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Characteristics of Spontaneous Ca^{2+} Oscillations in Bovine Aortic Endothelial Cells in Ca^{2+}-Free Solution

Jung-Mou Yang

Department of Physiology and Biophysics
National Defense Medical Center
Taipei, Taiwan, ROC

Abstract

In nominally Ca^{2+}-free solution, the incidence of spontaneous Ca^{2+} oscillations in the cultured bovine aortic endothelial (BAE) cells may occur during superfusion. Application of 3 μM ATP induced a single Ca^{2+} transient regardless of the prior absence or presence of spontaneous oscillations. The total mobilized [Ca^{2+}] depends on the incidence of spontaneous oscillations and the length of exposure in Ca^{2+}-free solution. The maximal rate of rise of the transient in cells induced by ATP immediately after an spontaneous spike was greater than that in cells without any spontaneous activity. But there was no difference in the recovery time constant. The sum of areas of spontaneous and ATP-induced transients in cells with spontaneous activity was not different from that of ATP-induced transient in cells without spontaneous activity. The results suggest that BAE cells, after growth to confluence, are subjected to spontaneous oscillations during superfusion in Ca^{2+}-free solution. The release of internal Ca^{2+} in response to ATP is enhanced in cells with spontaneous oscillations. The content of ATP-sensitive stores continually decreased when cells were superfused with Ca^{2+}-free solution, and most of the spontaneous release of Ca^{2+} was lost and not sequestrated back into ATP-sensitive stores.

Key Words: endothelial cell, Ca^{2+} oscillation, ATP

Introduction

Stimulation of endothelial cells can induce an increase of intracellular Ca^{2+} which causes release of active substance to exert function (12). The factors that activate the endothelial cells include mechanical stretch of cell membrane by osmotic change or shearing force of perfusing solutions, and receptor-mediated action by specific agonists, such as adenosine triphosphates (ATP) (1, 5, 18, 19). The production of inositol trisphosphate (IP_3) is assumed to regulate the release of intracellular Ca^{2+} mainly by operating on IP_3-sensitive Ca^{2+} channels (1, 4). The increase of intracellular Ca^{2+} after stimulation may be contributed from either influx from outside and/or release from internal stores (6, 9).

In studies of mobilization of intracellular Ca^{2+}, removal of extracellular Ca^{2+} and skinned cells are common maneuvers to eliminate the factor of Ca^{2+} influx from extracellular milieus. Our previous study has demonstrated that IP_3, but not ryanodine, can induce Ca^{2+} release from internal stores in permeabilized BAE cells, suggesting that in the experimental conditions at our laboratory BAE cells, different from other species, show no ryanodine-sensitive channels (11,17). BAE cells have been found to have spontaneous Ca^{2+} oscillations in Ca^{2+}-free solution (9,15). However, the source of Ca^{2+} responsible for oscillations and whether the released Ca^{2+} could be sequestrated back are not clear. Also, whether there is a difference in the agonist-induced Ca^{2+} mobilization between cells with and without the occurrence of spontaneous release of Ca^{2+} is not known. The present work was carried out to analyze the characteristics of spontaneous oscillations. The agonist, adenosine triphosphate (ATP), which can activate purine-receptors to increase the product of IP_3, was applied to test whether the spontaneous release of Ca^{2+} was associated with the IP_3-sensitive internal stores.

Corresponding author: Dr. Jung-Mou Yang, Department of Physiology and Biophysics, National Defense Medical Center, P.O. Box 90048-503, Taipei, Taiwan, ROC. Tel: (02)2365-0267, E-mail: jmyang@ndmc1.ndmctsgh.edu.tw

Materials and Methods

Cell Preparation

Endothelial cells were prepared from bovine aorta obtained from a local abattoir. After both ends of the vessel were ligated, the lumen was washed in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing penicillin (50 IU/ml)/streptomycin (50 μg/ml). Twenty milliliter of the washed solution containing 0.6 mg/ml collagenase (type 2; Sigma Chemical Co.) was injected into the vessel and incubated at 37°C for 15 min. Endothelial cells were harvested from the solution by centrifugation at 1000 rpm for 3 min. After being washed twice, the cells in Dulbecco's modified Eagle's culture medium (DMEM) containing 10% fetal calf serum, penicillin/streptomycin and glucose (4.8 mM) were plated on to cleaned glass coverslips in low density and were incubated under an atmosphere of 95% air-5% CO_2 at 37°C. Cells were fed with fresh media every 24 h, and used after 72 h when cells grew to confluence.

