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Prolonged Ca^{2+} transients in ATP-stimulated endothelial cells exposed to 50 Hz electric fields

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Abstract

Human umbilical vein endothelial cells were exposed to sinusoidal electric fields of 0.3 or 30 kV/m, 50 Hz, for 24 h. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) induced by ATP-stimulation in the absence of extracellular Ca^{2+} were observed in individual cells. No differences were observed between the exposure and sham-exposure groups in $[Ca^{2+}]_i$ resting level before ATP-stimulation, or in the $[Ca^{2+}]_i$ peak levels induced by stimulation. However, the duration of the initial transients in $[Ca^{2+}]_i$ following an ATP stimulus was significantly prolonged by exposure to a 30 kV/m field. The inositol trisphosphate receptor inhibitor, xestospongin C, inhibited the ATP-induced elevation in $[Ca^{2+}]_i$ in both the exposure and sham-exposure groups. The ATP-receptor P2Y appeared to play an important role in the increase of $[Ca^{2+}]_i$. The present results suggest that an extremely low-frequency electric field affects the function of vascular endothelial cells by a mechanism involving activation of P2Y.

Keywords: Low frequency electric fields; Intracellular Ca^{2+}; Adenosine triphosphate (ATP); Endothelial cells; Fluo3

1. Introduction

Biological effects of electric fields in vitro have been reported by many investigators (ICNIRP, 1998; Misakian et al., 1993). However, few reports describe the effects of extremely low frequency (ELF) electric fields on vascular endothelial cells.

In a wide range of cell types, Ca^{2+} is an important messenger in signal transduction (Meyer, 1991; Rasmussen et al., 1984). Endothelial cells exhibit changes in $[Ca^{2+}]_i$ in response to vasoactive substances such as adenosine triphosphate (ATP) (Jacob, 1990; Yumoto et al., 1995). ATP receptors were considered in the 1990s to belong to two major families, the ligand-gated ion channel P2X and the G protein-coupled P2Y (Abbracchio and Burnstock, 1994). The amplitude of elevation by ATP-stimulation in pre-activated HL-60 cells was decreased after exposure to electric fields (Kim et al., 1998), suggesting that electric fields might affect the ATP receptors of the endothelium.

We have reported that the effect of an electric field on changes in $[Ca^{2+}]_i$ was agonist-specific (ATP) in confluent monolayers of human umbilical vein endothelial cells (HUVEC) (Takahashi et al., 2002). The area under the curve (AUC) of increased $[Ca^{2+}]_i$ after induction by 100 μM ATP in the absence of extracellular Ca^{2+} was significantly greater after exposure to a 30 kV/m field. However, it was not clear whether the electric field affected the $[Ca^{2+}]_i$ peak level or the duration of $[Ca^{2+}]_i$ elevation. If the peak level is increased, the field

Abbreviations: Ca^{2+}-free Tyrode's solution (FT); G protein-coupled ATP receptor (P2Y).

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may enhance the efficacy of ATP receptors in triggering the Ca^{2+} signal. However, if the duration is prolonged, the effect of the field may be associated with an increased period of intracellular signal transduction. In the present study, the effects of 50 Hz ELF electric fields on $[Ca^{2+}]_i$ peak levels and on the duration of Ca^{2+} transients were evaluated using Fluo3 fluorescence in individual HUVEC stimulated by ATP. To ascertain whether the influence of the electric fields on the Ca^{2+} signal was related to the release of Ca^{2+} from intracellular stores, the effects of an inositol phosphate receptor inhibitor were also studied.

2. Materials and methods

2.1. Electric fields

The apparatus for exposing cultured cells to electric fields, described elsewhere (Takahashi et al., 2002), is shown in Fig. 1. Briefly, an electric field was generated using an arrangement of parallel stainless-steel plates. Two units for exposure and sham-exposure were placed in the same incubator. The maximum applied voltage in this apparatus was 3 kV. The distance between electrodes was 10 cm. Voltages of 0.03 kV or 3 kV were applied sinusoidally (50 Hz), resulting in air fields of 0.3 or 30 kV/m, respectively. The current induced in the medium by a 30 kV/m air field was 0.28 μA, which corresponded to a current density of 0.42×10^{-3} A/m^2. The magnetic fields in the chambers were $\leq$0.2 μT. A second unit in which the plates were short-circuited was used for sham-exposure. Neither corona nor ozone was detected in the incubator.

