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Dynamic regulation of intracellular Ca^{2+} concentration in aortic endothelial cells

Masahiro Oike *, Yushi Ito

Department of Pharmacology, Faculty of Medicine, Kyushu University, Kyushi 812, Japan

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Abstract

In non-excitable cells, a Ca^{2+} entry pathway is opened after the depletion of intracellular Ca^{2+} store sites. We have tried to estimate the sensitivity of this pathway to Ca^{2+} release using bovine aortic endothelial cells. Single application of a high concentration (30 μM) of ATP released almost all stored Ca^{2+} in Ca^{2+}-free extracellular solution, whereas a low concentration of ATP (30 nM) produced a partial ($57.3 \pm 3.0\%$) release of Ca^{2+}. By 10 min of Ca^{2+} re-perfusion, the Ca^{2+} store site was reloaded to 97.1% of its initial filling state. When thapsigargin was applied to this cell in Mn^{2+} solution, Mn^{2+}-induced quenching of fura-2 dye started when $19.3 \pm 5.3\%$ of Ca^{2+} release, produced by 30 nM ATP, had occurred. Therefore, Ca^{2+} release required for Mn^{2+} entry was estimated as $11.1 \pm 3.0\%$ of stored Ca^{2+}. These results indicate that intracellular Ca^{2+} concentration is controlled dynamically by simultaneously occurring Ca^{2+} release and entry in bovine aortic endothelial cells.

Keywords: Ca^{2+} release; Ca^{2+} entry; Endothelial cell; ATP; Thapsigargin

1. Introduction

In order to achieve functions of vascular endothelial cells such as producing vasoactive substances or regulating vascular permeability, elevation of intracellular Ca^{2+} concentration is essential (Parsaee et al., 1992; Watanabe et al., 1992; Yanagisawa et al., 1988). Vascular endothelium, however, does not have voltage-dependent Ca^{2+} channels (for review, see Revest and Abbott, 1992) but there are a few sources for Ca^{2+} mobilization, i.e., release from the intracellular Ca^{2+} store site (Himmel et al., 1994; Jacob, 1990), agonist- (Himmel et al., 1994; Jacob, 1990; Nilius et al., 1993) or stretch- (Demer et al., 1993; Naruse and Sokabe, 1993; Schwartz et al., 1992) induced Ca^{2+} entry through Ca^{2+}-permeable non-selective cation channels, Na^{+}/Ca^{2+} exchange (Sage et al., 1991) and Ca^{2+} release-activated Ca^{2+} entry (Jacob, 1990; Oike et al., 1994). Ca^{2+} release-activated (or capacitative) Ca^{2+} entry has been considered to play an important role to refill the depleted intracellular Ca^{2+} store site (for review, see Penner et al., 1993; Putney, 1991). However, only a little is known about the details of this mechanism in vascular endothelium. Furthermore, it has not been clarified how sensitive this entry pathway to Ca^{2+} release is, which would provide a good understanding about the Ca^{2+} homeostasis of vascular endothelium. To address this problem, we used two methods to release Ca^{2+} from the intracellular Ca^{2+} store site: Ca^{2+} release by either high or low concentrations of ATP, which releases Ca^{2+} via $InsP_3$-mediated pathway (Himmel et al., 1994; Purkiss et al., 1994), followed by the re-perfusion of extracellular Ca^{2+}. Secondly, a tumor promoter, thapsigargin, which blocks Ca^{2+}-ATPase and slowly depletes the intracellular Ca^{2+} store site (Thastrup et al., 1991), in order to evaluate the timing of Ca^{2+} entry. Results obtained were compatible with the idea that the release of about one-tenth of stored releasable Ca^{2+} is enough to activate Ca^{2+} release-activated Ca^{2+} entry, thereby indicating the dynamic and real time control of intracellular Ca^{2+} concentration in endothelial cells.

2. Materials and methods

2.1. Cell culture

Bovine thoracic aorta of a 1-year-old calf was obtained from the local slaughterhouse. Endothelial cells were

* Corresponding author. Tel.: (81-92) 641-1151, ext. 3303; Fax: (81-92) 633-6790; e-mail: moike@pharmaco.med.kyushu-u.ac.jp

scraped off from the intima with the edge of a razor. Collected endothelial cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO_2 and 95% air at 37°C. Cells of the 3rd to 4th subculture were used for the present experiment. Cells were grown on coverslips, and single non-confluent cells were used. Identification of endothelial cells was confirmed by the specific uptake of acetylated low density lipoprotein.

