

**Soichiro Kanoh, Mitsuko Kondo, Jun Tamaoki, Hideki Shirakawa, Kazutetsu Aoshiba, Shunichi Miyazaki, Hideo Kobayashi, Naokazu Nagata and Atsushi Nagai**  
*Am J Physiol Lung Cell Mol Physiol* 276:891-899, 1999.

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

SOICHIRO KANO<sup>1</sup>, MITSUKO KONDO<sup>2</sup>, JUN TAMAOKI<sup>2</sup>, HIDEKI SHIRAKAWA<sup>3</sup>,  
KAZUTETSU AOSHIBA<sup>2</sup>, SHUNICHI MIYAZAKI<sup>3</sup>, HIDEO KOBAYASHI<sup>1</sup>,  
NAOKAZU NAGATA<sup>1</sup> AND ATSUSHI NAGAI<sup>2</sup>

<sup>1</sup>Third Department of Medicine, National Defense Medical College, Saitama 359;  
and <sup>2</sup>First Department of Medicine and <sup>3</sup>Department 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 Ca<sup>2+</sup> oscillations in airway epithelium, we investigated cultured cow tracheal epithelial cells with a Ca<sup>2+</sup> image-analysis system. ATP (1 μM) induced long-lasting Ca<sup>2+</sup> 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 Ca<sup>2+</sup> oscillations, whereas cyclosporin A, a calcineurin inhibitor, did not. Treatment of cells with FK506 decreased Ca<sup>2+</sup> content in thapsigargin-sensitive stores, suggesting that the partial depletion of the stores causes the inhibition of Ca<sup>2+</sup> 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 Ca<sup>2+</sup> oscillations. Therefore, FK506 possesses an inhibitory action on the Ca<sup>2+</sup> 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 Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) (19, 22, 24), which mediates various cell functions including ion transport (8, 19) and mucus secretion (14). Repetitive spikes of [Ca<sup>2+</sup>]<sub>i</sub> (Ca<sup>2+</sup> oscillations) are observed during agonist stimulation in a wide variety of electrically nonexcitable and excitable cells and are considered as physiologically significant Ca<sup>2+</sup> kinetics that regulate cellular functions (2, 11, 20). Airway epithelial cells have been reported to show Ca<sup>2+</sup> oscillations in response to mechanical stimulation (4), acetylcholine (25), and neutrophil elastase (18). Although the mechanism of Ca<sup>2+</sup> oscillations is not fully understood, the intracellular Ca<sup>2+</sup> release channel (CRC) of the endoplasmic reticulum (ER) such as the inositol 1,4,5-trisphosphate (IP<sub>3</sub>) receptor (IP<sub>3</sub>R) 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 Ca<sup>2+</sup>-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 IP<sub>3</sub>R and that disrupting this complex by FK506 results in alternation of CRC conductance (6). However, the effect of FK506 on Ca<sup>2+</sup> oscillations remains unknown. In the present study, we demonstrate Ca<sup>2+</sup> 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 × 10<sup>5</sup> cells/cm<sup>2</sup> 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 Ca<sup>2+</sup> responses between these different culture conditions.

**Measurement of [Ca<sup>2+</sup>]<sub>i</sub> 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. Ca<sup>2+</sup> 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 ( $256 \times 256$  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  $\sim 40$  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  $\mu$ 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  $\mu$ g/lane) from the supernatants were separated by 15% SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride membrane. Recombinant human FKBP12 (0.3  $\mu$ 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  $123.1 \pm 1.5$  nM ( $n = 1,881$  cells). After stimulation with 100  $\mu$ 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  $\mu$ 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  $\sim 10$  and 30% at optimal ATP concentrations of 10 and 1  $\mu$ 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  $116.7 \pm 2.4$  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  $\mu$ 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

