Subconfluent (4-day culture) and confluent (8-day culture) cells plated on collagen-coated dishes were scraped and homogenized in radioimmuno-precipitation assay buffer (150 mM NaCl, 50 mM Tris·HCl, 0.5% Nonidet P-40, 0.1% SDS, and 2 mM EDTA, pH 7.4) containing 10 μ g/ml each of aprotinin, leupeptin, and phenylmethylsulfonyl fluoride followed by centrifugation (12,000 *g* for 30 min) at 4°C. Equal amounts of protein (75 μ g/lane) from the supernatants were separated by 15% SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride membrane. Recombinant human FKBP12 (0.3 μ g) was also loaded on the gel as a positive control. The membrane was blocked with blocking buffer (150 mM NaCl, 50 mM Tris, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk and 1% goat serum at 4°C overnight. Subsequently, the membrane was incubated with a 1:200 dilution of monoclonal anti-FKBP12 antibody (2C1) for 1 h at room temperature. As a negative control, a piece of membrane was cut along the interlane and incubated with blocking buffer instead of the anti-FKBP12 antibody. The membranes were then incubated with a 1:2,500 dilution of peroxidase-conjugated anti-mouse IgG goat antibody for 1 h at room temperature. The blots were developed with an enhanced chemiluminescent substrate (Pierce, Rockford, IL). Protein band densities were measured with a densitometer (ATTO densitograph, Atto, Tokyo, Japan), and the values are expressed in arbitrary optical density units.

Drugs. DMEM, Ham's F-12 medium, and nonessential amino acids were purchased from GIBCO BRL (Tokyo, Japan). Fura 2-AM was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals, FITC-conjugated or

peroxidase-conjugated anti-mouse IgG goat antibody, and recombinant human FKBP12 were obtained from Sigma (St. Louis, MO). FK506 and anti-FKBP12 antibody (2C1) were gifts from Fujisawa Pharmaceutical (Osaka, Japan). FK506, rapamycin, and cyclosporin A were dissolved in ethanol and used at a final ethanol concentration of <0.1%, and 0.1% ethanol-HEPES-buffered HBSS was employed as a vehicle control.

Statistics. Data are expressed as means \pm SE. Statistical analysis was performed by two-tailed paired or unpaired Student's *t*-test, and a *P* value of <0.05 was considered significant.

RESULTS

ATP-induced Ca^{2+} responses. At the subconfluent stage in culture, the mean resting $[Ca^{2+}]_i$ in single epithelial cells from cow tracheae was 123.1 ± 1.5 nM ($n = 1,881$ cells). After stimulation with 100 μ M ATP, >95% of the cells showed a rapid elevation in $[Ca^{2+}]_i$. This Ca^{2+} response was biphasic, consisting of an initial transient rise and a following sustained elevation (Fig. 1A). When the concentrations of ATP were decreased to 1–10 μ M, some cells showed repetitive Ca^{2+} spikes (i.e., Ca^{2+} oscillations). These Ca^{2+} oscillations could be divided into two groups with respect to their response patterns. One was a decaying pattern in which Ca^{2+} oscillations, consisting of at least three obvious $[Ca^{2+}]_i$ peaks, were gradually attenuated in amplitude and frequency and abolished within 5 min (Fig. 1B). The other was a long-lasting pattern (Fig. 1C). This oscillatory response showed discrete Ca^{2+} spikes arising from a steady $[Ca^{2+}]_i$ level, namely a transient pattern of Ca^{2+} oscillations. These Ca^{2+} oscillations had almost constant intervals (20–40 s) and peak values (300–400 nM) in individual cells and lasted for at least 20–30 min.

The Ca^{2+} -response data for the individual cells under subconfluent culture conditions are summarized in Fig. 2A. In subconfluent cells, the percentage of cells that showed a decaying pattern and a long-lasting pattern of Ca^{2+} oscillations was ~ 10 and 30% at optimal ATP concentrations of 10 and 1 μ M, respectively. In contrast, the percentage of cells that showed a transient $[Ca^{2+}]_i$ response was increased as the concentration of ATP increased. Similar experiments were performed in confluent epithelial cells (Fig. 2B). Under this condition, the mean resting $[Ca^{2+}]_i$ was 116.7 ± 2.4 nM ($n = 782$ cells), and the percentage of cells that showed a transient $[Ca^{2+}]_i$ response was increased in proportion to the concentration of ATP as with subconfluent cells. However, long-lasting Ca^{2+} oscillations were rarely observed and the percentage was <1%, whereas decaying Ca^{2+} oscillations were recognized in $\sim 25\%$ of cells at 10 μ M ATP.