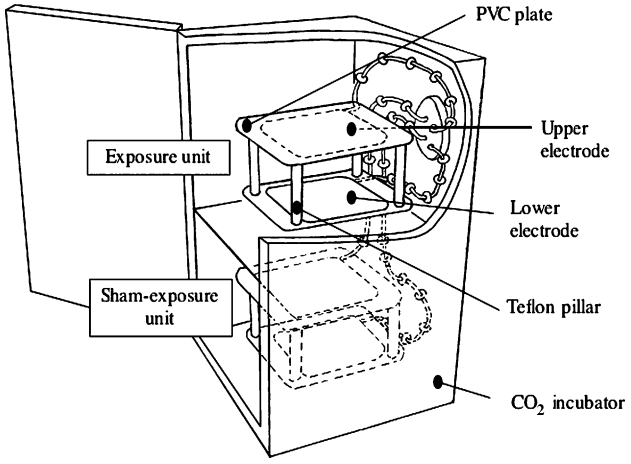

Fig. 1. Apparatus for exposing cultured cells to electric fields. Stainless-steel electrodes (1 mm thickness, 290 mm width, 370 mm depth) were insulated with polyvinyl chloride (PVC) and separated by Teflon insulation pillars. To produce the electric field, a voltage was applied to the upper electrode via a constant-voltage power supply; the lower electrode was grounded. The electrodes in the sham-exposure unit were short-circuited. The culture chamber was put on the midsection over 100 mm away from the edge of the lower electrode.

2.2. Cell culture

HUVEC were isolated from human umbilical cord according to method of Jaffe et al. (1973). The cells were seeded in type IV collagen-coated 4-well plates (Lab-Tek, chambered cover glass, Nunc, New York, USA) at 5×10^4 cells/well from the 7th to the 9th passage, and were incubated for 2 days at 37 °C in an atmosphere of 5% CO_2 and 95% air, using 700 μl/well KFSM (Gibco BRL, New York, USA) containing 10% newborn calf serum (Mitsubishi Chemical Corp., Tokyo, Japan) and 10 ng/ml basic fibroblast growth factor (Strathmann Biotec AG, Hamburg, Germany). The medium was changed and the cells were incubated for 24 h while being exposed continuously to electric fields or to sham conditions.

2.3. Fluorescence measurements

Fluo3-AM (Dojindo Labratories, Kumamoto, Japan) was used as fluorescent dye for $[Ca^{2+}]_i$. Fluorescence images of HUVEC loaded with Fluo3-AM were recorded using a laser scanning microscope (LSM 410, Carl Zeiss Jena GmbH, Jena, Germany). After electric field exposure, each chamber was washed with culture medium and then incubated in 500 μl of medium containing 5 mM Fluo3-AM for 1 h. The chambers were washed with Ca^{2+}-free Tyrode's solution (FT) (NaCl 139 mM, KCl 5.4 mM, $MgCl_2$ 1 mM, EGTA 1 mM, HEPES 5 mM, glucose 10 mM, buffered by NaOH to pH 7.4) and filled with 300 μl of FT. ATP-stimulation was performed as previously described (Takahashi et al., 2002) with the following modification: 300 μl of 200 μM ATP in FT was added into each well (100 μM, 600 μl/well). Fluorescence images were obtained every 1.5 s during ATP-stimulation using a laser scanning microscope at 488 nm excitation and 515 nm emission. Changes in fluorescence intensity in each cell were evaluated using an image processing program (Image J version 1.30, National Institute of Health, Maryland, USA).

2.4. Xestospongin C load

After loading with Fluo3-AM the chambers were washed with FT and filled with 300 μl of FT containing 10 μM xestospongin C (Wako Pure Chemical, Ohsaka, Japan) to inhibit the inositol 1,4,5-trisphosphate receptor, and then incubated for 10 min. ATP-stimulation was carried out as described above using ATP-solution containing 10 μM xestospongin C.