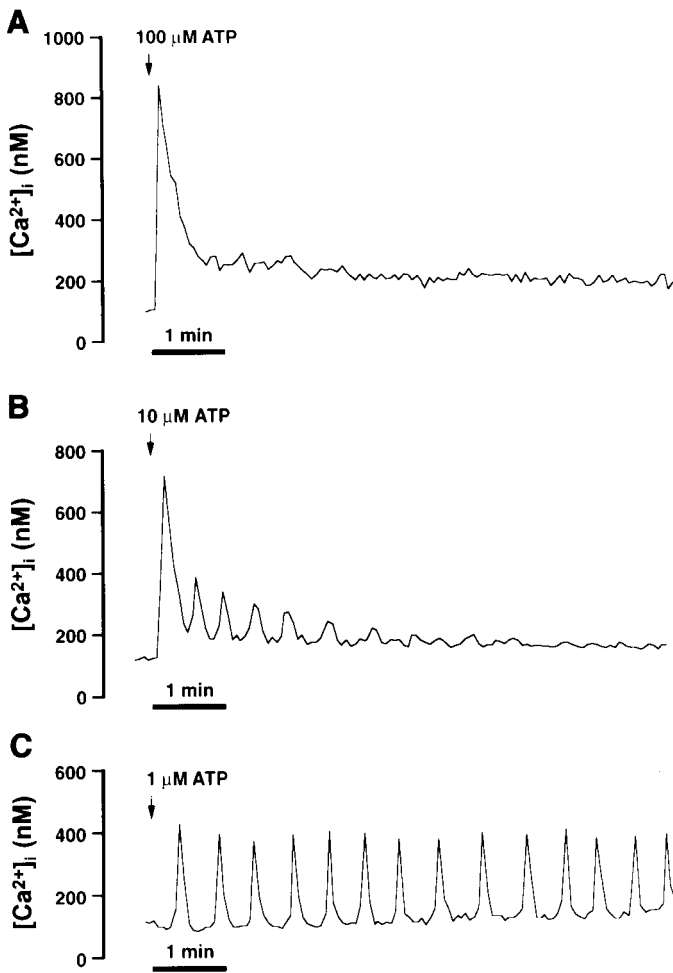


Fig. 1. Representative recordings of ATP-induced  $Ca^{2+}$  responses in single cow tracheal epithelium under subconfluent culture condition. *A*: 100  $\mu$ M ATP induced a transient rapid increase in intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) followed by a sustained elevation. *B*: decaying  $Ca^{2+}$  oscillations induced by 10  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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  $\mu$ 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-

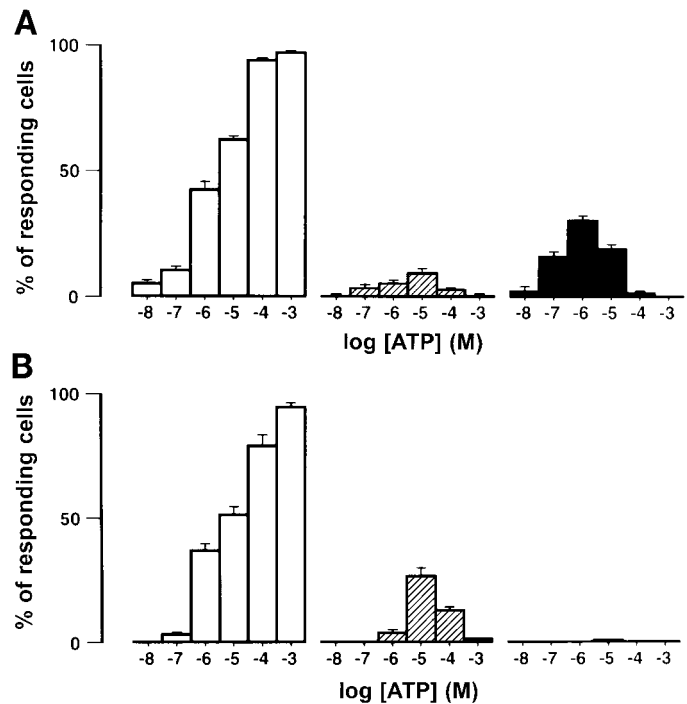


Fig. 2. Relationship between ATP concentration ( $[ATP]$ ) and percentage of responding cells that showed transient  $Ca^{2+}$  rise (open bars), decaying  $Ca^{2+}$  oscillations (hatched bars), and long-lasting  $Ca^{2+}$  oscillations (solid bars) under different culture conditions. Data are means  $\pm$  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  $>100$   $\mu$ M ATP, whereas greatest percentage of long-lasting oscillating cells was observed at 1  $\mu$ 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  $\mu$ M ATP.

