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Troglitazone inhibits the capacitative Ca^{2+} entry in endothelial cells

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Abstract

To investigate the effects of troglitazone on the capacitative Ca^{2+} entry, we monitored changes in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) induced by thapsigargin in fura-2-loaded porcine endothelial cells in situ and in primary culture. In aortic valve endothelial cells in situ, thapsigargin induced sustained elevation of $[Ca^{2+}]_i$. Both troglitazone and SKF 96365 inhibited the steady state increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. At 30 μM, troglitazone and SKF 96365 inhibited the $[Ca^{2+}]_i$ elevation to 19.4 ± 3.6% and 43.9 ± 4.5%, respectively. In aortic endothelial cells in primary culture, both troglitazone (10 μM) and SKF 96365 (100 μM) completely inhibited the thapsigargin-induced $[Ca^{2+}]_i$ increase. The EC_{50} value of troglitazone (1.4 ± 0.1 μM) was lower than that of SKF 96365 (10.0 ± 3.3 μM). We suggest that troglitazone would be a useful tool to investigate the capacitative Ca^{2+} entry.

Keywords: Ca^{2+} entry; Capacitative; Endothelial cell; Troglitazone; Thapsigargin; SKF 96365

1. Introduction

Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) play a major role in the signal transduction systems for diverse cellular functions such as muscle contraction, cell proliferation, exocytosis, angiogenesis, production of physiologically active substances and apoptosis both in excitable and non-excitable cells. One of the important mechanisms to induce a $[Ca^{2+}]_i$ elevation is the Ca^{2+} influx from the extracellular space. In excitable cells, the Ca^{2+} influx is mainly mediated by voltage-operated Ca^{2+} channels (VOCs) activated directly by depolarization of membrane potential and/or indirectly by the agonist-induced second messengers (Nelson et al., 1988; Karaki et al., 1997). On the contrary, in non-excitable cells, where VOCs are not expressed, dominant Ca^{2+} influx pathways include the capacitative Ca^{2+} entry pathways (Putney, 1990) and the so-called receptor-operated Ca^{2+} channels (Bolton, 1979). The capacitative Ca^{2+} entry pathways are activated by the depletion of the intracellular Ca^{2+} store sites. It was reported that thapsigargin (Thastrup et al., 1990) and cyclopiazonic acid (Seidler et al., 1989) inhibit the Ca^{2+}–ATPase of the store sites and induce the sustained elevation of $[Ca^{2+}]_i$ in many types of cells including endothelial cells (Schilling et al., 1992; Higuchi et al., 1996). In these cases, the inhibition of the Ca^{2+}–ATPase may deplete the stored Ca^{2+}, which result in the activation of the capacitative Ca^{2+} entry. Recently, cloning and functional expression of a mammalian homologue of the *Drosophila* eye-specific *trp* gene implied that TRP proteins form Ca^{2+} permeable channels linked to the capacitative Ca^{2+} entry (Wes et al., 1995; Philipp et al., 1996; Zhu et al., 1996). It was shown that endothelial cells express the message of this gene (Chang et al., 1997).

Troglitazone, (±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy) benzyl]-2,4-thiazolidinedione, one of the thiazolidinediones, is a newly developed oral antidiabetic drug (Bressler and Johnson, 1997). Troglitazone was shown to improve the response of fibroblasts to insulin impaired by high glucose and to restore impaired autophosphorylation of insulin receptors in response to insulin (Kellerer et al., 1994). In addition to the antidiabetic effect, troglitazone induced vasorelaxation by inhibit-

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ing VOCs in vascular smooth muscle cells (Song et al., 1997). We observed that troglitazone inhibited the Ca^{2+} influx activated by U46619, a thromboxane A_2 analogue, more potently than that induced by high K^+ depolarization in porcine coronary artery (Kawasaki et al., 1998). This finding suggests that troglitazone might inhibit the Ca^{2+} channels other than VOCs. Since it is difficult to discriminate the contribution of VOCs in the Ca^{2+} entry from that of the capacitative Ca^{2+} entry in smooth muscle cells, the effect of troglitazone on the capacitative Ca^{2+} entry has to be examined in non-excitable cells lacking VOCs, such as endothelial cells.

In the present study, using fura-2 fluorometry and endothelial cells in situ and in primary culture, we investigated the effect of troglitazone on the Ca^{2+} entry stimulated by thapsigargin (Thastrup et al., 1990) and agonists (Putney, 1997) such as endothelin-1 and bradykinin. We found that troglitazone strongly inhibits the capacitative Ca^{2+} entry which may play a major role in the Ca^{2+} influx in endothelial cells. Troglitazone inhibits this Ca^{2+} entry much more potently than SKF 96365, a well-known inhibitor of capacitative Ca^{2+} entry.

2. Materials and methods

2.1. Preparation of strips of porcine aortic valves (endothelial cells in situ)

The strips of porcine aortic valves were prepared as previously described (Aoki et al., 1994). The porcine aortic valves were isolated from fresh hearts at a local slaughterhouse immediately after animals had been slaughtered. All tissue specimens were placed in ice-cold physiological salt solution (PSS) and brought back to the laboratory. The aortic valve leaflets were handled with care in order to keep their surface intact. The valve leaflets were cut into strips in an axial direction (approximately 3 mm wide, 6 mm long, and 0.18 mm thick). The endothelial cells on the aortic valvular strips thus obtained were active in uptake of fluorescent acetylated-low density lipoprotein and expressed endothelial constitutive nitric oxide synthase, as evidenced by reverse transcription-polymerase chain reaction analysis (data not shown).