2.2. Measurement of intracellular Ca^{2+} concentration

For the measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) from a single bovine aortic endothelial cell, cells were loaded with 2 μM of the acetoxymethylester form of the Ca^{2+} fluorescent dye fura-2 (fura-2/AM, Wako, Osaka, Japan) dissolved in DMEM for 20 min at room temperature and thereafter for another 20 min at 37°C. The coverslip with fura-2-loaded cells was placed in a chamber of 0.5 ml volume and mounted on an inverted-microscope (Diaphot TMD with special optics for epifluorescence; Nikon, Tokyo, Japan). The cell was excited with two alternative excitation wavelengths, 340 and 380 nm (each slit 5 nm) applied by a spectrometer (Spex, Edison, NJ, USA). The obtained fluorescent intensity data (F_{340} and F_{380}, respectively) were analyzed using customized software provided by Spex (DM-3000CM). The ratio of fura-2 fluorescence intensities (R) excited by two ultraviolet lights, F_{340}/F_{380}, was calculated after subtraction of the background fluorescence. The background fluorescence was taken from a cell-free area of the same coverslip just beside the examined cell after each experiment in Krebs solution. It was less than 10% of the fura-2 signals at either excitation wavelength. We calculated apparent $[Ca^{2+}]_i$ using the equation:

$$[Ca^{2+}]_i = K_{eff} \cdot (R - R_{min})/(R_{max} - R)$$

where K_{eff} is the 'effective binding constant', R_{min} the fluorescent ratio at zero calcium and R_{max} that at high

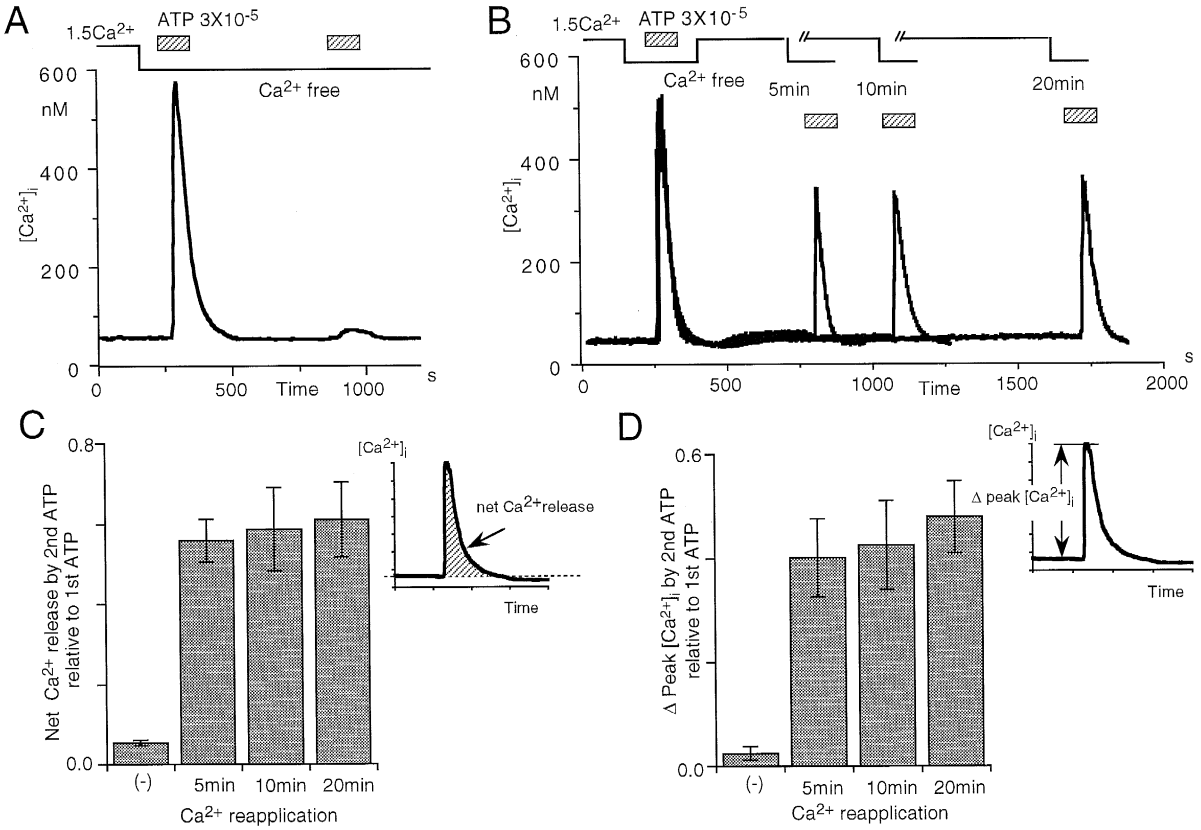

Fig. 1. Effects of repeated application of a high concentration of ATP (30 μM) on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in bovine aortic endothelial cells. (A) Effect of repeated application of 30 μM ATP on $[Ca^{2+}]_i$ in Ca^{2+}-free solution. ATP was applied two times for 2 min each at the times indicated by the hatched bars with an interval of 7 min. Note that little $[Ca^{2+}]_i$ elevation was evoked by the second application. (B) Between two applications of 30 μM ATP, 1.5 mM Ca^{2+} solution was perfused for 5, 10 or 20 min. Representative traces are shown. Ca^{2+}-containing solution was perfused as indicated by the steps. ATP was applied for 2 min at the times indicated by the hatched bars. (C) The net amount of release of Ca^{2+} by the second application of 30 μM ATP with 0, 5, 10 or 20 min of Ca^{2+} re-perfusion was measured by calculating the area under the $[Ca^{2+}]_i$ curve as shown in the inset (hatched area). Areas are expressed as relative to that produced by the first application of ATP in each cell. Each bar is the mean ± S.E.M. of four to five experiments. (D) The peak $[Ca^{2+}]_i$ induced by the second application of 30 μM ATP expressed relative to that produced by the first application. Inset shows how the values were measured. Each bar is the mean ± S.E.M. of four to five experiments.

