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Troglitazone and pioglitazone attenuate agonist-dependent Ca^{2+} mobilization and cell proliferation in vascular smooth muscle cells

[1,2]Michiko Asano, *,[1]Toshiaki Nakajima, [1]Kuniaki Iwasawa, [1]Toshihiro Morita, [1]Fumitaka Nakamura, [1]Hiroyuki Imuta, [1]Keigo Chisaki, [2]Nobuhiro Yamada, [1]Masao Omata & [2]Yukichi Okuda

[1]Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan and [2]Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-0005, Japan

1 The effects of troglitazone and pioglitazone on agonist-induced Ca^{2+} mobilization and cell proliferation were studied using fluorescent Ca^{2+} indicator fura-2 AM and incorporation of [^{3}H]-thymidine in rat aortic smooth muscle cells. The patch clamp techniques were also employed.

2 Vasopressin and platelet-derived growth factor-BB (PDGF) caused a transient elevation in $[Ca^{2+}]_i$ by Ca^{2+} mobilization from intracellular stores, followed by a sustained rise due to Ca^{2+} entry. Nicardipine partly inhibited the sustained phase, but La^{3+} completely abolished it.

3 Troglitazone and pioglitazone did not significantly affect the transient rise elicited by these agonists, but preferentially inhibited the sustained phase of $[Ca^{2+}]_i$.

4 Under voltage clamp conditions, troglitazone and pioglitazone inhibited voltage-dependent L-type Ca^{2+} current ($I_{Ca.L}$). They also inhibited nonselective cation channels (I_{cat}) elicited by vasopressin in a concentration-dependent manner. The half maximal inhibitory concentrations of troglitazone on $I_{Ca.L}$ and I_{cat} were 4.6 and 5.7 μM, respectively. On the other hand, nifedipine and nicardipine did not inhibit I_{cat}.

5 Vasopressin and PDGF increased incorporation of [^{3}H]-thymidine, and nifedipine and nicardipine partly suppressed it. However, the inhibitory effects of La^{3+} and exclusion of extracellular Ca^{2+} were more potent than the Ca^{2+} blocking agents. Troglitazone and pioglitazone also inhibited it concentration-dependently.

6 These results suggest that troglitazone and pioglitazone preferentially inhibited agonist (vasopressin and PDGF)-induced Ca^{2+} entry and proliferation in rat vascular smooth muscle cells, where the inhibitory effects of thiazolidinediones on $I_{Ca.L}$ and I_{cat} might be partly involved. Thus, thiazolidinediones may exert hypotensive and antiatherosclerotic effects.

Keywords: Thiazolidinediones; troglitazone; pioglitazone; receptor-mediated Ca^{2+} entry; vasopressin; aortic smooth muscle cell; cell proliferation; ionic currents; nonselective cation currents; voltage-dependent L-type Ca^{2+} currents; prostaglandin J_2

Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol bis-(β-aminoethylether) N,N,N′,N′-tetraacetic acid; I_{Ba}, Ba^{2+} current through $I_{Ca.L}$; $I_{Ca.L}$, voltage-dependent L-type Ca^{2+} current; I_{cat}, non-selective cation channels; PDGF, platelet-derived growth factor; PPAR-γ, the peroxisome proliferator-activated receptor-γ

Introduction

Troglitazone and pioglitazone, insulin-sensitizing agents, belong to a novel group of oral hypoglycemic drugs classified as thiazolidinediones (Whitcomb & Saltiel, 1995; Schwartz *et al*., 1998; Inzucchi *et al*., 1998), and increase insulin sensitivity and responsiveness of target organs in patients with several chronic disorders such as non-insulin-dependent diabetes mellitus (NIDDM), atherosclerosis, and hypertension where insulin resistance is involved (Kolterman *et al*., 1981; Modan *et al*., 1985; Ferrannini *et al*., 1987; Sowers *et al*., 1994; Nolan *et al*., 1994). Thiazolidinediones increase peripheral insulin-mediated glucose consumption and reduce hepatic glucose production by enhancing tissue sensitivity to insulin, and increase the number of cellular glucose transporters without affecting number or affinity of insulin receptors (Yoshioka *et al*., 1989; Ciaraldi *et al*., 1990). In vascular smooth muscle cells, insulin attenuates agonist (vasopressin and angiotensin-II)-induced intracellular Ca^{2+} rise and modulates voltage-dependent L-type Ca^{2+} channels (Standley *et al*., 1991; Anderson & Mark, 1993; Ram *et al*., 1993; Kahn *et al*., 1993), and antagonizes vasopressin-activated nonselective cation currents in A7r5 cells (Standley *et al*., 1991). Therefore, thiazolidinediones may improve these vascular actions of insulin, then indirectly reduce vascular contractility and lower blood pressure (Landin *et al*., 1991; Ogihara *et al*., 1995; Kotchen, 1996). On the other hand, the direct effects of pioglitazone on vascular smooth muscle cells have been reported (Buchanan *et al*., 1995). Also, troglitazone and pioglitazone by themselves inhibit voltage-dependent L-type Ca^{2+} channels (Zhang *et al*., 1994; Song *et al*., 1997; Nakamura *et al*., 1998). Additionally, troglitazone antagonizes the contraction elicited by noradrenaline (Song *et al*., 1997), and ciglitazone decreases platelet-derived growth factor (PDGF)-induced Ca^{2+} entry in vascular smooth muscle cells (Pershadsingh *et al*., 1993), but the detailed mechanisms of thiazolidinediones on intracellular Ca^{2+} mobilization and ionic mechanisms remain to be clarified.