2.2. Primary cell culture of porcine aortic endothelial cells

Primary cultures of the porcine aortic endothelial cells were obtained as previously described (Hirano et al., 1993), with minor modifications. After rinsing with sterilized phosphate-buffered saline (PBS), the aortas were transferred from a local slaughterhouse to our laboratory in ice-cold sterile PBS. The aortas were opened longitudinally and the endothelial lining was gently scraped off with a scalpel blade. Cellular sheets thus obtained were collected by centrifugation at 1000 rpm for 5 min, suspended in growth medium and mechanically dispersed into smaller clumps by pipetting. These cells were plated onto sterilized round glass coverslip (diameter = 25 mm; Matsunami, Osaka, Japan) in 35-mm culture dishes (Becton Dickinson, Lincoln Park, NJ, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin. The growth medium was changed every 2–3 days. For experiments, cultured endothelial cells at 7–10 days after plating, when the cells just reached confluence, were used. Phase-contrast microscopic feature at confluence was a typical cobblestone monolayer of endothelial cells. The cultured endothelial cells were active in uptake of fluorescent acetylated-low density lipoprotein and expressed endothelial constitutive nitric oxide synthase and endothelin-converting enzyme, as determined by reverse transcription-polymerase chain reaction analysis (data not shown).

2.3. Fura-2 loading

The valvular strips were loaded with Ca^{2+} indicator dye, fura-2, by incubating them in an oxygenated (a mixture of 95% O_2 and 5% CO_2) DMEM containing 25 μM fura-2/AM (an acetoxymethyl ester form of fura-2), 1 mM probenecid and 5% fetal bovine serum for 1.5 h at 37°C (Aoki et al., 1994). Probenecid was added to prevent extrusion of fura-2 (Di Virgilio et al., 1989). After loading with fura-2, the strips were equilibrated in PSS for at least 1 h at 25°C before starting the measurements, in order to remove the dye in the extracellular space.

The primary cultured endothelial cells were incubated in DMEM containing 5 μM fura-2/AM for 1 h at 37°C as described (Hirano et al., 1993). After being loaded with fura-2, the cells were washed and incubated in HEPES-buffered saline (HBS) for at least 1 h before starting the measurement.

2.4. Fura-2 fluorometry of endothelial cells in situ and in primary culture

Front-surface fura-2 fluorometry of aortic valvular strip was performed by using a fluorometer specifically designed (CAM-OF3, Japan Spectroscopie, Tokyo, Japan) (Aoki et al., 1994). Strips were mounted vertically in the quartz organ bath filled with normal PSS. Changes in fluorescence (500 nm) intensity at 340 nm and 380 nm excitation of the aortic valvular strips were monitored and their ratio was recorded as an indicator of $[Ca^{2+}]_i$. Fura-2 fluorometry of the cultured endothelial cells was performed using a fura-2 microfluorometer as described (Hirano et al., 1993). Changes in fluorescence of the

cultured cells were monitored by using an inverted fluorescent microscope (TMD 56, Nikon, Tokyo, Japan), equipped with a fluorescence-spectrophotometer (CAM 220, Japan Spectroscopie, Tokyo, Japan). Fluorescence intensities (500 nm) of the cells on the coverslip at 340 nm and 380 nm excitation were monitored and their ratio was recorded as an indicator of $[Ca^{2+}]_i$.

All measurements both in situ and in primary culture were performed at 25°C to prevent sequestration of the fluorescent dye (Kobayashi et al., 1986). Experiments with valvular strips were performed in PSS bubbled with a mixture of 5% CO_2 and 95% O_2. Experiments with the primary culture were performed in HBS. The fluorescence ratio data were expressed as percentages, assigning the values at rest and at the peak response obtained with 10 μM ATP to be 0% and 100%, respectively (Aoki et al., 1994). In the experiments to assess the effects of inhibitors on the sustained phase of $[Ca^{2+}]_i$ elevation induced by thapsigargin in the presence of extracellular Ca^{2+}, the level of fluorescence ratio obtained at 30 min after the application of thapsigargin (just before the application of inhibitors) was assigned to be 100%.