Ca^{2+}. Because precise in vivo calibration of $[Ca^{2+}]_i$ was difficult to perform, it should be noted that the calculated value is not an accurate intracellular concentration.

In some experiments we used Ca^{2+}-independent fluorescence (F_{iso}) as an indicator of Mn^{2+}-induced quenching of fura-2 dye. F_{iso} was calculated from F_{340} and F_{380} as previously described (Chiavaroli et al., 1994). All experiments were carried out at room temperature (20–25°C).

2.3. Materials

Modified Krebs solution (1.5 mM Ca^{2+} solution) was used as the standard extracellular solution, containing (in mM): 132 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 11.5 glucose, 11.5 Hepes; pH was adjusted to 7.3 with NaOH. Ca^{2+}-free solution and 1.5 mM Mn^{2+}-containing, Ca^{2+}-free solution were made by substituting $CaCl_2$ of Krebs solution with 1 mM EGTA and 1.5 mM $MnCl_2$, respectively. The bath was perfused continuously with these solutions at a rate of 1.5 ml/min.

ATP (Sigma, St. Louis, MO, USA), thapsigargin (Sigma) and ionomycin (Sigma) were used to release Ca^{2+} from the intracellular store site.

2.4. Data analysis

The net released Ca^{2+} by ATP or thapsigargin was calculated by integrating the elevated component of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$); $\int \Delta[Ca^{2+}]_i \cdot dt$. Pooled data are given as mean ± standard error of the mean, and statistical significance was determined using Student's unpaired *t*-test. Probabilities less than 5% ($P < 0.05$) were regarded as significant.

3. Results

3.1. Endothelial intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to high concentration of ATP with and without Ca^{2+} reapplication

Application of a high concentration (30 μM) of ATP for 2 min induced marked elevation of $[Ca^{2+}]_i$ in Ca^{2+}-free