Morphological examination. Because an apparent morphological difference was not observed with light microscopy between cells that showed oscillatory and nonoscillatory Ca^{2+} responses, ultrastructural examination was performed with electron microscopy. As shown in Fig. 3, subconfluent cells cultured for 4 days had microvilli and tight junctions, which are typical characteristics of epithelial cells, although they were flattened

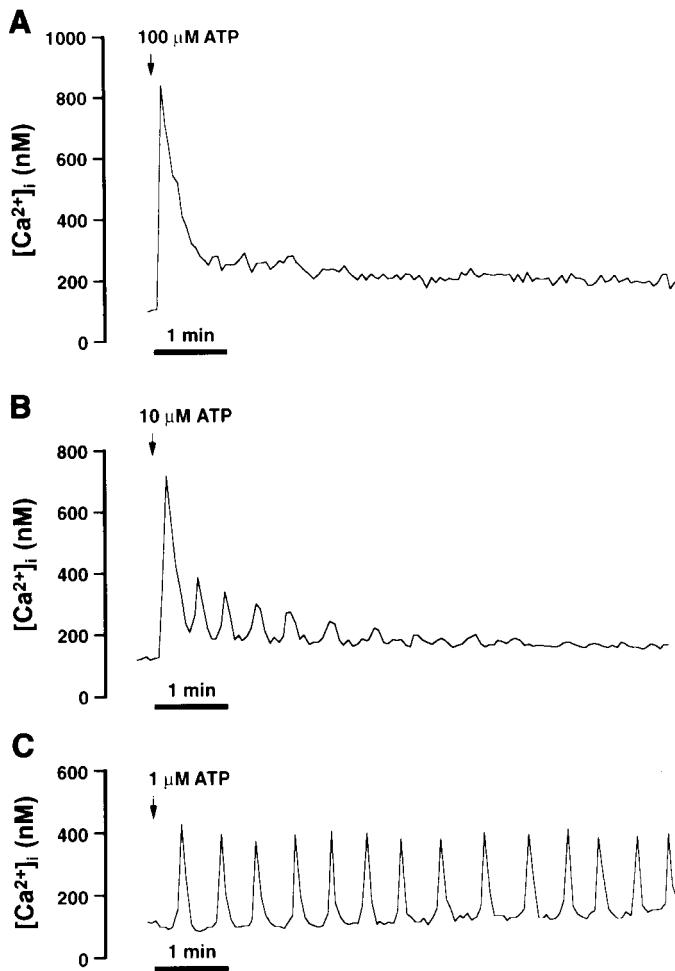


Fig. 1. Representative recordings of ATP-induced Ca^{2+} responses in single cow tracheal epithelium under subconfluent culture condition. *A*: 100 μ M ATP induced a transient rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by a sustained elevation. *B*: decaying Ca^{2+} oscillations induced by 10 μ M ATP in which oscillations were gradually attenuated in amplitude and frequency and abolished within 5 min. *C*: long-lasting Ca^{2+} oscillations induced by 1 μ M ATP. In this pattern, repetitive spikes of $[Ca^{2+}]_i$ lasted for at least 20–30 min. Arrows, addition of ATP.

in appearance. Similarly, confluent cells cultured for 8 days had slightly longer and more apparent microvilli in addition to the above findings. However, both cultured cells lacked cilia and secretory granules and showed an undifferentiated appearance.

Effect of FK506 on ATP-induced Ca^{2+} oscillations. Cultured airway epithelial cells showed several patterns of Ca^{2+} responses according to ATP concentrations and culture conditions. In this experiment, we selected 1 μ M ATP and subconfluent cells to examine the effect of FK506 on Ca^{2+} oscillations because this ATP concentration and culture condition seemed optimal to induce long-lasting Ca^{2+} oscillations. Vehicle alone added during Ca^{2+} oscillations had no effect on ongoing Ca^{2+} spikes (data not shown). After the addition of 1 μ M FK506 during Ca^{2+} oscillations, the first Ca^{2+} spike became slightly larger, but the subsequent Ca^{2+} oscillations were gradually attenuated in amplitude and frequency and were eventually abolished

(Fig. 4A). Likewise, the addition of 10 μ M FK506 produced a more pronounced inhibition of ongoing Ca^{2+} oscillations (Fig. 4B). This inhibitory effect was prevented when excess recombinant human FKBP12 was added to the extracellular milieu before FK506 (Fig. 4C). Rapamycin (1 μ M), another immunosuppressant drug that binds FKBP with high affinity, showed a similar inhibitory effect on ongoing Ca^{2+} oscillations as FK506 (Fig. 4D), whereas cyclosporin A (1 μ M), a specific calcineurin inhibitor that does not bind FKBP, had little effect (data not shown).

Effect of FK506 on Ca^{2+} content in thapsigargin-sensitive stores. Thapsigargin (TG), a specific ER Ca^{2+} -pump inhibitor (27), added during Ca^{2+} oscillations caused a sustained Ca^{2+} release and thereafter abolished the oscillations (Fig. 4E). Moreover, as shown in Fig. 5A, the addition of TG evoked a rise in $[Ca^{2+}]_i$, an effect that may be due to the depletion of intracellular Ca^{2+} stores, and inhibited the subsequent elevation in ATP (100 μ M)-induced $[Ca^{2+}]_i$, implying that the response to ATP is attributable to mobilization of Ca^{2+} from TG-sensitive stores. We thus examined the effect of FK506 on Ca^{2+} content in TG-sensitive stores to elucidate the site of action of FK506. To do so, EGTA (5 mM) was added to the medium to chelate extracellular Ca^{2+} 30 s before the addition of TG because deple-