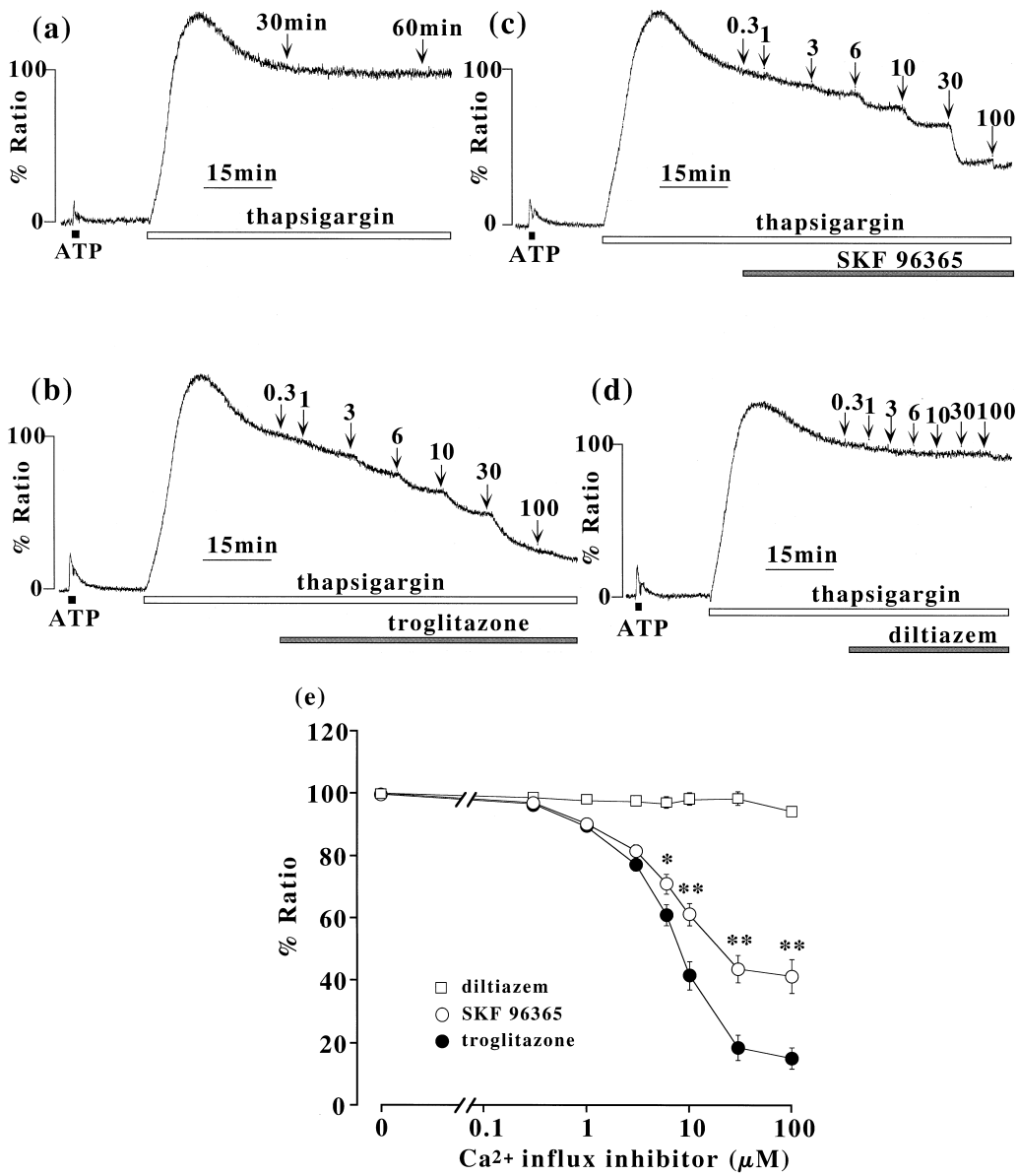

Fig. 1. Effects of troglitazone, SKF 96365 and diltiazem on the capacitative Ca^{2+} entry in endothelial cells in situ. (a–d) Representative recordings of 1 μM thapsigargin-induced $[Ca^{2+}]_i$ elevation in the presence of extracellular Ca^{2+} (a) without and with the cumulative applications of (b) troglitazone, (c) SKF 96365 and (d) diltiazem. At 30 min after thapsigargin application, when percentage fluorescence ratio ($[Ca^{2+}]_i$) was assigned as 100%, cumulative application of drugs was started. The final concentrations (μM) of drug are shown at the arrows. (e) Concentration-dependent effect of troglitazone, SKF 96365 and diltiazem on the sustained elevation of $[Ca^{2+}]_i$ induced by thapsigargin. Data are means ± S.E.M. ($n = 6–7$). $^{**}P < 0.01$; $^{*}P < 0.05$ compared with the values obtained with troglitazone.

2.5. Drugs and solutions

The composition of normal PSS was as follows (mM): 123 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.25 $CaCl_2$ and 11.5 D-glucose. PSS was aerated with a mixture of 95% O_2 and 5% CO_2, with the resulting pH being 7.4. The composition of HBS was as follows (mM): 133.9 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.25 $CaCl_2$, 11.5 D-glucose, 10 HEPES (including 4.1 NaCl), pH 7.4 at 25°C. Therefore, concentrations of total Na^+, K^+, Mg^{2+}, Ca^{2+} and D-glucose were similar in two buffers. Ca^{2+}-free version of the buffer was prepared by adding 2 mM EGTA in place of $CaCl_2$.

Fura-2/AM and EGTA were purchased from Dojindo (Kumamoto, Japan). Troglitazone [MW 441.55, dissolved in dimethylsulfoxide (DMSO)] was donated by Sankyo (Tokyo, Japan). SKF 96365 (1-{β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride) was purchased from Calbiochem (La Jolla, CA, USA). ATP was purchased from Boehringer Mannheim (Tokyo, Japan). Thapsigargin, bradykinin, diltiazem hydrochloride, probenecid and cyclopiazonic acid were purchased from Sigma (St. Louis, MO, USA). Endothelin-1 was purchased from Peptide Institute (Osaka, Japan).

2.6. Data analysis

The values were expressed as means ± standard error of the mean (S.E.M.). Student's *t* test was used to determine significant differences between two groups, and analysis of variance (ANOVA) was used to determine the concentration-dependent effect of troglitazone and SKF 96365 on the $[Ca^{2+}]_i$. Scheffe's test was used to determine statistical significance between four groups shown in Figs. 1 and 3. *P* values of less than 0.05 were considered to be statistically significantly different. All data were collected using a computerized data acquisition system (MacLab; Analog Digital Instruments, Australia, and Macintosh; Apple Computer, USA).