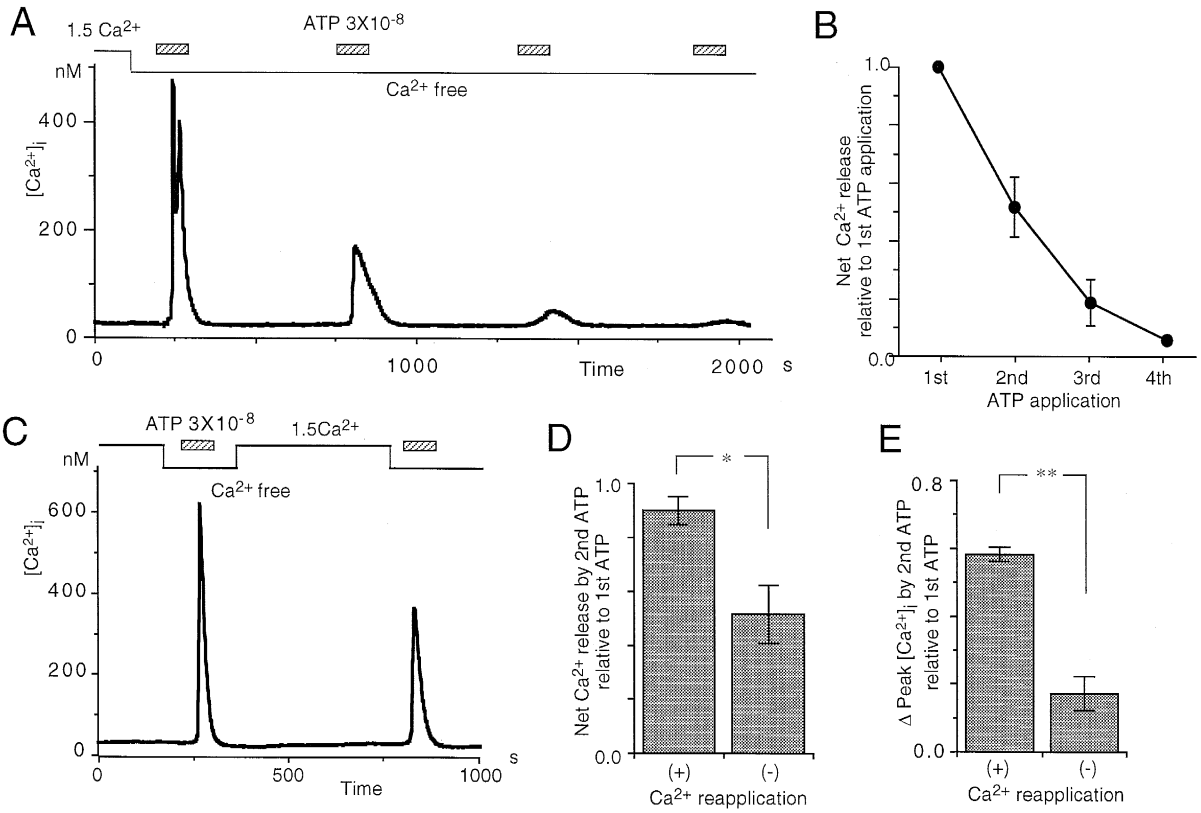

Fig. 2. Effects of repeated application of a low concentration of ATP (30 nM) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells. (A) Effect on $[Ca^{2+}]_i$ in Ca^{2+}-free solution. ATP was applied four times for 2 min each at the times indicated by hatched bars at 7 min intervals. Note that about half of the releasable Ca^{2+} has not been released by the first application of ATP. (B) Net Ca^{2+} release by each ATP application relative to the first ATP application. Each point is the mean ± S.E.M. of four experiments. (C) Effect of repeated application of 30 nM ATP on $[Ca^{2+}]_i$ with 5 min of Ca^{2+} re-perfusion. (D) The net amount of released Ca^{2+} by the second application of 30 nM ATP with (+) or without (−) Ca^{2+} re-perfusion was measured as in Fig. 1C. Values are expressed as relative to that produced by the first application of ATP. Each bar is the mean ± S.E.M. value of four experiments. * $P < 0.05$. (E) The peak $[Ca^{2+}]_i$ by the second application of 30 nM ATP with (+) or without (−) Ca^{2+} re-perfusion. Each bar is the mean ± S.E.M. of four experiments. ** $P < 0.01$.

solution, indicating the release of Ca^{2+} from the intracellular Ca^{2+} store site. Repeated application of the same concentration of ATP induced little further calcium release when the cell was kept in Ca^{2+}-free solution throughout (Fig. 1A). Thus it seems that almost complete depletion of the intracellular Ca^{2+} store site was obtained during the first application of 30 μM ATP and little of the released Ca^{2+} was taken back into the store site.

On the other hand, when 1.5 mM Ca^{2+} solution was perfused in the bath for 5, 10 or 20 min between two ATP applications, the $[Ca^{2+}]_i$ curve showed a little elevation during Ca^{2+} re-perfusion, and Ca^{2+}-releasing ability of the cell was markedly restored (Fig. 1B). ATP was completely washed out before starting Ca^{2+} re-perfusion, and the solution used, containing high Na^+ concentration, was far from that for the activation of Na^+/Ca^{2+} exchange (Sage et al., 1991). Furthermore, the cell membrane was not stretched by a slow continuous flow used at the present experiment. Therefore, the reloading of the Ca^{2+} store site was not due to either agonist-induced Ca^{2+} entry, Na^{2+}/Ca^{2+} exchange or stretch-activated channel, but by the mechanisms of Ca^{2+} release-activated Ca^{2+} entry. Fig. 1C and D indicate the restoration of Ca^{2+} releasing ability by reperfusion of 1.5 mM Ca^{2+} solution. We used two factors to describe Ca^{2+} release quantitatively, i.e., 'net Ca^{2+} release' and 'Δpeak $[Ca^{2+}]_i$' (see insets of Fig. 1C and D). Both values reached the maximum level within 5 min of Ca^{2+} reperfusion. Furthermore, the refilling process of the intracellular Ca^{2+} store site ceased when the Ca^{2+} level was about 60% of that released by the first application of ATP.

These results indicate that the Ca^{2+} release-activated Ca^{2+} entry pathway is activated by depleting the intracellular store site in bovine aortic endothelial cells and the reloading process of the depleted store site is almost completed within 5 min.

3.2. Endothelial $[Ca^{2+}]_i$ in response to low concentration of ATP with and without Ca^{2+} reapplication

To examine whether partial release of stored Ca^{2+} also can induce Ca^{2+} release-activated Ca^{2+} entry, we repeated similar experiments to the above but with a low concentration of ATP (30 nM).

Application of 30 nM ATP in Ca^{2+}-free solution induced a rise in $[Ca^{2+}]_i$, indicating this concentration of ATP also released Ca^{2+} from the intracellular Ca^{2+} store site. In some cells, application of 30 nM ATP showed oscillatory Ca^{2+} release (see Fig. 2A and Fig. 5). Upon repeated application of 30 nM ATP, $[Ca^{2+}]_i$ was elevated but decreasingly even when the cell was kept in Ca^{2+}-free solution throughout, suggesting that the first 30 nM ATP application did not deplete the Ca^{2+} store site (Fig. 2A). Fig. 2B summarizes the net Ca^{2+} release by each ATP application relative to that by the first one. Because the fourth application produced almost no Ca^{2+} release, we can assume that almost all the released Ca^{2+} was excluded from the cell by the fourth application, and was not taken into the store site. This was confirmed by the fact that ionomycin (3 μM), applied after the fourth application of 30 nM ATP, induced little further $[Ca^{2+}]_i$ elevation ($n = 3$, not shown). So we can calculate that released Ca^{2+} summed to $57.3 \pm 3.0\%$, $86.5 \pm 4.8\%$ and $96.6 \pm 4.7\%$ ($n = 5$) of initially releasable Ca^{2+} on average by the first, second and third application of 30 nM ATP, respectively.