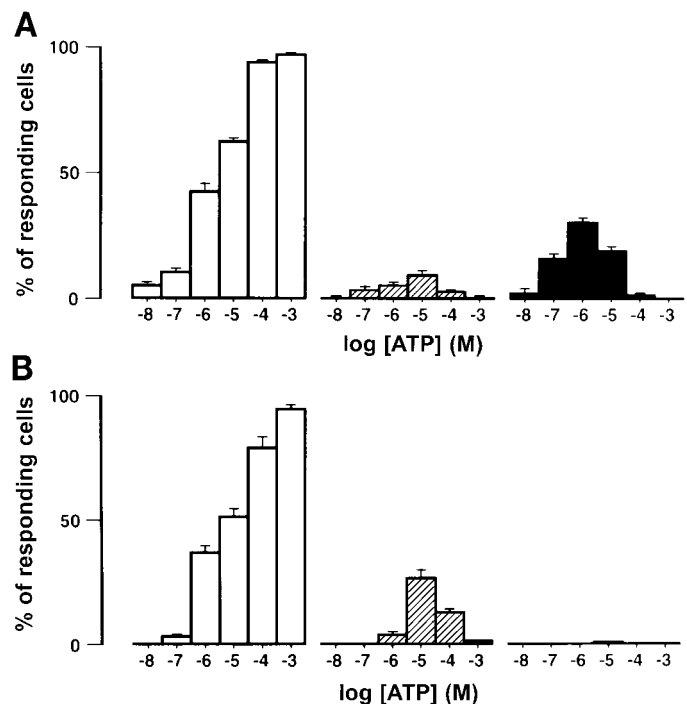


Fig. 2. Relationship between ATP concentration ($[ATP]$) and percentage of responding cells that showed transient Ca^{2+} rise (open bars), decaying Ca^{2+} oscillations (hatched bars), and long-lasting Ca^{2+} oscillations (solid bars) under different culture conditions. Data are means \pm SE; $n = 4$ –20 experiments/ATP concentration where 30–40 cells were observed in individual experiments. *A*: under subconfluent culture conditions, nearly all cells showed transient Ca^{2+} rise in response to >100 μ M ATP, whereas greatest percentage of long-lasting oscillating cells was observed at 1 μ M ATP. *B*: under confluent culture conditions, long-lasting Ca^{2+} oscillations were rarely observed and percentage was $<1\%$, although decaying Ca^{2+} oscillations were recognized in $\sim 25\%$ cells at 10 μ M ATP.

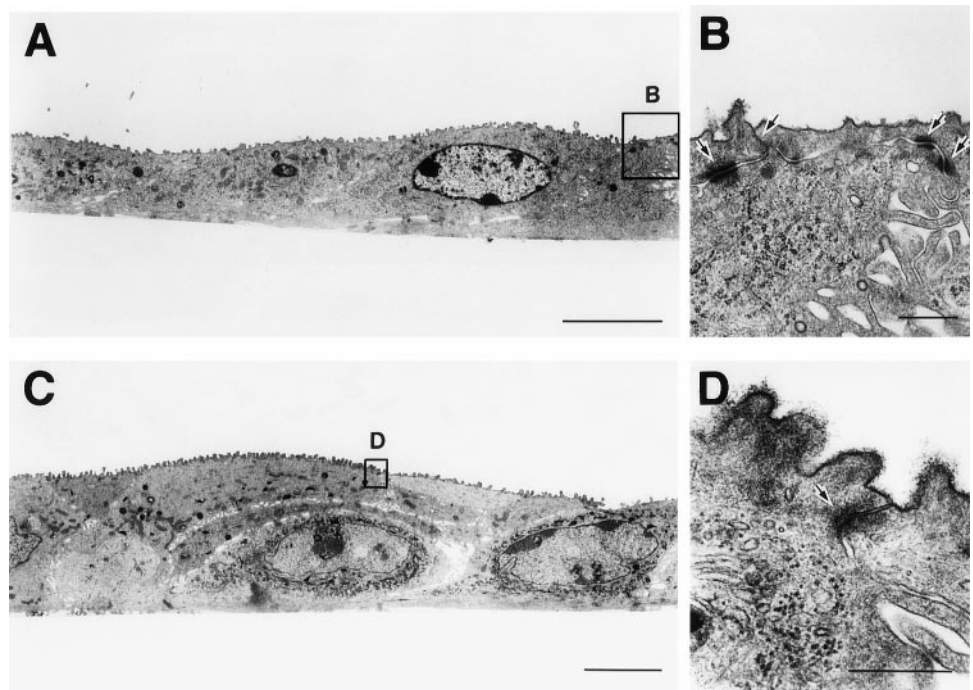


Fig. 3. Transmission electron micrographs of cow tracheal epithelial cells cultured for 4 [A and B (enlargement of box in A)] and 8 days [C and D (enlargement of box in C)]. Both cultured cells had typical epithelial characteristics such as microvilli and junctional complexes (arrows) but lacked cilia and secretory granules. Bars: 5 μ m in A and C; 500 nm in B and D.

tion of Ca^{2+} in ER causes Ca^{2+} influx from the external solution through the capacitative Ca^{2+} entry pathway (23), and the area beneath the records of the rise in TG-evoked $[Ca^{2+}]_i$ above the basal level was integrated. Examples of estimated Ca^{2+} content in TG-sensitive stores treated with vehicle alone and with 1 μ M FK506 for 5 min are demonstrated in Fig. 5, B and C, insets, respectively. Similar experiments were carried out at various time points (1, 3, 5, and 10 min) after the addition of vehicle alone or FK506, and the results were compared with those in the cells with no drugs added (control; 100%). As shown in Fig. 6, vehicle alone had no effect, but FK506 (1 μ M) significantly diminished the ER Ca^{2+} content to 69.6 ± 6.3 and $70.0 \pm 6.5\%$ at 5 and 10 min, respectively, after the addition ($P < 0.01$; $n = 60$ cells/group).