2.5. Statistical analysis

A Mann–Whitney *U*-test was used for statistical analysis of all the data. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Fluorescence of nuclear and cytosolic regions in cells

Almost all cells responded to ATP-stimulation (exposure 192/198, sham-exposure 161/169). In fluorescence images, the contours of individual cells and the borders between adjacent cells were obscure, while the contours of the nuclei were relatively clear (Fig. 2). $[Ca^{2+}]_i$ in the cytosolic and nuclear regions changed synchronously in measurements at intervals of 1.5 s, but the amplitude of change was greater in the nuclear region (Fig. 3). Fluorescence in the nuclear region was used for comparison between the study groups. The patterns of change in fluorescence were classified into two major types: (1) one to several spikes; (2) slow decay after a rapid initial rise (Fig. 3).

3.2. Fluorescence of $[Ca^{2+}]_i$ at resting and peak levels

There were no significant differences between exposure and sham-exposure groups in $[Ca^{2+}]_i$ resting or peak levels (Fig. 4). Xestospongin C inhibited the elevation in $[Ca^{2+}]_i$induced by ATP-stimulation in both the exposure and sham-exposure groups (Fig. 5).

3.3. Duration of the initial Ca^{2+} transients

The overall duration of the initial transients was defined as the time from the first detectable rise above reference level (which was twice the average of the resting level of the sham-exposure group) until return to below the reference level. The duration was significantly prolonged by exposure of cells to a 30 kV/m field (Fig. 6). This prolongation was also observed after classification into spiking and slowly decaying types. The prolongation induced by exposure to the 0.3 kV/m field was detected in the spiking type but not observed in the total. In $[Ca^{2+}]_i$ spiking cells, the time required for the fluorescence intensity of the final spike (intervals 10–15 s) to fall below the reference level was evaluated. $[Ca^{2+}]_i$ oscillations that rarely occurred after a long resting level (30 s or longer) were not included in this evaluation. No recognizable morphological changes were observed between 30 kV/m exposed and sham-exposed cells using phase contrast microscopy after ATP-stimulation (data not shown).

4. Discussion

After 24 h exposure of HUVEC to a sinusoidal 30 kV/m electric field at 50 Hz, the initial Ca^{2+} transients induced by a 100 μM ATP stimulus in the absence of extracellular Ca^{2+} were significantly prolonged. Xestospongin C inhibited the increase in $[Ca^{2+}]_i$ after the exposure.

The $[Ca^{2+}]_i$ resting level was lower in the nuclear than in the cytosolic region of each endothelial cell. The overall fluorescence intensity in each region seemed to increase in proportion to the space available for the Ca^{2+}-Fluo3 complex in that region and to Ca^{2+}

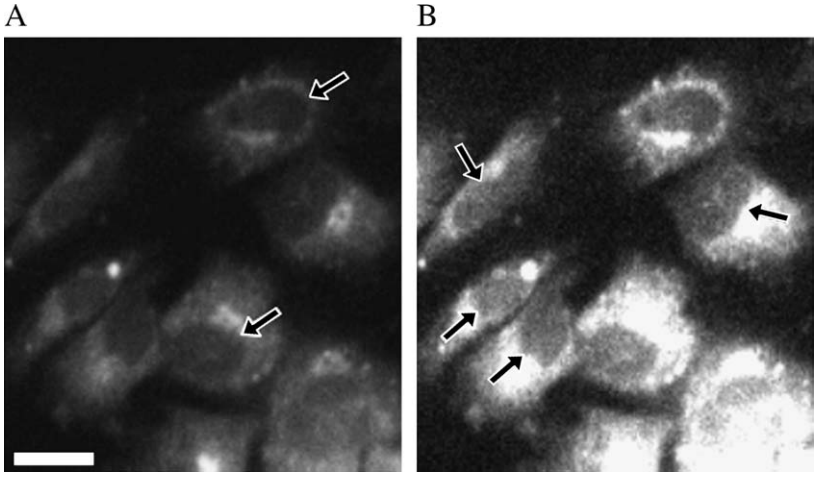

Fig. 2. Fluo3 fluorescence images at $[Ca^{2+}]_i$ resting level in HUVEC. (A) The contours of cell nuclei in the original images were more readily identifiable than the cell contours. (B) In images with adjusted black and white contrast, the nuclear contours were clearer. The original images were used for the evaluation of fluorescence intensity. The adjusted images were used to discern the nuclear regions. Bar indicates 20 μm.