3. Results

3.1. Effects of troglitazone, SKF 96365 and diltiazem on thapsigargin-induced $[Ca^{2+}]_i$ increase in endothelial cells in situ

To induce a capacitative Ca^{2+} influx in the endothelial cells in situ, porcine aortic valvular strips were stimulated with 1 μM thapsigargin in normal PSS. Thapsigargin induced an initial rapid increase in $[Ca^{2+}]_i$. At 1 μM, the initial peak was obtained at 9.9 ± 0.5 min ($n = 6$), and the level of $[Ca^{2+}]_i$ was 541.5 ± 87.8% ($n = 6$) of that induced by 10 μM ATP. The $[Ca^{2+}]_i$, after reaching its peak, slightly declined to a sustained phase within 30 min. The sustained phase of $[Ca^{2+}]_i$ increase was maintained for more than 30 min (Fig. 1a). The levels of $[Ca^{2+}]_i$ at 30 min and 60 min after application of thapsigargin were 390.4 ± 60.3% and 381.9 ± 53.7% of that induced by 10 μM ATP, respectively. Thapsigargin-induced elevation of $[Ca^{2+}]_i$ was concentration-dependent, with the minimum concentration required to induce the maximum response to be 1 μM (data not shown). On the other hand, in the absence of extracellular Ca^{2+}, thapsigargin induced only a transient elevation of $[Ca^{2+}]_i$ with a peak at 5.6 ± 0.2 min ($n = 8$) (Fig. 2). Thus, the sustained phase observed in the presence of extracellular Ca^{2+} was considered to be due to an influx of Ca^{2+}. The effects of troglitazone on the capacitative Ca^{2+} influx were investigated during this sustained phase.

Fig. 1b, c and d show the effects of troglitazone, SKF 96365, widely used as an inhibitor of capacitative Ca^{2+} entry (Merritt et al., 1990; Schilling et al., 1992) and diltiazem, an L-type Ca^{2+} channel inhibitor, on the sustained increases in $[Ca^{2+}]_i$ induced by 1 μM thapsigargin. When troglitazone was applied cumulatively during the sustained phase of $[Ca^{2+}]_i$ elevation induced by thapsigargin, the $[Ca^{2+}]_i$ level decreased in a concentration-dependent manner (Fig. 1b and e). The significant inhibition of the thapsigargin-induced Ca^{2+} influx by troglitazone was observed at 1 μM and higher concentrations. The maximum inhibition was obtained with 30 μM, and $[Ca^{2+}]_i$ was 19.4 ± 3.6% ($n = 7$), assigning the level of $[Ca^{2+}]_i$ just before the cumulative application of troglitazone to be 100%. The concentration of troglitazone to induce 50% of the maximum inhibition (IC_{50}) was 7.0 ± 0.5 μM ($n = 7$). SKF 96365 also inhibited the $[Ca^{2+}]_i$ elevation induced by 1 μM thapsigargin (Fig. 1c and e). The inhibitory effect of SKF 96365 was significant at 1 μM and higher concentrations, and the maximum inhibition was obtained at 30 μM. The IC_{50} value for SKF 96365 was 6.9 ± 0.8 μM ($n = 7$). However, 30 μM SKF 96365 decreased $[Ca^{2+}]_i$ only to 43.9 ± 4.5% ($n = 7$) of the level observed just before the application, which was significantly higher than that obtained with 30 μM troglitazone. At concentrations of 6 μM and higher, the levels of $[Ca^{2+}]_i$ obtained with SKF 96365 were significantly higher ($P < 0.05$) than those obtained with troglitazone (Fig. 1e). On the contrary, diltiazem, an L-type Ca^{2+} channel blocker, had no effect at all on the $[Ca^{2+}]_i$ elevation induced by thapsigargin even at 100 μM (Fig. 1d and e).

Effects of troglitazone on the Ca^{2+} release induced by thapsigargin in the Ca^{2+}-free PSS were examined (Fig. 2). Fig. 2a and b are representative recordings showing 1 μM thapsigargin-induced increases in $[Ca^{2+}]_i$ in the absence and presence of 10 μM troglitazone in the Ca^{2+}-free PSS. When the strips were exposed to the Ca^{2+}-free PSS containing 2 mM EGTA, the resting level of $[Ca^{2+}]_i$ gradually decreased. When troglitazone was applied 5 min before the exposure to the Ca^{2+}-free PSS, there was no difference in the decreases in the resting $[Ca^{2+}]_i$ levels between the