Effect of FK506 on basal $[Ca^{2+}]_i$ level. To determine the effect of FK506 itself on basal $[Ca^{2+}]_i$ level, 1 μ M FK506 was added to cultured airway epithelial cells and the response was compared with that of the vehicle control. Ca^{2+} images were acquired every minute to avoid the influence of UV light for excitation of the cells and fura 2. Basal $[Ca^{2+}]_i$ before the addition of FK506 was 124.3 ± 3.1 nM ($n = 120$ cells) and was significantly elevated 3 min after the addition (125.2 ± 3.2 nM; $P < 0.05$). The increase in $[Ca^{2+}]_i$ 5 min after FK506 (130.5 ± 3.3 nM; $P < 0.001$) was >5 nM, and it reached a plateau thereafter, whereas vehicle alone had no such effect (Fig. 7).

Effect of FK506 on ATP-induced transient Ca^{2+} rise. To examine whether the reduction in Ca^{2+} content in TG-sensitive stores by FK506 has an effect on the Ca^{2+} response induced by a high concentration of ATP, 100 μ M ATP was added after treatment with 1 μ M FK506 or vehicle alone for 10 min. As shown in Fig. 8, the peak $[Ca^{2+}]_i$ level was smaller in FK506-treated cells than in

vehicle-treated cells. The increases in $[Ca^{2+}]_i$ from the basal level were 494.4 ± 12.4 and 647.2 ± 18.8 nM, respectively ($P < 0.001$; $n = 120$ cells/group).

Detection of FKBP12. To assess whether FKBP12 is present in our cultured tracheal epithelial cells, immunocytochemical staining with a monoclonal anti-FKBP12 antibody was performed. As shown in Fig. 9, immunofluorescence was observed in the cytoplasm of subconfluent cells, indicating that epithelial cells possess FKBP-like immunoreactivities. The localization of FKBP12 was likewise found in confluent cells (data not shown). To further investigate whether the amount of FKBP12 is different according to culture periods, a Western blotting technique was used. As shown in Fig. 10A, specific binding for FKBP12 was observed in both subconfluent and confluent cells (lanes 2 and 3, respectively), and densitometric estimation of the FKBP12 quantity revealed that subconfluent cells had more FKBP-like immunoreactivities than did confluent cells (Fig. 10B).

DISCUSSION

This study showed that low concentrations of ATP induce long-lasting Ca^{2+} oscillations in subconfluent cells and that FK506 inhibits Ca^{2+} oscillations in a dose-dependent manner. It is well known that inflammatory mediators such as bradykinin and ATP evoke an increase in $[Ca^{2+}]_i$ (19, 22, 24), but there are few reports regarding Ca^{2+} oscillations induced by mediators in airway epithelium (18, 23). The range of ATP concentrations that evoked oscillatory responses was limited, and >100 μ M ATP did not cause Ca^{2+} oscillations in most cells. We thus speculate that moderate stimulation might be appropriate for ongoing Ca^{2+} oscillations in individual cells. These observations are