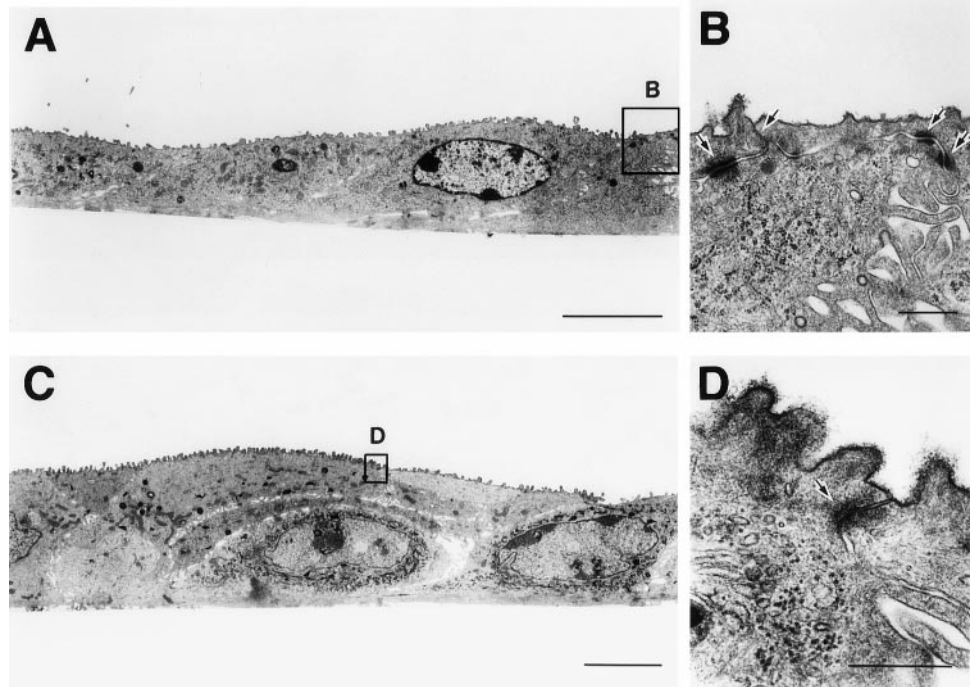


Fig. 3. Transmission electron micrographs of cow tracheal epithelial cells cultured for 4 [A and B (enlargement of box in A)] and 8 days [C and D (enlargement of box in C)]. Both cultured cells had typical epithelial characteristics such as microvilli and junctional complexes (arrows) but lacked cilia and secretory granules. Bars: 5  $\mu$ 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  $\mu$ 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  $\mu$ M) significantly diminished the ER  $Ca^{2+}$  content to  $69.6 \pm 6.3$  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  $\mu$ 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  $124.3 \pm 3.1$  nM ( $n = 120$  cells) and was significantly elevated 3 min after the addition ( $125.2 \pm 3.2$  nM;  $P < 0.05$ ). The increase in  $[Ca^{2+}]_i$  5 min after FK506 ( $130.5 \pm 3.3$  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  $\mu$ M ATP was added after treatment with 1  $\mu$ 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  $494.4 \pm 12.4$  and  $647.2 \pm 18.8$  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 (Janes 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  $>100$   $\mu$ 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