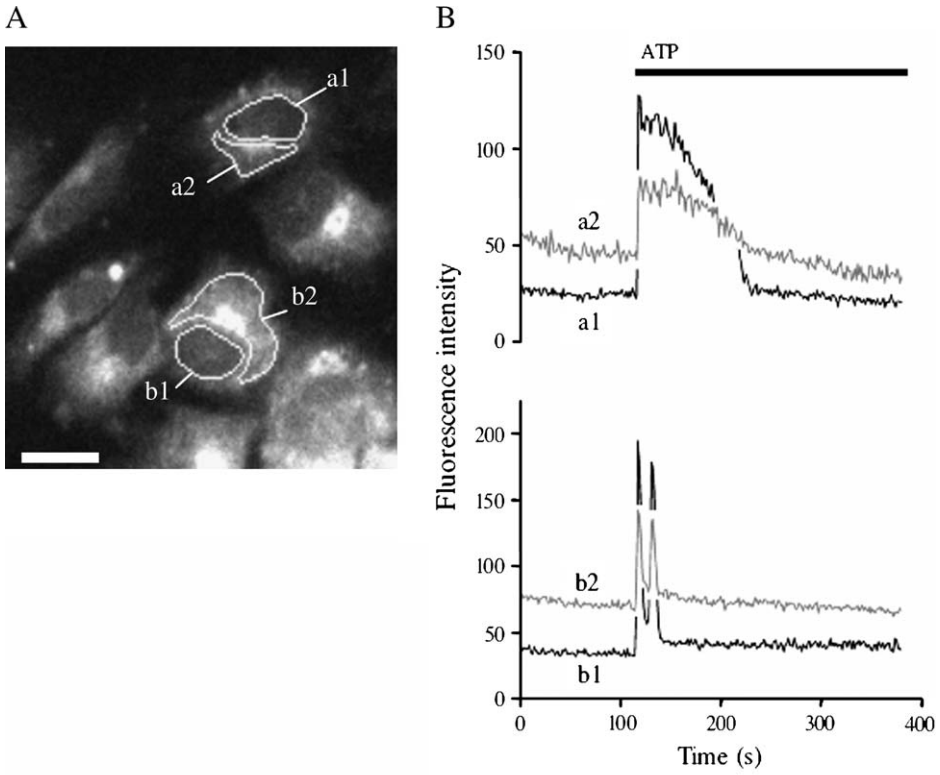

Fig. 3. Synchronization of $[Ca^{2+}]_i$ changes in the nuclear and cytosolic regions. (A) Fluorescence image at $[Ca^{2+}]_i$ resting level after adjustment of the contrast. Bar indicates 20 μm. (B) Typical changes in $[Ca^{2+}]_i$ after ATP-stimulation. Fluorescence in the nuclear and cytosolic regions (a1 and a2, b1 and b2) in the same cells changed synchronously in measurements at 1.5 s intervals. Patterns of changes in $[Ca^{2+}]_i$ were roughly classified into two types, one characterized by several spikes (b1, b2) and the other by a rapid rise followed by a gradual fall (a1, a2). $[Ca^{2+}]_i$ resting levels and peak levels were lower and higher, respectively, in the nuclear than the cytosolic region (a1 vs. a2, b1 vs. b2) of almost all cells.