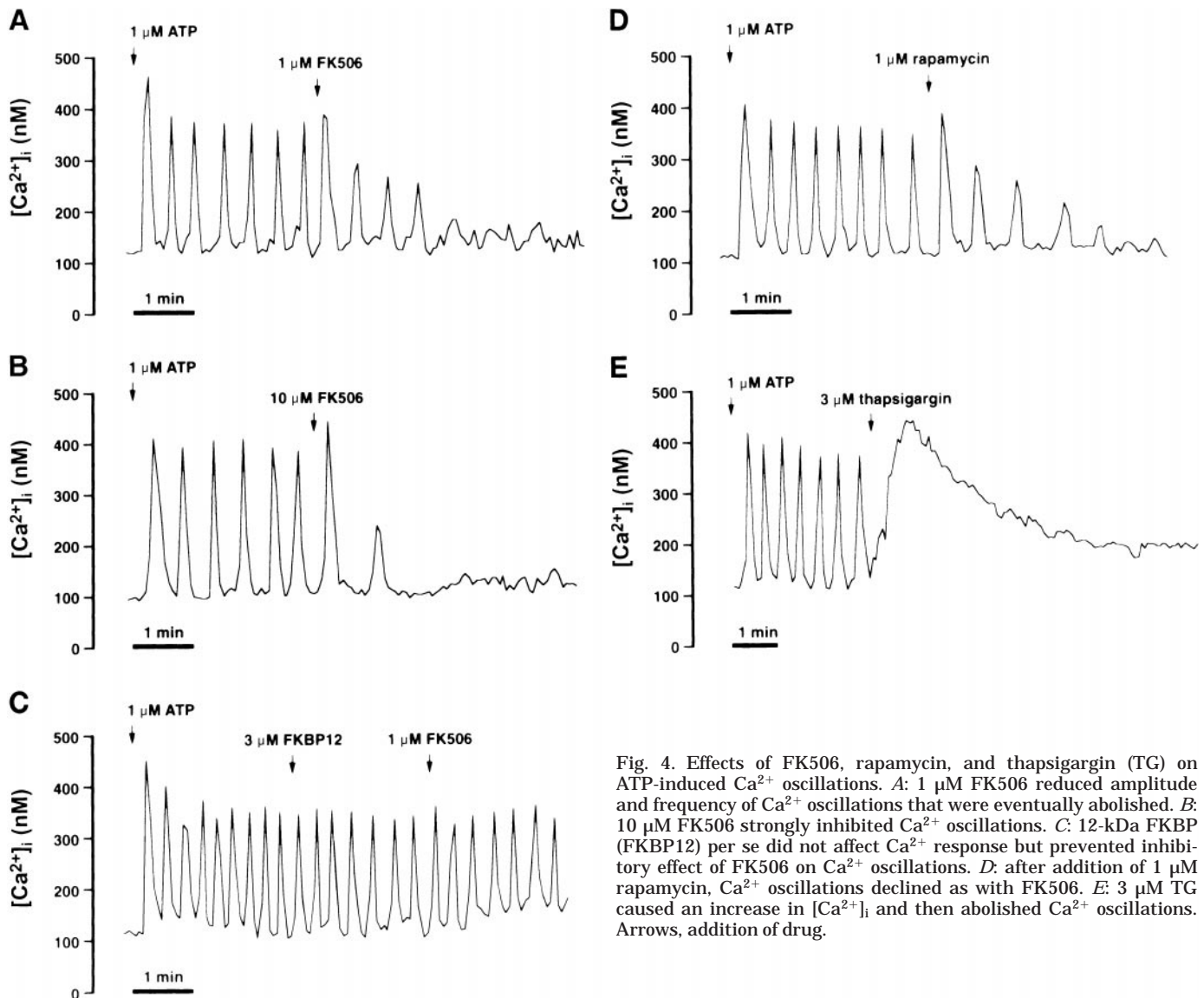


Fig. 4. Effects of FK506, rapamycin, and thapsigargin (TG) on ATP-induced Ca^{2+} oscillations. *A*: 1 μM FK506 reduced amplitude and frequency of Ca^{2+} oscillations that were eventually abolished. *B*: 10 μM FK506 strongly inhibited Ca^{2+} oscillations. *C*: 12-kDa FKBP (FKBP12) per se did not affect Ca^{2+} response but prevented inhibitory effect of FK506 on Ca^{2+} oscillations. *D*: after addition of 1 μM rapamycin, Ca^{2+} oscillations declined as with FK506. *E*: 3 μM TG caused an increase in $[\text{Ca}^{2+}]_i$ and then abolished Ca^{2+} oscillations. Arrows, addition of drug.

consistent with previous reports (28, 32) using different agonists on other cell types. In addition, even at an optimal concentration of ATP (1 μM), only 30% of the cells at the subconfluent stage showed long-lasting Ca^{2+} oscillations. However, we could not find the morphological difference with light microscopy between the cells that showed Ca^{2+} oscillations and those that did not (data not shown). Moreover, there was no difference in the ultrastructure between subconfluent that tended to show oscillatory responses and confluent cells that did not (Fig. 3). Regarding the difference in the incidence of agonist-induced Ca^{2+} oscillations according to different culture periods, Salathe and Bookman (25) showed that acetylcholine produced Ca^{2+} oscillations in sheep tracheal epithelial ciliated cells only early in culture (1–3 days). Therefore, the cells cultured for relatively short periods may have the ability to produce long-lasting Ca^{2+} oscillations. Although the reason for this is unknown, the difference in the amount of FKBP12 could be involved, as described later.

Extracellular ATP stimulates P2U receptor in the airway epithelium, activates phospholipase C, and produces IP_3 , which in turn mobilizes Ca^{2+} from the ER via the IP_3R (14). Intracellular Ca^{2+} pools play a role in the regulation of $[\text{Ca}^{2+}]_i$ and are profoundly involved in Ca^{2+} oscillations (2, 4, 11, 20). Some investigators (1, 3, 7, 13) have shown that FKBP12 associates with both skeletal and cardiac RyRs and modulate CRC function in lipid bilayers and that FK506 or rapamycin increases the open probability of the RyRs. Furthermore, Cameron et al. (6) demonstrated that the physiological role of FKBP is to stabilize the CRC function of IP_3R as well as of RyR by promoting optimal cooperativity among four subunits of the channel and that dissociation of FKBP12 from the IP_3R by FK506 and rapamycin causes leakiness in the gating properties of the channel. We therefore expected that FK506 added during Ca^{2+} oscillations would potentiate Ca^{2+} release. It is of interest, however, that FK506 gradually attenuated and abolished ATP-induced Ca^{2+} oscillations, although