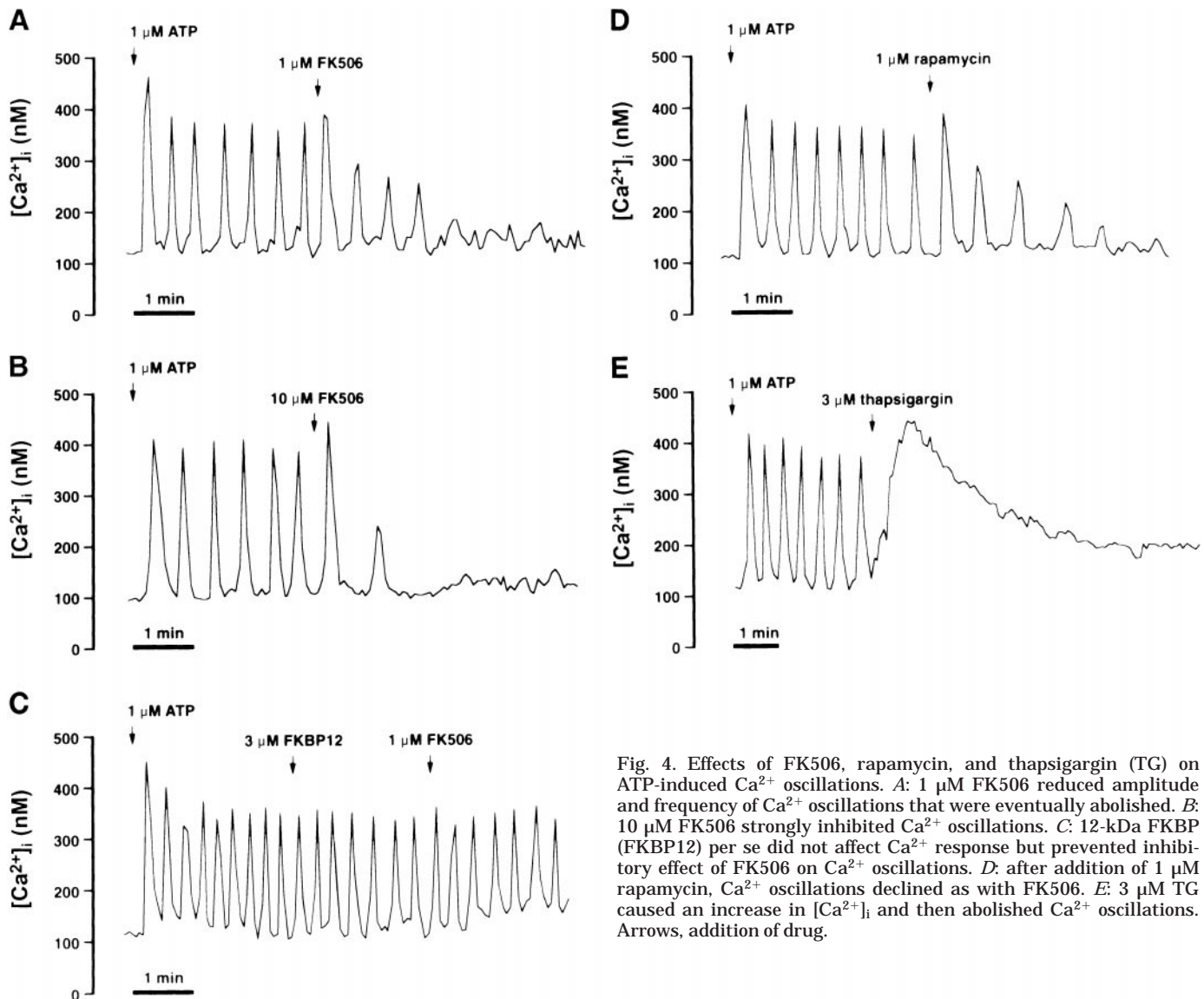


Fig. 4. Effects of FK506, rapamycin, and thapsigargin (TG) on ATP-induced  $\text{Ca}^{2+}$  oscillations. *A*: 1  $\mu\text{M}$  FK506 reduced amplitude and frequency of  $\text{Ca}^{2+}$  oscillations that were eventually abolished. *B*: 10  $\mu\text{M}$  FK506 strongly inhibited  $\text{Ca}^{2+}$  oscillations. *C*: 12-kDa FKBP (FKBP12) per se did not affect  $\text{Ca}^{2+}$  response but prevented inhibitory effect of FK506 on  $\text{Ca}^{2+}$  oscillations. *D*: after addition of 1  $\mu\text{M}$  rapamycin,  $\text{Ca}^{2+}$  oscillations declined as with FK506. *E*: 3  $\mu\text{M}$  TG caused an increase in  $[\text{Ca}^{2+}]_i$  and then abolished  $\text{Ca}^{2+}$  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  $\mu\text{M}$ ), only 30% of the cells at the subconfluent stage showed long-lasting  $\text{Ca}^{2+}$  oscillations. However, we could not find the morphological difference with light microscopy between the cells that showed  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  oscillations according to different culture periods, Salathe and Bookman (25) showed that acetylcholine produced  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{IP}_3$ , which in turn mobilizes  $\text{Ca}^{2+}$  from the ER via the  $\text{IP}_3\text{R}$  (14). Intracellular  $\text{Ca}^{2+}$  pools play a role in the regulation of  $[\text{Ca}^{2+}]_i$  and are profoundly involved in  $\text{Ca}^{2+}$  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  $\text{IP}_3\text{R}$  as well as of RyR by promoting optimal cooperativity among four subunits of the channel and that dissociation of FKBP12 from the  $\text{IP}_3\text{R}$  by FK506 and rapamycin causes leakiness in the gating properties of the channel. We therefore expected that FK506 added during  $\text{Ca}^{2+}$  oscillations would potentiate  $\text{Ca}^{2+}$  release. It is of interest, however, that FK506 gradually attenuated and abolished ATP-induced  $\text{Ca}^{2+}$  oscillations, although