Dye Loading and Ca^{2+} Measurement

Cells were loaded with the Ca^{2+}-sensitive dye Fura-2 by incubation in culture medium containing 5 μM acetoxymethyl ester form of the dye at 37°C for 30 min. Coverslips with growing cells were then mounted in a small perfusion chamber on the stage of a microspectrofluorimeter which of a Nikon Diaphot EPI fluorescent microscope fitted with a computer-driven mechanical filter exchanger and a cooled, integrating charge coupled device (CCD) imaging system (8, 16). Cells were irradiated alternatively with light at 350 and 380 nm wavelength. Fluorescence measurements were taken at each wavelengths over 0.5 s intervals. The emitted light was filtered through a 520 nm long-pass filter. The ratio was measured every 1.1 s and calculated to give an estimate of cytoplasmic calcium.

This system was calibrated using 10 μM solutions of the penta potassium salts of the dyes in buffers of known Ca^{2+} concentrations with the addition to 10 % glycerol to increase their viscosity. These solutions were placed in the perfusion chamber. Concentration-ratio curves were constructed using this data. This type of calibration cannot be used to estimate absolute Ca^{2+} concentrations in cells loaded with the acetoxymethyl ester forms of the dyes (21). No account was taken of intracellular protein binding or the uptake of the dye into intracellular organelles. However, this method could be used to detect transients within a single cell and to compare relative changes in Ca^{2+}. For the purposes of the experiments presented here which related to time-dependent changes in Ca^{2+}, no attempt was made to make more accurate calibrations. Therefore, the maximal rate of rise and area of the Ca^{2+} transient were analyzed in ratio units to present relative changes in $[Ca^{2+}]$. Time constant for the rate of decay of Ca^{2+} transients were calculated using a least mean squares single exponential best fist. Integration of the area under the fluorescence ratio-time curve was calculated to estimate total $[Ca^{2+}]$ mobilized.

The composition of the basic solution contained (mM)140 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 Na_2HPO_4, 10 Glucose, 5 $NaHCO_3$, 20 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4). In nominally Ca^{2+}-free solution, CaCl2 was deleted or replaced with 0.2 mM -ethylenebis(oxonitrilo) tetraacetate (EGTA). Adenosine triphosphate (ATP) was purchased from Sigma Chemical Company. The solution was preheated to 34-36°C and was superfused through the experimental chamber at a rate of 2-3 ml/min.

Where appropriate, data were expressed as means±SEM with statistical analyses carried out using a Student's *t*-test.

Results

Spontaneous Activities in Ca^{2+}-Free Mediums

The BAE cells usually grew to confluence after three days in culture medium. In cultured BAE cells, removal of extracellular Ca^{2+} caused a rapid decrease in basal Ca^{2+} level. Some cells were kept unexcited, while others were spontaneously excited to show a burst of Ca^{2+} spikes. Fig. 1 showed three common patterns of response in Ca^{2+}-free solution. a) Cells remained unexcited (Fig. 1A); b) and c) cells showed bursts of Ca^{2+} spikes but with different characteristics (Fig. 1B and C). In Fig. 1B, the series of Ca^{2+} spikes were relatively even similar in size and maximal rate of rise but with different recovery time constants. In Fig. 1C, the total mobilized $[Ca^{2+}]$ (area) and maximal rate of rise of the Ca^{2+} oscillations were larger at the beginning and then gradually decreased, recovery time constant was slightly increased sequentially. The averaged parameters of six cells in each group were shown in Fig. 2. Spikes that were irregularly fused as the last two spikes in Fig. 1C are not included in the analysis.