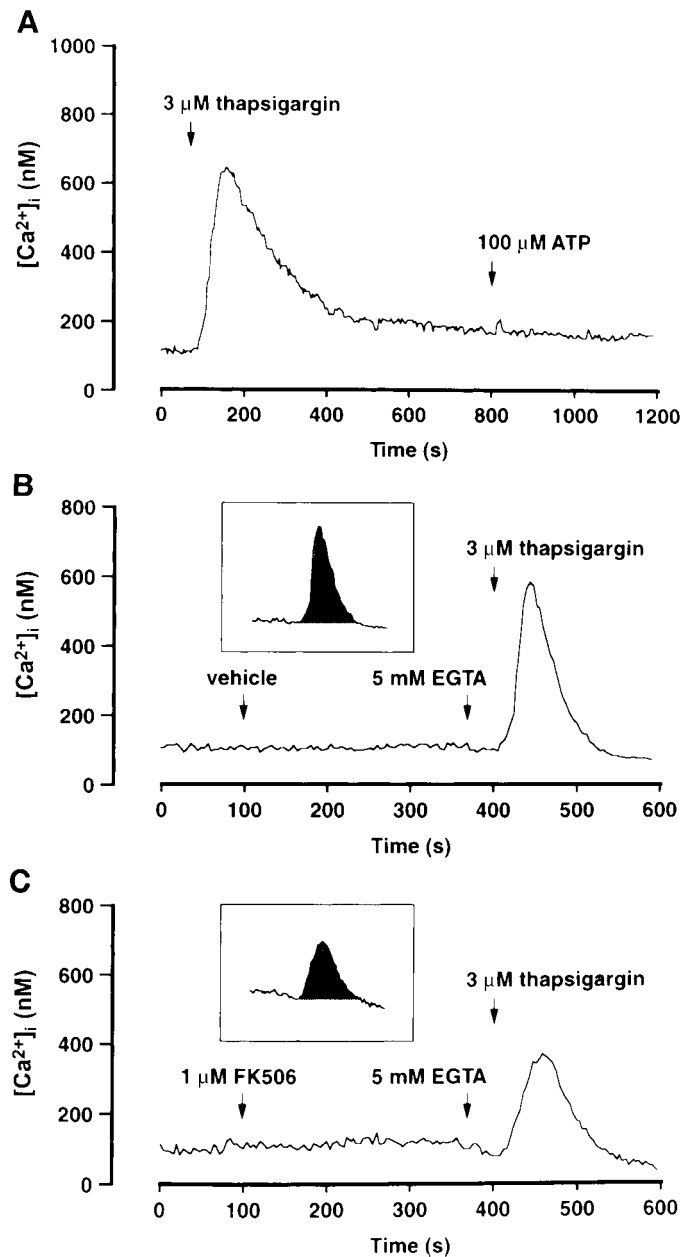


Fig. 5. Inhibition of ATP-induced increase in $[Ca^{2+}]_i$ by TG and effect of FK506 on TG-evoked $[Ca^{2+}]_i$ rise. A: TG evoked a transient $[Ca^{2+}]_i$ rise followed by a sustained response. Subsequent addition of ATP did not cause a substantial increase in $[Ca^{2+}]_i$. After treatment with vehicle alone (B) or FK506 (C) for 5 min, TG was added in presence of EGTA. $[Ca^{2+}]_i$ showed transient increase and then declined to below basal level. As an index of Ca^{2+} content in TG-sensitive stores, area beneath records of TG-evoked $[Ca^{2+}]_i$ rise above basal level was integrated (insets, solid areas).

the first Ca^{2+} spike was potentiated after the addition of FK506. One possible explanation for this observation would be that the slightly larger Ca^{2+} spike reflects the increased leakiness of CRC, and the following suppression of Ca^{2+} oscillations may be exerted by the decrease in the net Ca^{2+} accumulation of intracellular Ca^{2+} stores. To confirm this, we examined the Ca^{2+} content in TG-sensitive stores in FK506-treated cells because the source of ATP-stimulated Ca^{2+} release appeared to

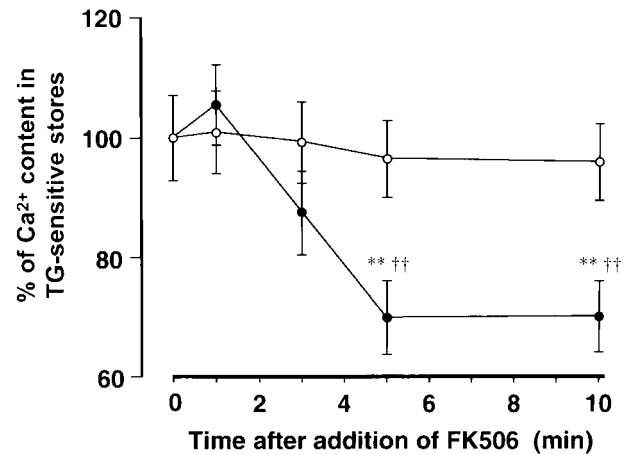


Fig. 6. Effect of FK506 on Ca^{2+} content in TG-sensitive stores. Cells were pretreated with vehicle alone (\circ) or FK506 (1 μ M; \bullet) for indicated times, and Ca^{2+} content in TG-sensitive stores was determined. Values are means \pm SE of percentage of control response (no drug added); $n = 60$ cells/time point. Vehicle alone had no effect, whereas FK506 significantly diminished Ca^{2+} content 5 min later. Significantly different ($P < 0.01$) from: ** control value; $\dagger\dagger$ corresponding response to vehicle alone.