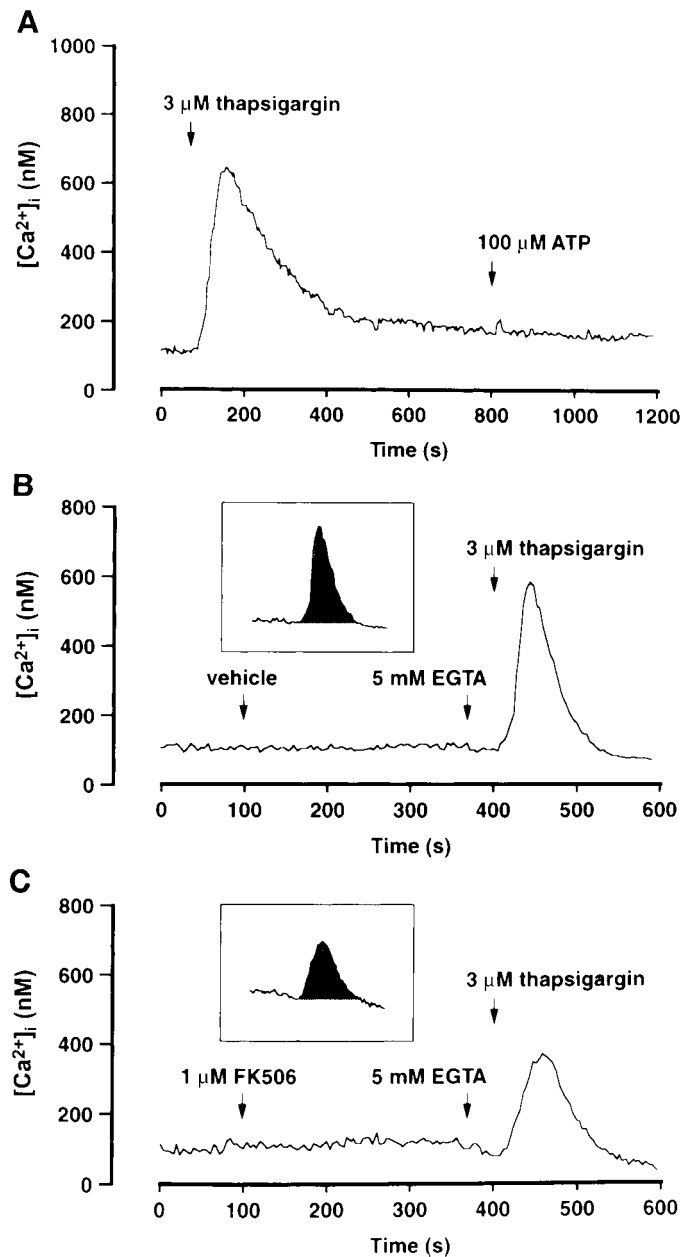


Fig. 5. Inhibition of ATP-induced increase in  $[Ca^{2+}]_i$  by TG and effect of FK506 on TG-evoked  $[Ca^{2+}]_i$  rise. A: TG evoked a transient  $[Ca^{2+}]_i$  rise followed by a sustained response. Subsequent addition of ATP did not cause a substantial increase in  $[Ca^{2+}]_i$ . After treatment 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

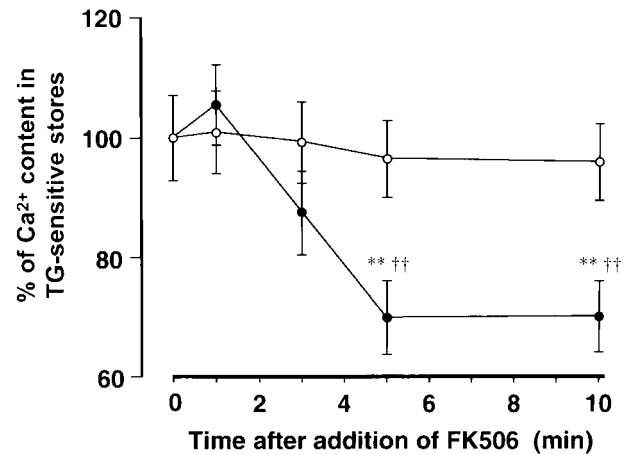


Fig. 6. Effect of FK506 on  $Ca^{2+}$  content in TG-sensitive stores. Cells were pretreated with vehicle alone (○) or FK506 (1  $\mu$ M; ●) for indicated times, and  $Ca^{2+}$  content in TG-sensitive stores was determined. Values are means  $\pm$  SE of percentage of control response (no drug added);  $n = 60$  