Effects of ATP

In Ca^{2+}-free solution, 3 μM ATP always caused one single spike. The size of the Ca^{2+} transient depended on the occurrence of spontaneous activity and the time of exposure of cells in Ca^{2+}-free solution before ATP application. As shown in Fig. 3, the

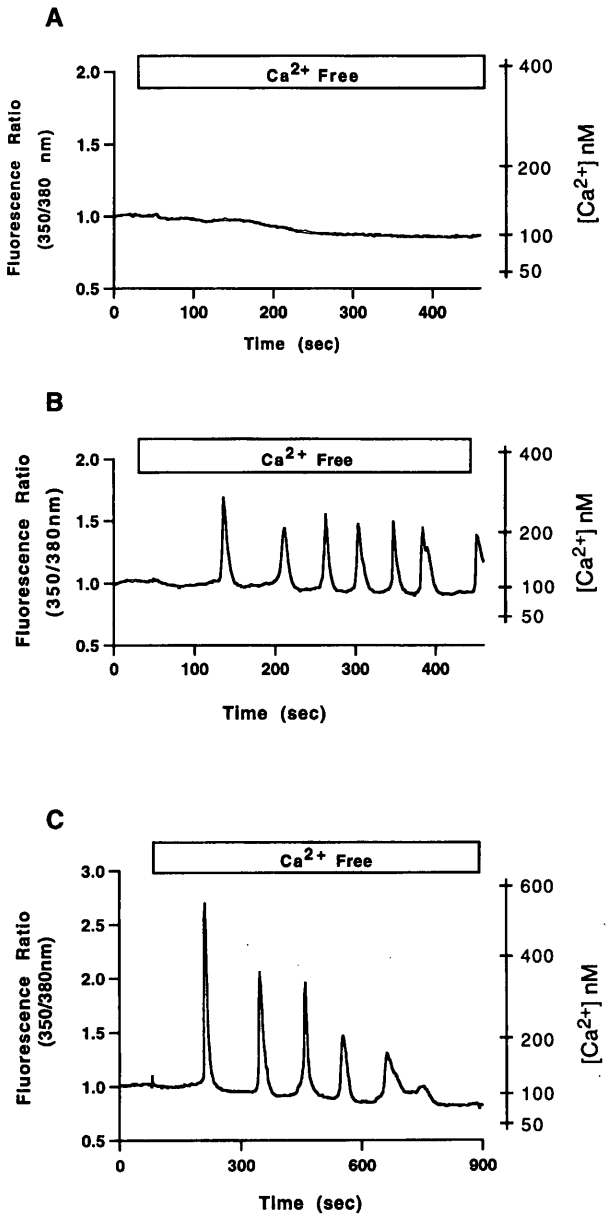

Fig. 1. Responses of BAE cells to the absence of external Ca^{2+}. No spontaneous oscillation (A), note the decrease of basal Ca^{2+}, and with bursts of Ca^{2+} spikes (B and C) Noted that the patterns were different between B and C (detail in text).