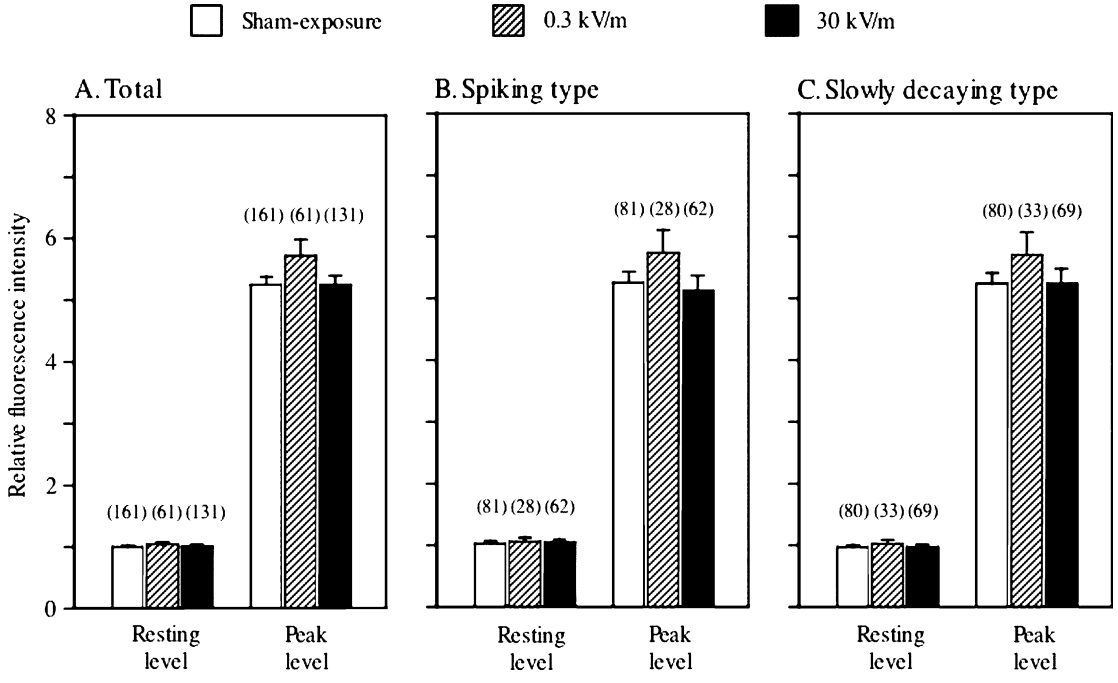

Fig. 4. $[Ca^{2+}]_i$ resting and peak levels. (A) No significant differences between study groups were observed in the resting and ATP-induced peak levels. Nor were any significant differences observed after the cells were classified into (B) spiking and (C) slowly decaying types based on the changes in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ level was expressed as relative fluorescence intensity, which is intensity divided by the average fluorescence before ATP-stimulation of the sham-exposure group. Each datum represents the mean ± SEM from 28–161 cells.

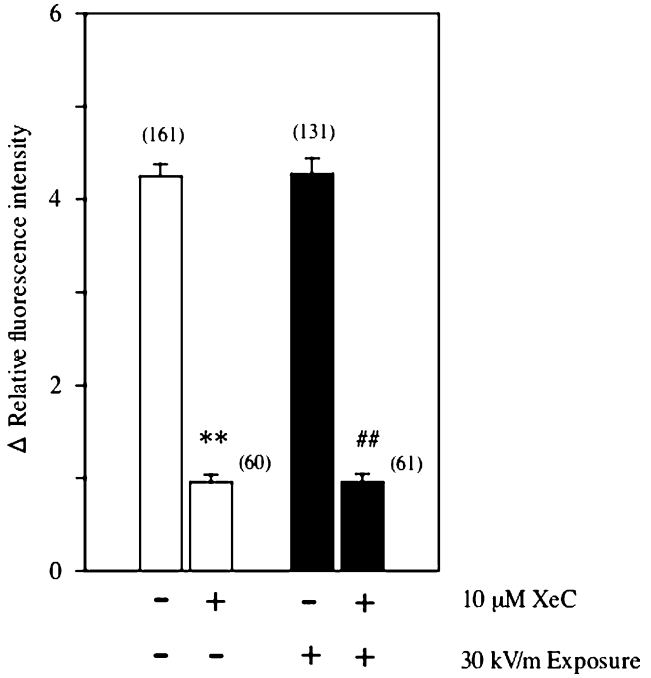

Fig. 5. Mean peak increases in $[Ca^{2+}]_i$ induced in HUVEC by ATP-stimulation. Xestospongin C (XeC) inhibited peak $[Ca^{2+}]_i$ in both the exposure and sham-exposure groups. Elevation in $[Ca^{2+}]_i$ level was expressed as Δ(relative fluorescence intensity), which is the elevation in intensity divided by the average fluorescence before ATP-stimulation of the sham-exposure group without XeC. Each datum represents the mean ± SEM from 60–161 cells. $^{**}p<0.01$ as compared with absence of XeC in sham-exposure. $^{\#\#}p<0.01$ as compared with absence of XeC in 30 kV/m exposure.