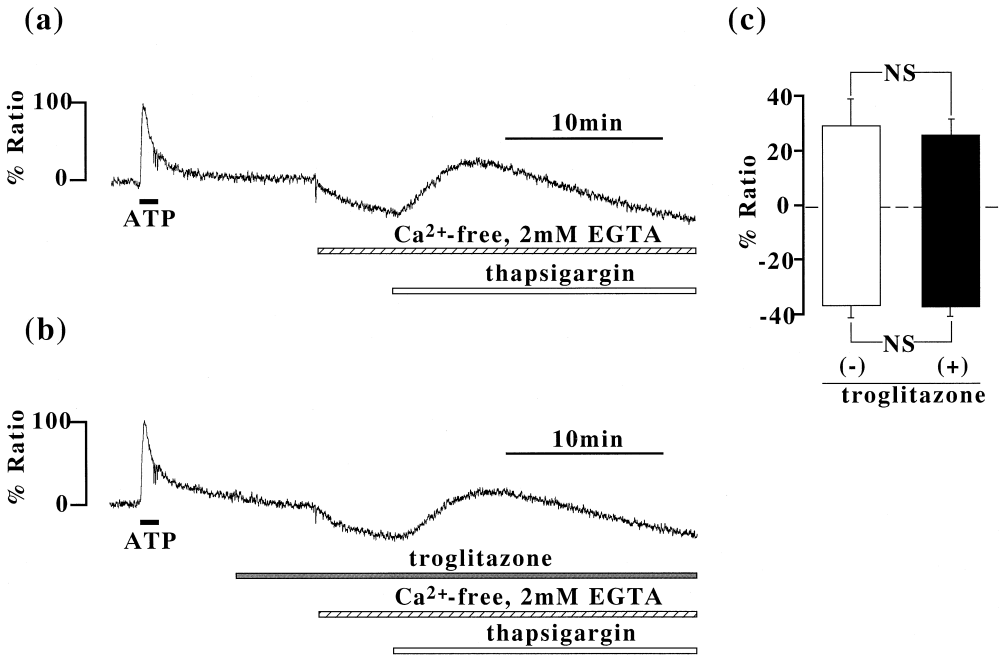

Fig. 2. Effects of troglitazone on the thapsigargin-induced $[Ca^{2+}]_i$ elevation in the Ca^{2+}-free media in endothelial cells in situ. (a,b) Representative recordings of the increases in $[Ca^{2+}]_i$ induced by 1 μM thapsigargin in Ca^{2+}-free solution containing 2 mM EGTA in the (a) absence and (b) presence of 10 μM troglitazone. The level of percentage fluorescence ratio ($[Ca^{2+}]_i$) observed with 10 μM ATP in the presence of extracellular Ca^{2+} was assigned as 100%. (c) The level of $[Ca^{2+}]_i$ elevated by thapsigargin in the absence and presence of 10 μM troglitazone. The bottom and the top of each column represent the level of $[Ca^{2+}]_i$ just before and at the peak obtained by the application of thapsigargin, respectively. Data are means ± S.E.M. ($n = 8$) of measurements as shown in (a) and (b). N.S., not significant.

absence ($-36.8 \pm 4.2\%$, $n = 8$) and presence ($-37.0 \pm 3.6\%$, $n = 8$) of troglitazone (Fig. 2c). Subsequent application of thapsigargin increased $[Ca^{2+}]_i$ to a similar extent both in the absence ($29.2 \pm 9.7\%$, $n = 8$) and presence ($25.5 \pm 6.0\%$, $n = 8$) of troglitazone (Fig. 2c). Thus, troglitazone had no effect on the Ca^{2+} release induced by thapsigargin in the absence of extracellular Ca^{2+}.

3.2. Effects of troglitazone, SKF 96365 and diltiazem on thapsigargin-induced $[Ca^{2+}]_i$ increase in vascular endothelial cells in primary culture

The application of 1 μM thapsigargin also induced an elevation of $[Ca^{2+}]_i$ in the endothelial cells of the primary culture in the presence of extracellular Ca^{2+}. Thapsigargin induced an initial rapid increase in $[Ca^{2+}]_i$ which reached a peak at 5.4 ± 0.9 min ($n = 6$). The level of $[Ca^{2+}]_i$ at the peak was $535.0 \pm 82.5\%$ of that induced by 10 μM ATP. After reaching the peak level, $[Ca^{2+}]_i$ demonstrated a slight gradual decline. The levels of $[Ca^{2+}]_i$ were $270.0 \pm 70.0\%$ and $224.5 \pm 65.8\%$ at 30 min and 45 min after the application of thapsigargin. This decline in $[Ca^{2+}]_i$ level in the primary cultured endothelial cells contrasted with the sustained $[Ca^{2+}]_i$ level in endothelial cells in situ.

We tested for the possible inhibitory effect of the solvent used, 0.1% DMSO (the final concentration of solvent of troglitazone). When 0.1% DMSO was applied at 30 min after the application of thapsigargin, the $[Ca^{2+}]_i$ gradually declined as observed in the absence of DMSO. Assigning the level of $[Ca^{2+}]_i$ at rest and at 30 min after the application of thapsigargin to be 0% and 100%, respectively, the level of $[Ca^{2+}]_i$ at 15 min after the application of 0.1% DMSO was $79.2 \pm 5.0\%$ ($n = 6$) (Fig. 3a). Thus, 0.1% DMSO per se had no effect on the thapsigargin-induced $[Ca^{2+}]_i$ elevation in endothelial cells in primary culture. When 10 μM troglitazone was applied at 30 min after the application of thapsigargin, $[Ca^{2+}]_i$ rapidly decreased to the resting level within 15 min (Fig. 3b). Similarly, 10 μM SKF 96365 rapidly decreased thapsigargin-induced $[Ca^{2+}]_i$ elevation (Fig. 3c). However, the level of $[Ca^{2+}]_i$ did not completely return to the resting level, but remained at a slightly, but significantly ($P < 0.05$) higher level. On the other hand, 10 μM diltiazem had no effect on the thapsigargin-induced elevation of $[Ca^{2+}]_i$ (Fig. 3d).

Troglitazone and SKF 96365 inhibited the thapsigargin-induced elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 3e). Both troglitazone and SKF 96365 completely inhibited the slightly declining phase of elevations of $[Ca^{2+}]_i$. The minimum concentrations of troglitazone and SKF 96365 required to induce significant inhibition of the thapsigargin-induced $[Ca^{2+}]_i$ increase were 1 μM and 10 μM, respectively, compared with the values obtained with the vehicle (Fig. 3e). The maximum