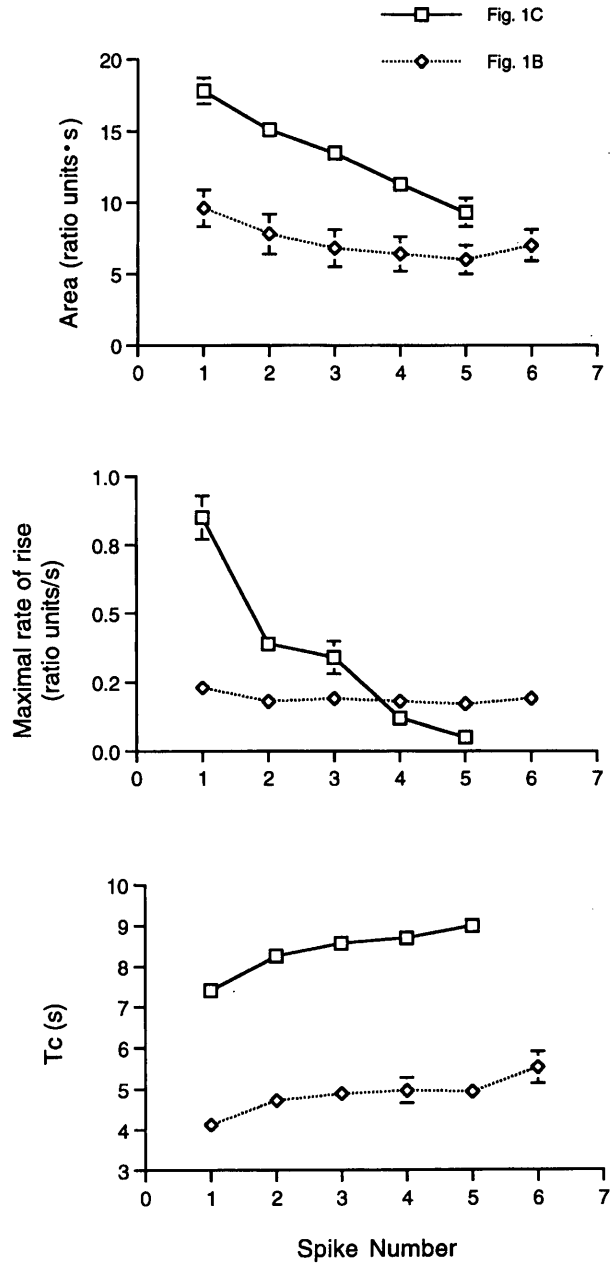

Fig. 2. An analysis of Ca^{2+} spikes in different types of spontaneous oscillations as shown in Fig 1B and C. The mobilized $[Ca^{2+}]$ were determined from area under the fluorescence ratio/time curve. The maximal rate of rise is presented as a function of time. The recovery time constant (Tc) for the rate of decay of Ca^{2+} transients is measured using a least mean squares single exponential best fit. Each group has six cells.

average size of Ca^{2+} transient in response to 3 μM ATP in cells without spontaneous activity was dependent on the time of administration after removal of extracellular Ca^{2+}. The earlier the cells were exposed to 3 μM ATP, the larger size of the Ca^{2+} transient could be induced.

In another series, 3 μM ATP was applied to a group of sixteen cells in Ca^{2+}-free solution at the same time. Six cells of the sixteen cells were spontaneously excited with a single spike before ATP while the other ten cells were not. In average, the mobilized $[Ca^{2+}]$ of the spontaneous spike was 18.8±1.7 ratio units·s. The maximal rate of rise and the recovery time constant were 0.202±0.006 ratio units/s and 8.3±0.9 s, respectively. The effects of 3 μM ATP on the Ca^{2+} transient were shown in Fig. 4. Fig. 4A shows an example of superimposed recordings of two cells, one with and another without a spontaneous spike before the addition of ATP. The analysis shows that cells without spontaneous transient had a larger area of the

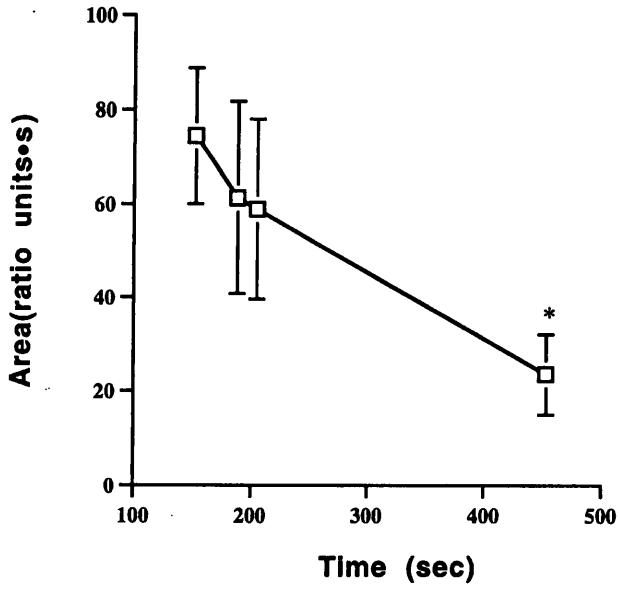

Fig. 3. The effect of time on ATP-induced Ca^{2+} mobilization as determined from the area under the fluorescence ratio/time curve in Ca2+-free solution. The mobilized $[Ca^{2+}]$ induced by 3 μM ATP was shown at different time after superfusion of Ca^{2+}-free solution, 150 sec (n=9), 188 sec (n=15), 205 sec (n=13), 453 sec (n=15). Cells analyzed did not oscillate before the administration of ATP. *$p<0.05$ in comparison with other groups.

Ca^{2+} spike induced by 3 μM ATP. However, in cells with spontaneous activity, the total mobilized $[Ca^{2+}]$ (sum of areas of spontaneous and ATP-induced Ca^{2+} transients) was not different from those without spontaneous activity (Fig. 4B). The maximal rate of rise of the transient induced by 3 μM ATP in cells with spontaneous activity was greater than that without spontaneous activity but recovery time constant was the same (Fig. 4C and D).