be TG-sensitive stores (Figs. 4E and 5A). In the presence of EGTA, the rise in $[Ca^{2+}]_i$ induced by TG is supposed to reflect the release from intracellular Ca^{2+} stores (Fig. 5B). Under this condition, 1 μ M FK506 significantly decreased the ER Ca^{2+} content by 30% 5 min after being added (Figs. 5C and 6). If this partial depletion of ER Ca^{2+} content is due to the increased leakiness of CRC, it must be preceded by the elevation in $[Ca^{2+}]_i$ resulting from leaked Ca^{2+} . This hypothesis was supported by the finding that basal $[Ca^{2+}]_i$ was significantly elevated 3 min after the addition of 1 μ M FK506 and formed a new $[Ca^{2+}]_i$ level that was 5 nM higher than the initial level in 5 min (Fig. 7). These results are in accord with previous reports that the leakiness of CRC by FK506 or rapamycin causes partial depletion of Ca^{2+} in RyR-gated (3, 30, 31) or IP₃R-gated (6) stores. However, there seems to be a discrepancy in

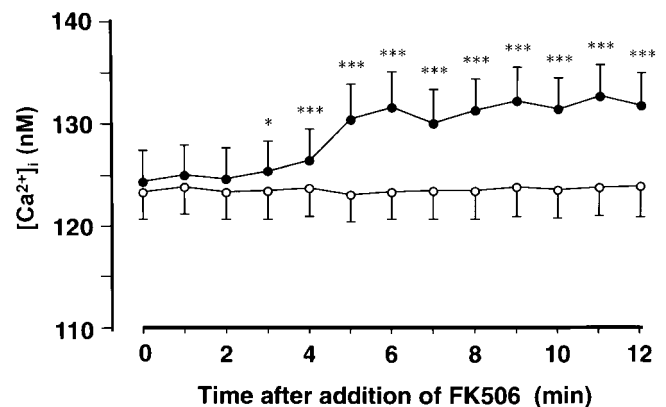


Fig. 7. Effect of FK506 on basal $[Ca^{2+}]_i$ level in airway epithelial cells. Vehicle alone (\circ) or FK506 (1 μ M; \bullet) was added, and $[Ca^{2+}]_i$ was measured every minute. Values are means \pm SE; $n = 120$ cells/time point. Basal $[Ca^{2+}]_i$ after addition of FK506 was significantly elevated 3 min later and reached a plateau at 5 min, whereas vehicle had no effect on basal $[Ca^{2+}]_i$. Significantly different from initial (0-min) baseline $[Ca^{2+}]_i$ value: * $P < 0.05$; *** $P < 0.001$.

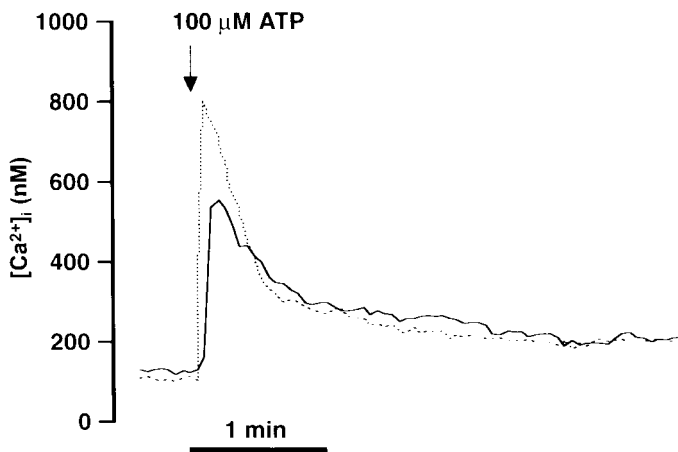


Fig. 8. Representative recordings of ATP-induced transient Ca^{2+} rise in cultured cow tracheal epithelium treated with FK506 (solid line) and vehicle alone (dotted line). After treatment with $1 \mu M$ FK506 or vehicle alone for 10 min, ATP was added (arrow).

the time course between the depletion of Ca^{2+} content and the immediate inhibition of Ca^{2+} oscillations by FK506. Brillantes et al. (3) have shown that when caffeine was applied successively to rapamycin-treated sarcoplasmic reticulum vesicles from rabbit skeletal muscle, Ca^{2+} release induced by the second caffeine addition was smaller than that by the first, indicating that the vesicles are partially depleted of Ca^{2+} . Timerman et al. (30) also reported that the time to load Ca^{2+} into terminal cisternae vesicles treated with FK506 was distinctly prolonged and that their Ca^{2+} uptake rate was reduced because of the leakiness of RyRs. Taken together, the immediate inhibition of Ca^{2+} oscillations by FK506 may be due to insufficient time to take up Ca^{2+} fully into the ER. Because repetitive Ca^{2+} spikes depend on Ca^{2+} refilling into intracellular Ca^{2+} stores, the depleted stores are probably unable to keep Ca^{2+} oscillations. Prior addition of excess FKBP12 prevented the inhibitory effect of FK506. This suggests that extracellular FKBP12 may have trapped FK506 outside the cells, and hence FK506 could not penetrate into the cells and bind intracellular FKBP.