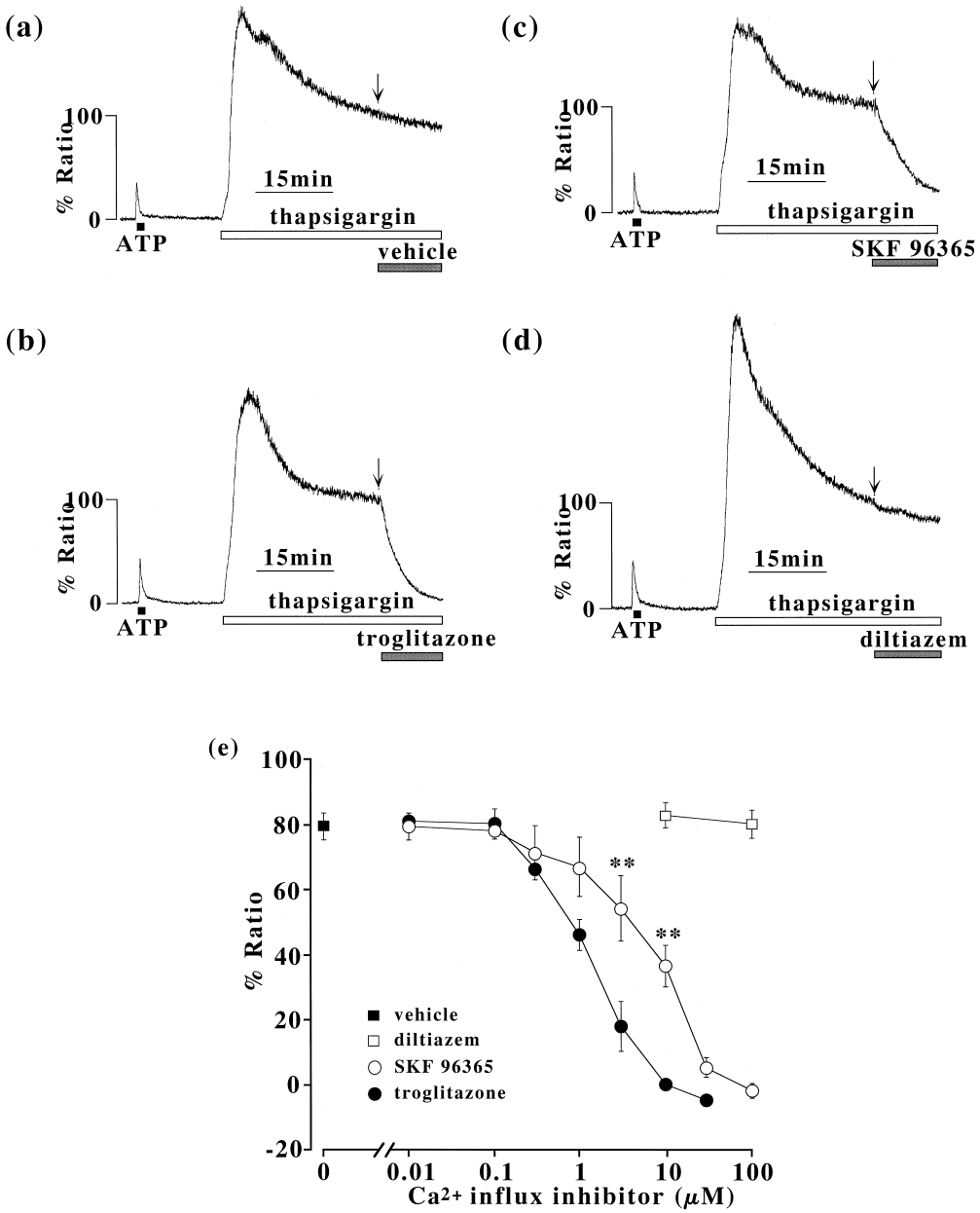

Fig. 3. Effects of troglitazone, SKF 96365 and diltiazem on capacitative Ca^{2+} entry in vascular endothelial cells in primary culture. (a–d) Representative recordings showing the effects of a vehicle (0.1% DMSO) as a (a) control, (b) 10 μM troglitazone, (c) 10 μM SKF 96365 and (d) 10 μM diltiazem on the $[Ca^{2+}]_i$ elevation induced by 1 μM thapsigargin. Each drug was applied at 30 min after application of TG. (e) Concentration-dependent effects of vehicle, troglitazone, SKF 96365 and diltiazem at 15 min after the application on the $[Ca^{2+}]_i$ elevation induced by 1 μM thapsigargin. Data are means ± S.E.M. ($n = 3$–6). $^{**}P < 0.01$ compared with the values obtained with troglitazone.

inhibition was obtained with 10 μM troglitazone and 100 μM SKF 96365. The IC_{50} values for troglitazone and SKF 96365 were 1.4 ± 0.1 μM and 10.0 ± 3.3 μM, respectively. At the concentration of 3 and 10 μM, the levels of $[Ca^{2+}]_i$ obtained with SKF 96365 were significantly higher than those obtained with troglitazone ($P < 0.01$). On the other hand, diltiazem, even at 100 μM, had no effect on thapsigargin-induced increase in $[Ca^{2+}]_i$ in the primary culture (Fig. 3e).

Cyclopiazonic acid, another inhibitor for Ca^{2+}–ATPase of the Ca^{2+} stores (Goeger et al., 1988; Seidler et al., 1989), induced a similar sustained elevation of $[Ca^{2+}]_i$ in the cultured endothelial cells (data not shown). Application of 30 μM cyclopiazonic acid induced an initial rapid increase in $[Ca^{2+}]_i$ (517.9 ± 75.6% of that induced by 10 μM ATP, $n = 4$) at 6.8 ± 2.3 min ($n = 4$), followed by a slight decline in $[Ca^{2+}]_i$ levels. The level of $[Ca^{2+}]_i$ at 30 min was 290.2 ± 47.5% ($n = 4$). Thus, cyclopiazonic acid-induced $[Ca^{2+}]_i$ elevation was similar to that observed with thapsigargin. When 10 μM troglitazone was applied 30 min after application of cyclopiazonic acid, the level of $[Ca^{2+}]_i$ rapidly declined to 0.21 ± 5.6% of that