Discussion

Mechanical stimulations, e.g., osmotic and shearing stress, have been found to increase intracellular IP_3 and to release endothelium-derived relaxing factor in endothelial cells (4, 18). In BAE cells, there is a varied Ca^{2+} induced Ca^{2+} release (CICR) process which appears to have pharmacological properties different from CICR mechanisms in other cell types. An operation of an IP_3-sensitive but ryanodine-insensitive CICR mechanism on the internal Ca^{2+} stores has been demonstrated (11, 17). In the present study, the absence of external Ca^{2+} might reduce the stability of cell membrane and the strength of intercellular adhesion, making these cells more sensitive to mechanical disturbances, e.g., fluid flow, that evoked the production of IP_3, especially after they have grown to confluence (19). The increase of IP_3 mobilizes Ca^{2+} from IP_3-sensitive internal stores, the so called IP_3-induced Ca^{2+} release (7). The exact mechanisms responsible for the different patterns of spontaneous Ca^{2+} oscillations are not fully understood. A complex interaction between the magnitude of an oscillatory increase of cytosolic IP_3 and the available content of intracellular IP_3-sensitive Ca^{2+} stores must contribute to the patterns of repetitive increase in $[Ca^{2+}]_i$ (2, 3, 6, 13).

ATP activates the production of IP_3 which involves the mobilization of $[Ca^{2+}]_i$ from IP_3-sensitive internal stores (1, 9, 20). In Ca^{2+}-free solution, a Ca^{2+} transient induced by ATP represents a balance among internal Ca^{2+} release, the loss of Ca^{2+} from the cell and the sequestration of Ca^{2+} into intracellular stores. The loss of Ca^{2+} leads to a reduction in store content, possibly through channels or mechanism of Na^+/Ca^{2+} exchange in the cell membrane (9). In the present study, the total mobilized $[Ca^{2+}]$ induced by ATP was significantly reduced after long exposure to Ca^{2+}-free solution or the repetitive occurrence of spontaneous oscillations. A decrease in the size of the transient indicates that the loss of Ca^{2+} involves the IP_3-sensitive stores because ryanodine-sensitive stores is not demonstrated in BAE cells. Thus, the content of Ca^{2+} stores continually decreases as long as cells remain in Ca^{2+}-free solution, resulting in a decrease in response to ATP. As illustrated in Fig. 4B, the sum of Ca^{2+} transients from spontaneous release and ATP stimulation in cells with spontaneous activity is the same as the transient of stimulated release from cells without spontaneous activity, suggesting that Ca^{2+} for the spontaneous Ca^{2+} oscillations released from IP_3-sensitive stores and is lost and not sequestrated back in Ca^{2+}-free solution.

The addition of ATP immediately after a spontaneous transient enhanced the rate of Ca^{2+} release even though the Ca^{2+} content of IP_3-sensitive Ca^{2+} store was decreased (Fig. 4). This indicates that cells with spontaneous activity become more sensitive to agonist stimulation. The possibility is that spontaneous activity, possibly due to mechanical stimulation, accompanied with ATP stimulation produces more IP_3 which enhances the dynamics of Ca^{2+} release via the IP_3 sensitive channels (1, 10, 14, 20).

In conclusion, our results provide some characteristic of spontaneous Ca^{2+} oscillation in cultured BAE cells. a) After grown to confluence, cells superfused with Ca^{2+}-free solution are subjected to spontaneous oscillations; b) The Ca^{2+} for the spontaneous oscillation is released from IP_3-sensitive internal stores; and c) In Ca^{2+}-free solution, intracellular Ca^{2+} with a low basal $[Ca^{2+}]_i$ level continually decreases. The decrease in basal $[Ca^{2+}]_i$ is associated with loss of IP_3-sensitive internal Ca^{2+} stores. From these results, one should consider the