concentration. The nuclear region is not sparse, because of the nuclear envelope and the internal contents, so the low resting level in this region may be attributable to the smaller space available relative to the cytosolic region. The greater change of fluorescence in the nuclear region after ATP-stimulation may also be attributable to these differences in available space. The main intracellular Ca^{2+} store is the endoplasmic reticulum, and the nuclear membranes resemble the endoplasmic reticulum in structure. If a large quantity of Ca^{2+} is released from the nuclear envelope lumen to the small available internal space then the Ca^{2+} concentration and, ultimately, the fluorescence intensity may become high relative to that in the cytosolic region. However, the change in $[Ca^{2+}]_i$ induced by ATP-stimulation is possibly associated with the cell nucleus.

The cytoplasm is shielded from externally applied electric fields by the cell membrane (Barnes, 1996). Changes in surface charge induced by applied electric and magnetic fields were observed in *Physarum polycephalum* (Marron et al., 1988). Receptor conformations might be changed by ELF electric fields owing to modulation of the cell membrane charge (Luben and Blank, 1995). Robertson and Astumian (1990) analyzed the effects of electric fields on conformational changes of membrane enzymes from the viewpoint of chemical kinetics and equilibrium, using an electroconformational coupling model (Tsong and Astumian, 1987). ATP receptors and calcium pumps, as membrane proteins, are implicated in the changes in $[Ca^{2+}]_i$