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; †† 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  $\mu$ 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  $\mu$ 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 IP<sub>3</sub>R-gated (6) stores. However, there seems to be a discrepancy in

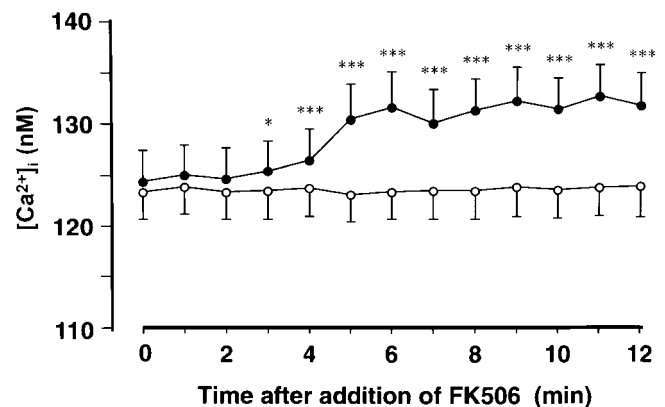


Fig. 7. Effect of FK506 on basal  $[Ca^{2+}]_i$  level in airway epithelial cells. Vehicle alone (○) or FK506 (1  $\mu$ M; ●) was added, and  $[Ca^{2+}]_i$  was measured every minute. Values are means  $\pm$  SE;  $n = 120$  cells/time point. Basal  $[Ca^{2+}]_i$  after addition of FK506 was significantly 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: \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ .

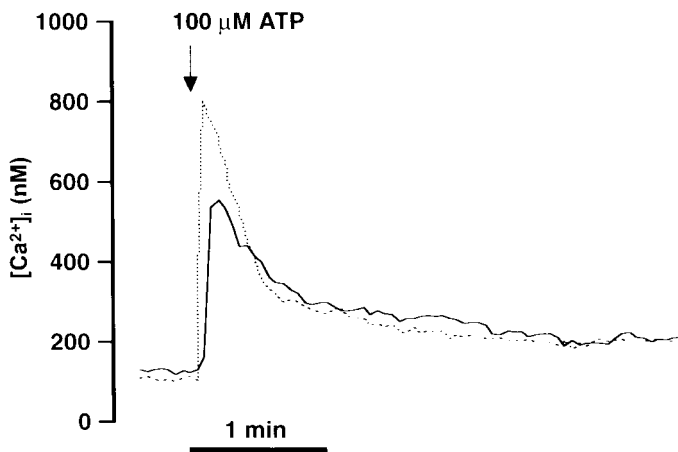


Fig. 8. Representative recordings of ATP-induced transient  $Ca^{2+}$  rise in cultured cow tracheal epithelium treated with FK506 (solid line) and vehicle alone (dotted line). After treatment with  $1 \mu M$  FK506 or vehicle alone for 10 min, ATP was added (arrow).

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.

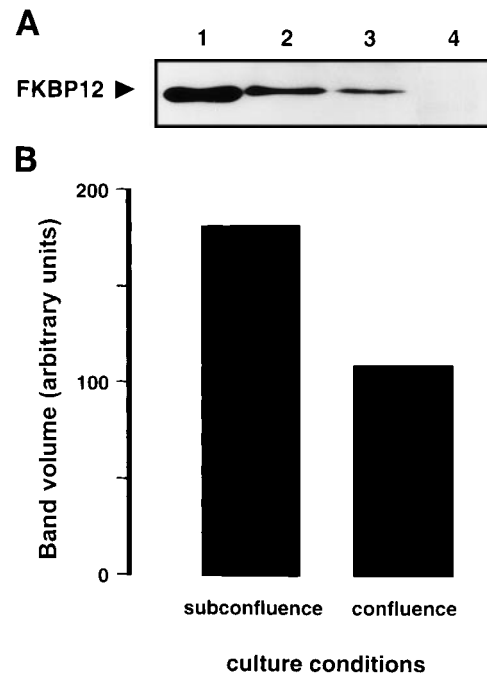


Fig. 10. Expression of FKBP12 in subconfluent and confluent cells as determined by Western blot analysis. *A*: recombinant human FKBP12 ( $0.3 \mu g$ ; lane 1) and equal amounts of extracted protein ( $75 \mu g$ ) from subconfluent (lanes 2 and 4) and confluent (lane 3) cells were subjected to 15% SDS-PAGE 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-

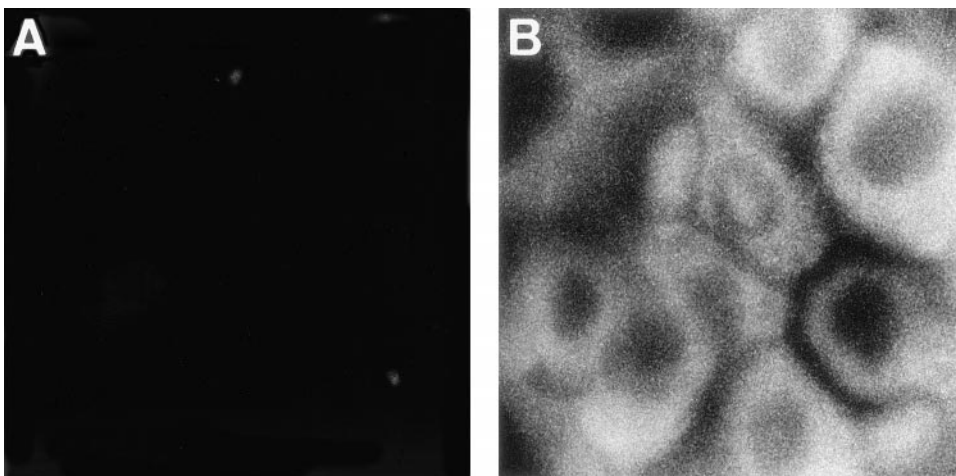


Fig. 9. Indirect immunofluorescence of FKBP12 in cow cultured tracheal epithelium at subconfluent stage in culture. Immunocytochemical staining was performed with (B) and without (A) anti-FKBP12 antibody as a first antibody. Original magnification,  $\times 400$ .

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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