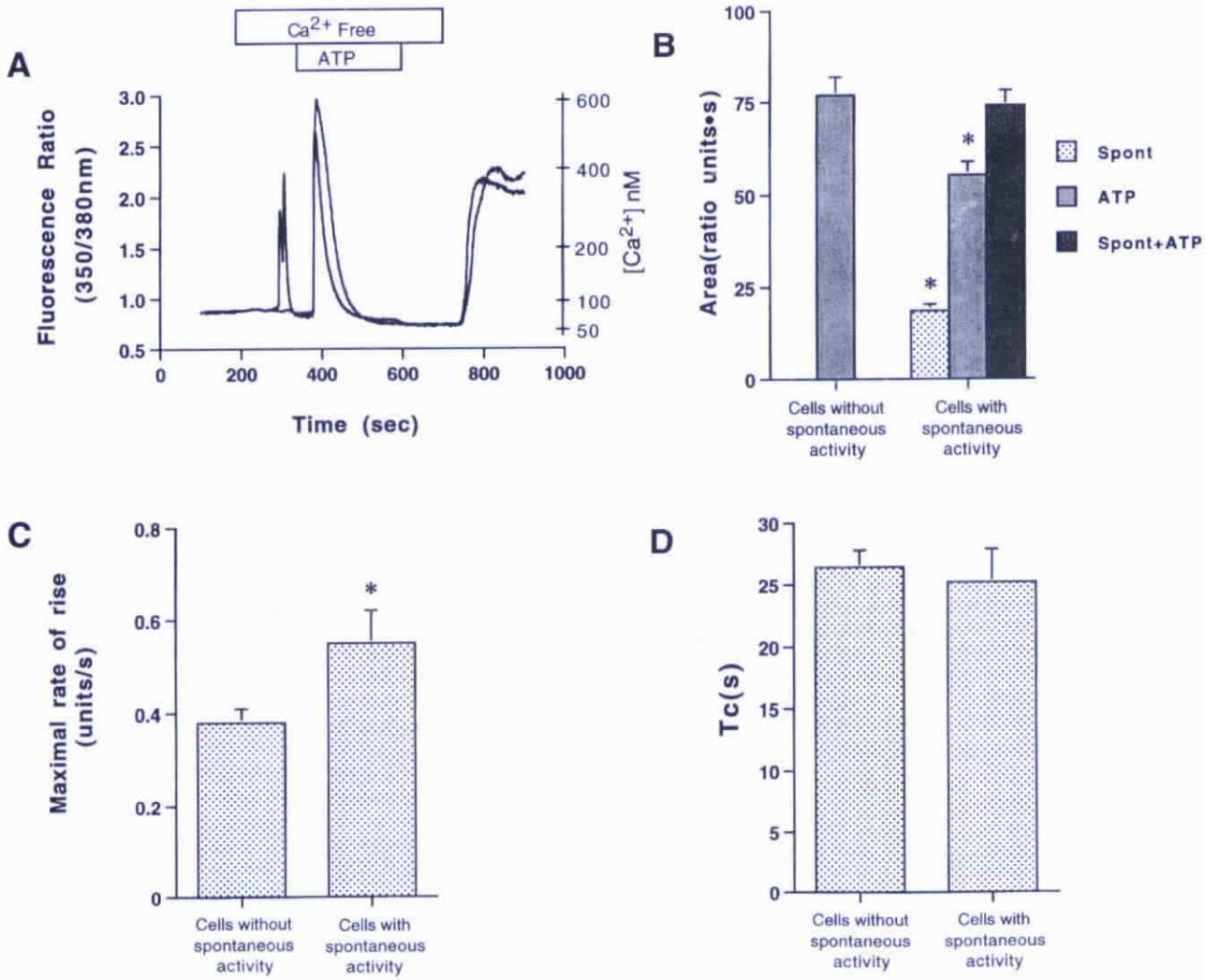

Fig. 4. A comparison of responses to ATP between cells with (n=6) and without (n=10) spontaneous activity in Ca^{2+}-free solution. Sixteen cells were measured at the same time. A, Superimposed changes of fluorescence ratio in response to 3 μM ATP in two cells. Note that the cell with a spontaneous spike had a smaller transient induced by ATP. B, Analysis of changes in area. C and D, the maximal rate of rise and the recovery time constant (Tc) of the Ca^{2+} transient induced by 3 μM ATP. *p<0.05

stability of cells and length of exposure in Ca^{2+}-free solution whenever the agonist-induced calcium mobilization is studied.

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