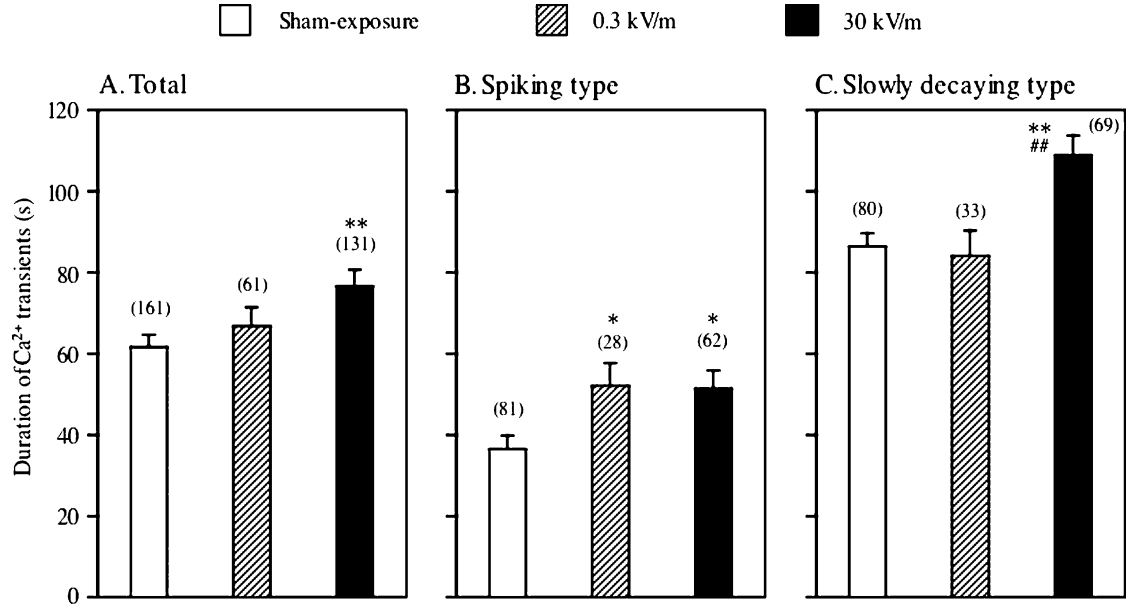

Fig. 6. Duration of Ca^{2+} transients in HUVEC induced by ATP-stimulation. (A) The average overall duration of the initial transients induced by the ATP-stimulus was significantly prolonged by exposure to 30 kV/m. Significant differences were also observed in the duration of (B) spiking and (C) slowly decaying types. The prolongation of transients induced by 0.3 kV/m was observed in the spiking type but not in the total. The duration was the time during which fluorescence intensity was above twice of the average resting level in the sham-exposure group. Each datum represents the mean ± SEM from 28–161 cells. $^{*}p<0.05$, $^{**}p<0.01$ as compared with sham-exposure. $^{\#\#}p<0.01$ as compared with 0.3 kV/m exposure.

induced by ATP. Calcium pumps are activated by increased $[Ca^{2+}]_i$. If the electric fields affected these pumps, the $[Ca^{2+}]_i$ peak level would be significantly changed. However, such changes were not observed. Therefore, the applied electric fields may have changed the conformation of the ATP receptors.

Prolongation of the Ca^{2+} transients after exposure to a 0.3 kV/m field was observed in the spiking type of cells but not in the slowly decaying type. However, the 30 kV/m field prolonged the transients in both types. These results suggest that the spiking type cells were more sensitive than the slowly decaying type to electric fields. The difference might be related to a period of intracellular signal transduction after the Ca^{2+} signal induced by ATP-stimulation. The diverse patterns of change in $[Ca^{2+}]_i$ induced by agonists in non-excitable cells have been reported by Fewtrell (1993). These patterns may indicate differences in mechanisms or cell status in signal transduction. The difference in sensitivity to field strength between spiking and slowly decaying cells might reflect such differences in mechanism or cell status.

Ca^{2+}-free Tyrode's solution was used as the extracellular buffer. Therefore, the observed elevation of $[Ca^{2+}]_i$ was attributable to Ca^{2+} release from intracellular stores, not to an influx of Ca^{2+}. This agrees with the finding that an ATP-induced $[Ca^{2+}]_i$ increase in endothelial cells was caused by release of Ca^{2+} from the intracellular stores via activation of P2Y (Ohata et al., 1997). Mobilization of intracellular Ca^{2+} via release of inositol 1,4,5-trisphosphate (IP_3) by activation of phospholipase C after G protein coupling is common to P2Y family mechanisms of intracellular signal transduction (Boarder et al., 1998; Communi et al., 2000; Kunapuli et al., 1998). Xestospongin C inhibits the IP_3-receptor-mediated release of Ca^{2+} from intracellular stores (Jiang et al., 2001; Miyamoto et al., 2000). In the present study, the inhibition of $[Ca^{2+}]_i$ elevation by xestspongin C suggests that P2Y played a major role in the response to ATP-stimulation, and supports the suggestion that prolonged duration by electric fields is related to the action of P2Y.

ATP receptors are predicted to have an important role in endothelium-dependent relaxation of vascular smooth muscle cells (Martin et al., 1985; O'Connor et al., 1991). For instance, an in situ study of rat mesenteric artery showed that vessels pre-contracted with norepinephrin were dilated by ATP-stimulation (Ralevic and Burnstock, 1991). A well-known endothelium-dependent relaxation factor, nitric oxide, is produced by nitric oxide synthetase (NOS). NOS activation is associated with an increase in $[Ca^{2+}]_i$ via IP_3 production in endothelial cells (Hirata and Emori, 1993). These findings suggest a relationship between G protein-coupled P2Y and vascular relaxation. Therefore, an electric field could affect vascular relaxation or suppression of vasoconstriction by acting on the endothelium-dependent relaxation mechanism. ATP receptors also appear on different cells of the endothelium, e.g. platelets, hepatocytes, myocytes, nerve cells (Abbracchio and Burnstock, 1994). The function of these cells is possibly affected by electric fields via the action of P2Y. Since intracellular Ca^{2+} is an important messenger in cellular function, more precise studies are required to understand the effects of electric fields through ATP receptors and intracellular Ca^{2+}.

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