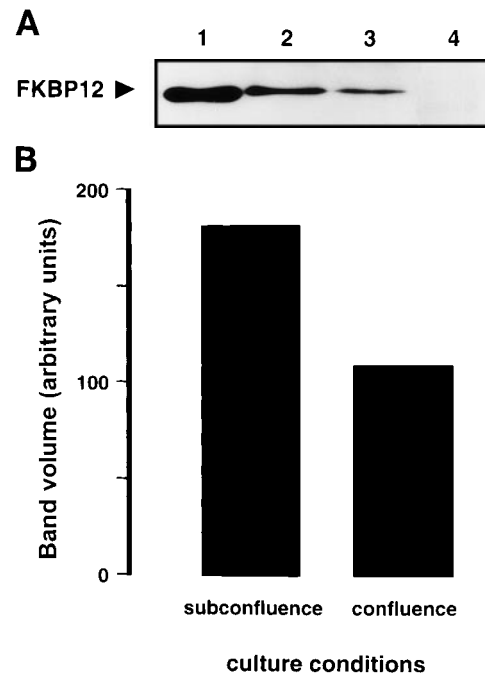


Fig. 10. Expression of FKBP12 in subconfluent and confluent cells as determined by Western blot analysis. *A*: recombinant human FKBP12 ($0.3 \mu g$; lane 1) and equal amounts of extracted protein ($75 \mu g$) from subconfluent (lanes 2 and 4) and confluent (lane 3) cells were subjected to 15% SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane, which was developed with (lanes 1–3) and without (lane 4) anti-FKBP12 antibody and peroxidase-conjugated anti-mouse IgG goat antibody. Blots were visualized with enhanced chemiluminescence. *B*: FKBP12 band densities of subconfluent and confluent cells were measured with a densitometer. Results are representatives of 3 independent experiments.

According to Cameron et al. (5), phosphorylation of IP_3R by protein kinase C increases CRC activity in response to IP_3 , whereas dephosphorylation by calcineurin that is anchored to the IP_3R via FKBP12 decreases CRC activity. They suggested that reversible phosphorylation cycles in the IP_3R are important as a mechanism of ongoing Ca^{2+} oscillations. We therefore assessed the effect of rapamycin and cyclosporin A to elucidate whether the effect of FK506 is due to inhibi-

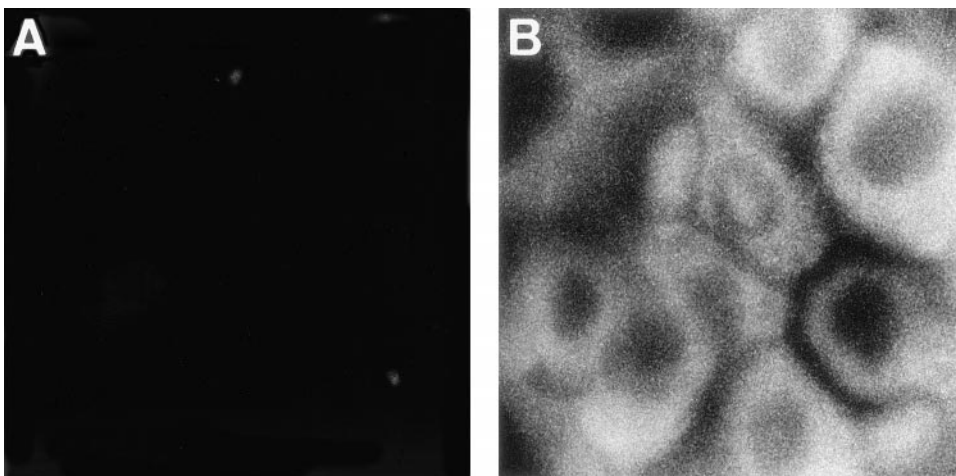


Fig. 9. Indirect immunofluorescence of FKBP12 in cow cultured tracheal epithelium at subconfluent stage in culture. Immunocytochemical staining was performed with (B) and without (A) anti-FKBP12 antibody as a first antibody. Original magnification, $\times 400$.

tion of calcineurin. The addition of rapamycin inhibited Ca^{2+} oscillations as did FK506, but cyclosporin A was without effect, suggesting that FK506 may exert its effect by disturbing FKBP- IP_3R association rather than by interfering with calcineurin-dependent dephosphorylation of IP_3Rs . Thus intracellular FKBP appears to be crucial for Ca^{2+} oscillations.

Positive staining with anti-FKBP12 antibody confirmed the existence of cytoplasmic FKBP in our cultured epithelium. However, it was difficult to find a significant difference in immunocytochemical staining between individual cells that showed oscillatory and nonoscillatory Ca^{2+} responses induced by ATP or between subconfluent and confluent cells. Thus a Western blotting technique was employed to further investigate the difference in FKBP12 expression between our cultured epithelial cells at a subconfluent stage, which tended to show long-lasting Ca^{2+} oscillations, and those at a confluent stage, which did not. We found that subconfluent cells had a greater amount of FKBP12 than confluent cells as shown in Fig. 9. Although this cannot readily explain the physiological relevance, we speculate that FKBP12 may be one of the factors that regulates the occurrence of Ca^{2+} oscillations.

In conclusion, FK506 is a potent immunosuppressant macrolide that prevents graft rejection and autoimmune disorders, and this drug has recently been shown to reduce allergic airway inflammation (16) and airway hyperreactivity (9, 21). Our present data may add new information about the pharmacological actions of FK506 that inhibit Ca^{2+} oscillations, which may result in the prevention of Ca^{2+} -mediated inflammatory responses in airway epithelium.

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Address for reprint requests and other correspondence: A. Nagai, First Dept. of Medicine, Tokyo Women's Medical College 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162, Japan (E-mail: a-nagai@tkd.att.ne.jp).

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