When 1.5 mM Ca^{2+} solution was perfused between two 30 nM ATP applications, the second application of ATP produced significantly larger Ca^{2+} release than in Ca^{2+}-free solution throughout (Fig. 2C, D and E). These results indicate that partial Ca^{2+} release, about half of stored releasable Ca^{2+}, from the intracellular Ca^{2+} store site can induce Ca^{2+} release-activated Ca^{2+} entry in vascular endothelial cells.

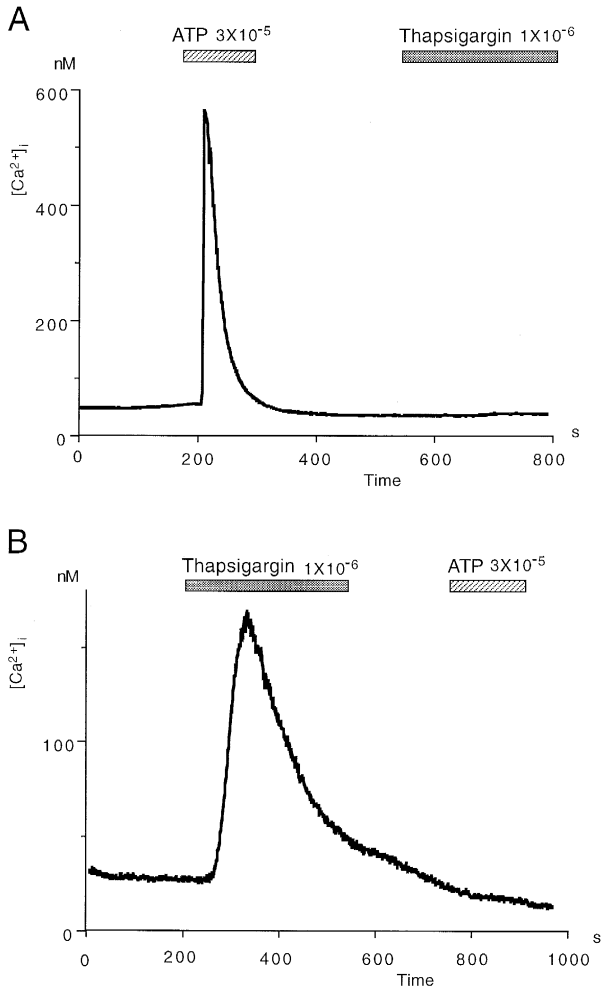

Fig. 3. Effect of sequential application of a high concentration of ATP (30 μM) and 1 μM thapsigargin on $[Ca^{2+}]_i$ in bovine aortic endothelial cells. ATP was applied for 3 min before (A) or after (B) application of thapsigargin for 6 min in Ca^{2+}-free solution.

3.3. Thapsigargin-induced change in $[Ca^{2+}]_i$ of vascular endothelial cells

Because ATP lower than 30 nM often failed to release Ca^{2+}, we used thapsigargin to passively deplete the intracellular Ca^{2+} store site. To examine whether Ca^{2+} release induced by thapsigargin and by ATP are from the same Ca^{2+} store site we applied 30 μM ATP and 1 μM thapsigargin sequentially. After the ATP-sensitive intracellular Ca^{2+} store site was depleted by the high concentration of ATP in Ca^{2+}-free solution, the following application of thapsigargin produced no further Ca^{2+} release (Fig. 3A). When 1 μM thapsigargin in Ca^{2+}-free solution was applied first, $[Ca^{2+}]_i$ started to elevate shortly after the application and declined to the base level over several minutes. The time course of Ca^{2+} release by thapsigargin was longer than by ATP. The following application of 30 μM ATP showed no response in this thapsigargin-pretreated cell (Fig. 3B). These results indicate that thapsigargin and ATP release Ca^{2+} from the same intracellular store site in bovine aortic endothelial cells but with different time courses.

We then recorded Ca^{2+} release-activated Ca^{2+} entry using thapsigargin. On perfusion of Ca^{2+} extracellularly, following release of Ca^{2+} by thapsigargin, $[Ca^{2+}]_i$ was again elevated, indicating that the Ca^{2+} release-activated Ca^{2+} entry pathway was activated also by thapsigargin (Fig. 4A). Because thapsigargin releases Ca^{2+} more gradually than ATP, we then tried to indicate the timing of the beginning of Ca^{2+} entry after Ca^{2+} release using this agent. When thapsigargin was applied in 1.5 mM Mn^{2+}-containing, Ca^{2+}-free solution, quenching of fura-2 dye by entered Mn^{2+} was clearly observed as a downward deflection of the Ca^{2+}-insensitive intensity curve (F_{iso}). The quenching started not after complete store site depletion but during the initial thapsigargin-induced Ca^{2+} releasing phase (Fig. 4B arrow; for comparison, see Fig. 4A). The time course of Ca^{2+} release by thapsigargin differed from cell to cell, with quenching starting 41–77 s after the beginning of Ca^{2+} release (mean ± S.E.M. = 53.8 ± 7.0 s, $n = 7$) and always during the initial Ca^{2+}-releasing phase. This result indicates that Ca^{2+} release-activated Ca^{2+} entry starts to be activated during the releasing process of Ca^{2+}.