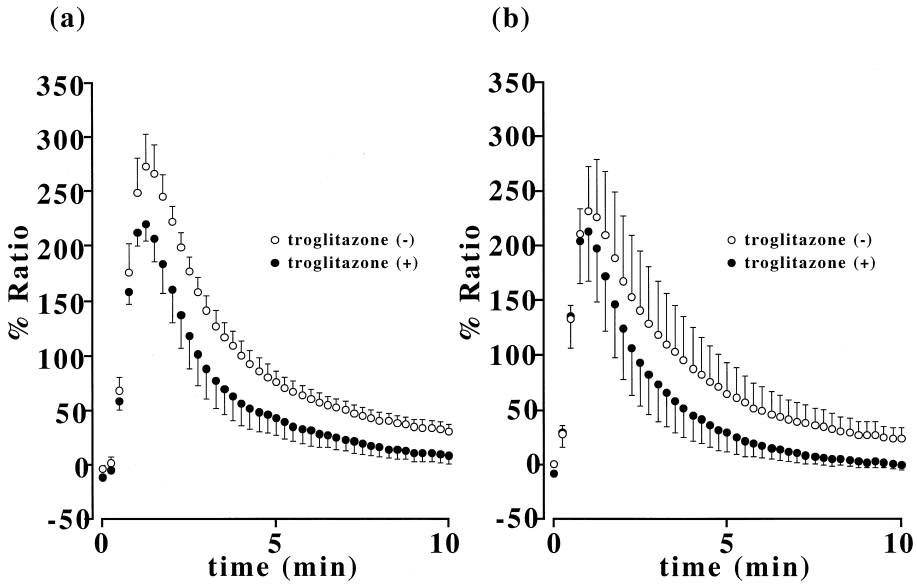

Fig. 4. Effects of troglitazone on agonist-induced $[Ca^{2+}]_i$ increase in valvular endothelial cells in situ. (a,b) Time courses of changes in $[Ca^{2+}]_i$ induced by 100 nM endothelin-1 (a, $n = 6$) and 100 nM bradykinin (b, $n = 5$) in the absence and presence of 10 μM troglitazone, respectively. Troglitazone was applied 10 min before and during the application of endothelin-1 and bradykinin. Data are means ± S.E.M.

before the application of troglitazone within 15 min ($n = 4$).

3.3. Effects of troglitazone on endothelin-1- and bradykinin-induced $[Ca^{2+}]_i$ increase in endothelial cell in situ

We investigated the effect of troglitazone on the Ca^{2+} influx induced by physiologically relevant agonists, endothelin-1 and bradykinin. Fig. 4a and b show the effects of troglitazone on 100 nM endothelin-1- and 100 nM bradykinin-induced $[Ca^{2+}]_i$ increase in the endothelial cells in situ in normal PSS, respectively. Both endothelin-1 and bradykinin abruptly increased the level of $[Ca^{2+}]_i$, which reached a peak at 1.4 ± 0.1 min and 1.0 ± 0.1 min, respectively, and then, progressively declined to reach a steady level. The peak $[Ca^{2+}]_i$ elevations induced by endothelin-1 and bradykinin were 275.2 ± 29.9% ($n = 6$) and 250.9 ± 46.6% ($n = 5$) of the response to 10 μM ATP, respectively. The levels of $[Ca^{2+}]_i$ at 10 min after the application of endothelin-1 and bradykinin were 30.9 ± 6.7% and 24.7 ± 10.3%, respectively. When 10 μM troglitazone was applied 10 min prior to and during the stimulation with endothelin-1 and bradykinin, the peak levels of $[Ca^{2+}]_i$ were 228.9 ± 18.4% ($n = 6$) and 218.5 ± 43.6% ($n = 5$), respectively. The peak responses to endothelin-1 and bradykinin in the presence of troglitazone were obtained at 1.3 ± 0.1 min and 0.89 ± 0.1 min, respectively. Thus, troglitazone had no significant effects on the peak $[Ca^{2+}]_i$ elevations induced by both endothelin-1 and bradykinin ($P > 0.05$). However, the levels of $[Ca^{2+}]_i$ at 10 min after the applications of endothelin-1 and bradykinin in the presence of troglitazone were 8.4 ± 7.2% and −1.7 ± 3.4%, respectively. These values were significantly smaller than those observed in the absence of troglitazone ($P < 0.05$).

4. Discussion

In the presence of the extracellular Ca^{2+}, both thapsigargin and cyclopiazonic acid induced a marked increase in $[Ca^{2+}]_i$ in endothelial cells. It is generally accepted that endothelial cells lack VOCs, with some exception of those derived from the microvascular bed (Bossu et al., 1989). The electrophysiological studies could not detect the activity of VOCs (L-type Ca^{2+} channels) in either freshly isolated (Busse et al., 1988) or cultured endothelial cells (Colden-Stanfield et al., 1987). In the present study, the endothelial cells of the aortic valve and in primary culture from aorta did not induce $[Ca^{2+}]_i$ elevations in response to external high K^+ solution (data not shown). Diltiazem, a Ca^{2+} channel blocker, had no effect on the extracellular Ca^{2+}-dependent $[Ca^{2+}]_i$ elevations induced by thapsigargin. These observations indicate that endothelial cells both in situ and in primary culture lack VOCs and that the inhibition of thapsigargin-induced Ca^{2+} influx by troglitazone was not due to the inhibition of VOCs. Since there was no difference in the transient $[Ca^{2+}]_i$ elevation induced by thapsigargin in Ca^{2+}-free media between in the presence and in the absence of troglitazone, it is unlikely that troglitazone specifically modulated the thapsigargin-induced inhibition of the Ca^{2+}-ATPase or Ca^{2+} depletion of the intracellular store sites. Rather we suggest that troglitazone acted as an inhibitor of Ca^{2+} influx pathway

activated by the depletion of Ca^{2+} stores due to the inhibition of Ca^{2+}-ATPase, namely the capacitative Ca^{2+} entry.