3.4. Evaluation of net Ca^{2+} release needed to induce Ca^{2+} release-activated Ca^{2+} entry

To evaluate how much Ca^{2+} release is required to start Ca^{2+} release-activated Ca^{2+} entry, we used the following

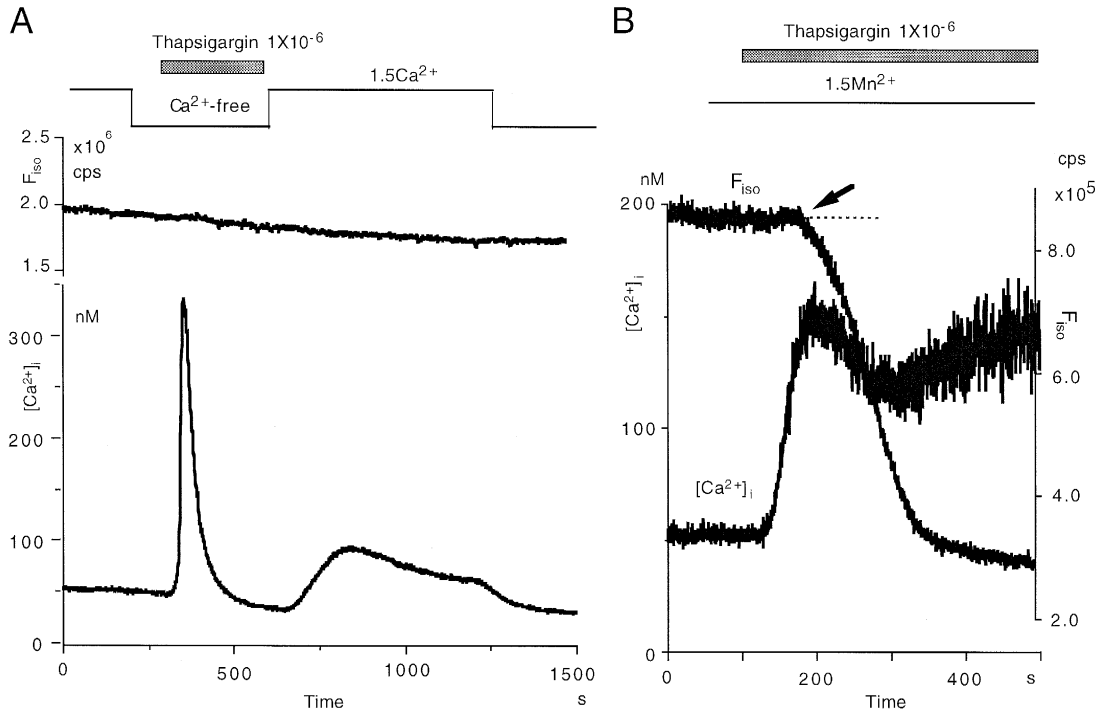

Fig. 4. (A) Effect of 1 μM thapsigargin on $[Ca^{2+}]_i$ in bovine aortic endothelial cells. Thapsigargin was applied in Ca^{2+}-free solution. After the elevated $[Ca^{2+}]_i$ had returned to the base level, 1.5 mM Ca^{2+} solution was re-perfused as indicated by the step. It is clear that the $[Ca^{2+}]_i$ follows the extracellular concentration of Ca^{2+}. Similar results were obtained with five other cells. Upper trace shows the Ca^{2+}-insensitive fluorescence intensity curve (F_{iso}) calculated from F_{340} and F_{380}. (B) Thapsigargin (1 μM) was applied as indicated by the hatched bar in 1.5 mM Mn^{2+}-containing, Ca^{2+}-free solution to the cell. The $[Ca^{2+}]_i$ curve and the F_{iso} curve are superimposed. Shortly after the beginning of the $[Ca^{2+}]_i$ curve elevation, a downward deflection of F_{iso} has started (at the time indicated by the arrow) because of the quenching of fura-2 by entered Mn^{2+}. The $[Ca^{2+}]_i$ curve is therefore no longer reliable from this point. The dotted line indicates the expected F_{iso} curve if it was not quenched (see panel A for comparison). Similar results were observed with six other cells.

protocol. After the first application of the low concentration of ATP (30 nM) which had been confirmed to release $57.3 \pm 3.0\%$ of total Ca^{2+}-releasing ability of the cell (see Fig. 2B), the intracellular Ca^{2+} store site was filled again by perfusing 1.5 mM Ca^{2+} solution extracellularly for 10 min. In Fig. 5A, the high concentration of ATP (30 μM) was then applied to deplete the Ca^{2+} store site. By taking Ca^{2+} release by the first 30 nM ATP as a control (100%), it was found in five experiments that 30 μM ATP released $169.8 \pm 34.5\%$ Ca^{2+}. Because 30 nM ATP-induced Ca^{2+} release is 57.3% of initially stored Ca^{2+}, and because 30 μM ATP can deplete the store site, 'release of 169.8% to control by 30 μM ATP' means that Ca^{2+} re-application for 10 min refilled the Ca^{2+} store site to 97.3% ($= 57.3 \times 169.8/100$) of its initial amount.

We then applied 1 μM thapsigargin in 1.5 mM Mn^{2+} solution to a cell pretreated with ATP (30 nM) followed by 1.5 mM Ca^{2+} solution as above (Fig. 5B). As a result of opening the Ca^{2+} release-activated Ca^{2+} entry pathway by thapsigargin-induced Ca^{2+} release, quenching of fura-2 dye by Mn^{2+} was observed with a similar delay as in Fig. 4B (delay time of 49.5 ± 6.1 s, $n = 4$; $P > 0.05$). The beginning of the downward deflection of the F_{iso} curve was always easily distinguishable. The net Ca^{2+} release needed to initiate Mn^{2+} entry was calculated by integrating the area of the $[Ca^{2+}]_i$ curve till the beginning of quenching (see Fig. 5B). If we again set the Ca^{2+} release by the first 30 nM ATP as 100%, Ca^{2+} release needed to start quenching was $19.3 \pm 5.3\%$. We estimated that 30 nM ATP releases 57.3% of initial stored Ca^{2+} on average, and the store site is refilled to 97.3% of the initial amount after Ca^{2+} re-perfusion for 10 min. So the control, 30 nM ATP-induced Ca^{2+} release, was not 57.3% but 58.9% ($= 57.3/97.3 \times 100$) of stored Ca^{2+} after Ca^{2+} re-perfusion. Therefore, the induction of fura-2 quenching by Mn^{2+} needed the release of 11.1% ($= 19.3 \times 57.3/100$) of stored Ca^{2+} on average. Calculating the standard error in the same way, we conclude that the amount of Ca^{2+} release which is required to induce Ca^{2+} release-activated Ca^{2+} entry is $11.1 \pm 3.0\%$ of stored Ca^{2+}.

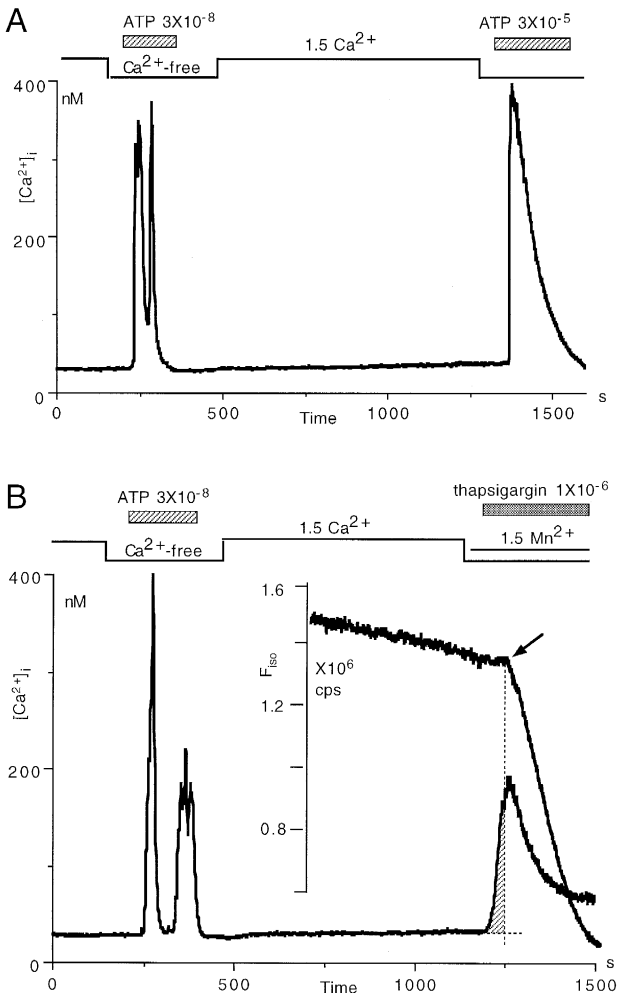

Fig. 5. (A) Sequential application of a low (30 nM) and high (30 μM) concentration of ATP. After 30 nM ATP was applied for 2 min in Ca^{2+}-free solution, 1.5 mM Ca^{2+} solution was re-perfused for 10 min to reload the Ca^{2+} store site. The effect of 30 μM ATP was then observed. (B) Thapsigargin (1 μM) was applied in 1.5 mM Mn^{2+}-containing, Ca^{2+}-free solution to a cell pretreated with 30 nM ATP and following re-perfusion of 1.5 mM Ca^{2+} solution for 10 min. The cell was, therefore, in the same condition as just before the application of 30 μM ATP in A. After the application of thapsigargin, transient elevation of $[Ca^{2+}]_i$ was observed. The Ca^{2+}-insensitive fluorescence intensity curve (F_{iso}) is superimposed. Note that F_{iso} shows a steeper downward deflection as indicated by the arrow. The hatched area indicates net Ca^{2+} release needed to induce Mn^{2+} entry.