SKF 96365 has been widely used as an inhibitor of receptor-operated Ca^{2+} entry both in excitable and non-excitable cells (Merritt et al., 1990), and also inhibited the capacitative Ca^{2+} entry (Schilling et al., 1992) and VOCs in smooth muscle (Merritt et al., 1990). The observations concerning SKF 96365 in the present study are consistent with the previous reports. Now, it has become apparent that troglitazone as well as SKF 96365 inhibits both the capacitative Ca^{2+} entry and VOCs. The efficacy and potency of troglitazone to inhibit the capacitative Ca^{2+} entry were greater than those of SKF 96365. Encabo et al. (1996) reported that LOE 908, a cation channel blocker, inhibited the thapsigargin-induced capacitative Ca^{2+} entry in cultured human endothelial cells ($IC_{50} = 2$ μM). Guse et al. (1997) reported that in Jurkat T-lymphocytes, LU 52396 inhibited the capacitative Ca^{2+} entry ($IC_{50} = 5$ μM). The inhibition by LU 52396 was observed only when $[Ca^{2+}]_i$ was low or the channel was in its closed state. Hopf et al. (1996) reported that in the cultured skeletal muscle cells, AN1043 and AN406, dihydropyridine compounds, inhibited the Ca^{2+} channels activated by cyclopiazonic acid; however, 10 μM AN1043 only partially inhibited thapsigargin-induced sustained $[Ca^{2+}]_i$ increase (64% inhibition). Importantly, none of these inhibitors showed the complete inhibition of the capacitative Ca^{2+} entry, as observed with troglitazone in the present study. In addition, despite the similarity in the inhibitory effect on the capacitative Ca^{2+} entry, there are no similarities in the chemical structure between troglitazone and these possible inhibitors.

There is a possibility that troglitazone inhibits the coupling between the store depletion and the activation of the Ca^{2+} influx. There are two mechanisms proposed for such coupling: the direct coupling mediated via cytoskeletal structure such as actin filaments (Holda and Blatter, 1997) and the indirect coupling mediated via second messengers. Many candidates have been proposed for the second messengers. These include Ca^{2+} influx factor, cyclic GMP, cytochrome P450 metabolites of arachidonic acids, heterotrimeric G-proteins, small G-proteins, tyrosine kinase, myosin light chain kinase and protein phosphatases (for review, see Parekh and Penner, 1997). However, there is no report regarding the effects of troglitazone on these signaling pathways except for tyrosine phosphorylation (Kellerer et al., 1994). There is a possibility that troglitazone might alter the state of protein phosphorylation which is responsible for mediating the activation of Ca^{2+} influx due to the store depletion. These possibilities remain to be elucidated.

There were slight differences in the thapsigargin-induced $[Ca^{2+}]_i$ elevation between in situ endothelial cells and the cultured cells, such as time course, the levels of sustained elevation (relative to the response to ATP) and the efficacy of SKF 96365 (incomplete inhibition in in situ cells vs. complete inhibition in the cultured cells). These observations suggest that the properties of the capacitative Ca^{2+} entry might be altered during the culture condition. However, both in in situ cells and in primary cultured cells, troglitazone inhibited the capacitative Ca^{2+} entry activated by thapsigargin. The study on the cultured endothelial cells suggested that receptor-mediated stimulations such as bradykinin (Schilling et al., 1992) and histamine (Merritt et al., 1990) activated the capacitative Ca^{2+} entry, since the sustained $[Ca^{2+}]_i$ elevations induced by these agonist were inhibited by SKF 96395. However, the contribution of the capacitative Ca^{2+} entry in the Ca^{2+} influx induced by agonist in in situ endothelial cells has not been examined. Here, we demonstrated that the sustained $[Ca^{2+}]_i$ elevations induced by endothelin-1 and bradykinin were inhibited by the troglitazone in endothelial cells in situ, and suggests the contribution of troglitazone-sensitive Ca^{2+} influx, i.e., capacitative Ca^{2+} entry in the agonist-induced Ca^{2+} entry.

Some possibilities other than direct inhibition of the capacitative Ca^{2+} entry could also be considered for the mechanism by which troglitazone decreased the extracellular Ca^{2+}-dependent elevation of $[Ca^{2+}]_i$, especially in the case of the agonist-induced Ca^{2+} influx. First, troglitazone might inhibit K^+ channels, which depolarizes membrane potential and thereby attenuates Ca^{2+} influx (Adams et al., 1989). Troglitazone was shown to inhibit ATP-sensitive K^+ channels in insulin-secreting cells (Lee et al., 1996). Secondly, troglitazone might activate extrusion of Ca^{2+} from the cytosol by activating cytoplasmic Ca^{2+}-ATPase and decrease $[Ca^{2+}]_i$. However, these possibilities remain to be elucidated.

Carboxyamidotriazole, an inhibitor of non-voltage-operated Ca^{2+} channels including receptor-operated Ca^{2+} channels, was shown to suppress angiogenesis of human endothelial cells, indicating a role for Ca^{2+} in the regulation of angiogenesis (Kohn et al., 1995). Troglitazone, therefore, may inhibit neovascularization seen in diabetic retinopathy. On the other hand, we previously demonstrated that the capacitative Ca^{2+} entry induced by cyclopiazonic acid caused endothelium-dependent vasorelaxation (Higuchi et al., 1996). Troglitazone may inhibit the production of endothelium-derived relaxing factors. Since the concentration of troglitazone we used in the present study was not far from the therapeutic range which was reported to be 2–3 μM (Horikoshi et al., 1994), the inhibition of the Ca^{2+} influx by troglitazone observed in the present study could be operable in the clinical use. However, the effects of troglitazone on the function of endothelial cells remain to be investigated.

5. Conclusion

The present study extended our previous findings in vascular smooth muscle cells that troglitazone inhibited

agonist-induced Ca^{2+} influx more potently than depolarization-induced Ca^{2+} influx, and demonstrated that troglitazone is an inhibitor of the capacitative Ca^{2+} entry in endothelial cells. This inhibitory effect is more potent than a widely used inhibitor SKF 96365.

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