4. Discussion

The mechanism of Ca^{2+} release-activated Ca^{2+} entry has been considered to play a role to refill the depleted Ca^{2+} store site in non-excitable cells such as vascular endothelium (Penner et al., 1993; Putney, 1991). We used a rather indirect method, measuring the change of $[Ca^{2+}]_i$, to detect the activation of Ca^{2+} entry, because it is very difficult to record this Ca^{2+} release-activated Ca^{2+} entry as a detectable membrane current by the patch-clamp method (Oike et al., 1994). Perfusion of Ca^{2+} extracellularly after the complete store depletion restored the ability of Ca^{2+} release, but both peak $[Ca^{2+}]_i$ and net released Ca^{2+} amount was not completely restored by up to 20 min of Ca^{2+} re-perfusion (Fig. 1B, C and D). This may be because of our experimental conditions, e.g., temperature or solution. Desensitization of the ATP-induced IP_3 production or its action is negligible because successive application of low and high concentrations of ATP seems to be able to release all stored Ca^{2+} (see Fig. 5A). A low concentration of ATP (30 nM) induced a partial release of Ca^{2+}, which has been reported to be because of Ca^{2+}-dependent inactivation of Ca^{2+} release (for review, see Missiaen et al., 1994). We assume here that repeated application of 30 nM ATP finally mobilized all potentially releasable Ca^{2+}, because the following application of iono-

mycin showed little $[Ca^{2+}]_i$ elevation. Thus we can calculate that the first application of 30 nM ATP released $57.3 \pm 3.0\%$ of total releasing ability (Fig. 2B). It was somewhat strange that only a little, in some cells no, elevation of $[Ca^{2+}]_i$ was seen during the re-perfusion of extracellular Ca^{2+} while the Ca^{2+} release-activated Ca^{2+} entry was activated. This can be explained by the fact that $[Ca^{2+}]_i$ elevation just beneath the plasmalemma is difficult to detect by fura-2 (Etter et al., 1994), so entered Ca^{2+} might be distributed very locally and taken into the intracellular Ca^{2+} store site before being captured by fura-2. On the other hand, when thapsigargin was used to deplete the Ca^{2+} store site, re-perfusion of Ca^{2+} produced marked $[Ca^{2+}]_i$ elevation (Fig. 4A), because entered Ca^{2+} would not be taken into the store site in thapsigargin-treated cells. Mn^{2+} ion has been known to induce quenching of fura-2 dye (Jacob, 1990). We found that the entry of divalent cation began during the initial elevation of fluorescent ratio by thapsigargin, using Mn^{2+}-induced quenching as a marker (Fig. 4B).

These results indicate that complete depletion or large amounts of Ca^{2+} release from the intracellular Ca^{2+} store site is not necessary to induce Ca^{2+} release-activated Ca^{2+} entry. Our estimate of the amount of Ca^{2+} release which is required to start Ca^{2+} entry after Ca^{2+} release revealed that Mn^{2+}-induced quenching was observed at much lower Ca^{2+} release than that by a single application of 30 nM ATP (Fig. 5B). Comparison of net Ca^{2+} release by 30 nM ATP with that which occurred before starting of quenching revealed that $11.1 \pm 3.0\%$ of stored Ca^{2+} was enough to activate Ca^{2+} release-activated Ca^{2+} entry. The possibility that Mn^{2+} entered via persistently opened, ATP-related pathway in this protocol is negligible because (i) there was no difference in time course of quenching with (Fig. 5B) and without (Fig. 4B) preceding ATP application, (ii) the refilling process of the store site ceased within 5 min after Ca^{2+} re-perfusion according to Fig. 1B. No generally accepted cellular messengers of Ca^{2+} release-activated Ca^{2+} entry have been proposed, so the latency from the beginning of Ca^{2+} release till the start of quenching may include the time required to produce this unknown messenger. Therefore this amount of Ca^{2+} release required to induce Ca^{2+} release-activated Ca^{2+} entry may still be an overestimate.

We started this study to estimate how much Ca^{2+} release is actually needed to open the Ca^{2+} release-activated Ca^{2+} entry pathway. We found that this Ca^{2+} entry mechanism is not a simple 'depletion-refilling' system but actively participates in real-time control of $[Ca^{2+}]_i$. This mechanism requires not the complete depletion of the Ca^{2+} store site but only a small amount, about one-tenth of total stored Ca^{2+}, and can be activated while the releasing process is still proceeding. Therefore, agonist-induced Ca^{2+} mobilization can be amplified by this Ca^{2+} release-activated Ca^{2+} entry and this amplification may be another important role of this mechanism. We conclude that in bovine aortic endothelial cells the Ca^{2+} release-activated Ca^{2+} entry mechanism is activated abruptly by a small amount of Ca^{2+} release from the intracellular Ca^{2+} store site and so $[Ca^{2+}]_i$ is controlled dynamically by Ca^{2+} release and entry.

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