*Author for correspondence.

Thiazolidinediones such as troglitazone and pioglitazone have been shown to inhibit cell proliferation elicited by vasoactive agents (epidermal growth factor, insulin and basic fibroblast growth factor) (Dubey *et al*., 1993; Law *et al*., 1996) or some pathophysiologic conditions (Yasunari *et al*., 1997; Shinohara *et al*., 1998). The basic mechanisms of thiazolidinediones-induced inhibition of cell proliferation have not been clarified, but several mechanisms, i.e. the peroxisome proliferator-activated receptor-γ (PPAR-γ) (Lehmann *et al*., 1995), have been proposed. Alternatively, calcium is considered as an intracellular signal implicated in the growth-regulatory control of a number of cell types (Berridge, 1995). DNA synthesis and cell proliferation are inhibited by Ca^{2+} channel antagonists such as verapamil and nifedipine in vascular smooth muscle cells (Kramsch *et al*., 1980; Block *et al*., 1989; Sperti & Colucci, 1991; Yang *et al*., 1993), and stimulation of calcium entry is required for DNA synthesis induced by PDGF (Mogami & Kojima, 1993). Thus, reduction of Ca^{2+} entry through Ca^{2+} channels may also be one of the mechanisms underlying antiproliferative effects of thiazolidinediones.

Therefore, to clarify the mechanisms underlying the effects of thiazolidinediones (troglitazone and pioglitazone) we examined the effects of these agents on agonist (vasopressin and PDGF)-mediated Ca^{2+} mobilization and DNA synthesis in vascular smooth muscle cells. Additionally, since troglitazone is known to be a potent activator of PPARγ (Lehmann *et al*., 1995), we have compared the effects of thiazolidinediones with those of prostaglandin J_2, another potent activator of PPAR-γ (Kliewer *et al*., 1995).

Methods

Cell preparation

A7r5 cells (ATCC-7), well established vascular smooth muscle cell lines obtained from embryonic rat aorta (Kimes & Brandt, 1976; Standley *et al*., 1991), were purchased from the American Type Culture Collection through Dainippon Seiyaku (Kyoto, Japan). Cultured cells were fed every second day with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum, 100 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin at 35°C in a fully humidified atmosphere of 5% CO_2. Cells subcultured to passage numbers 12–18 were grown as monolayers on glass slides, and confluent cells were detached from the culture flasks with 0.25% trypsin in 0.02% EDTA, and used for later experiments. Cell viability determined by trypan blue exclusion was approximately 91%. All experiments were performed at 35–37°C.

Solutions and drugs

The composition of the standard Tyrode solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, HEPES-NaOH buffer 5 (pH 7.4). The high K^+ solution contained (in mM): KCl 140, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, HEPES-KOH buffer 5 (pH 7.4). The pipette solution contained (in mM): CsCl 130, EGTA 0.15, $MgCl_2$ 2, Na_2ATP 3, guanosine 5′-triphosphate (GTP, sodium salt, Sigma) 0.1 and HEPES-CsOH buffer 5 (pH 7.2). To record membrane potential, the patch pipette contained (in mM): KCl 130, EGTA 0.01, $MgCl_2$ 2, Na_2ATP 3, GTP 0.1 and HEPES-KOH buffer 5 (pH 7.2). The Ba^{2+}-containing Tyrode solution was the same as the control bathing solution with the exception that $CaCl_2$ was replaced by $BaCl_2$ (5 mM). When extracellular or intracellular Cl^- concentration was changed, Cl^- was replaced with equimolar aspartic acid. Troglitazone and pioglitazone were obtained from Sankyo Co. Ltd. (Tokyo, Japan) and Takeda Co. Ltd. (Osaka, Japan). Troglitazone and pioglitazone were dissolved in dimethyl sulphoxide (DMSO) to give a stock solution of 1–100 mM, and the final concentration of DMSO applied to the bathing solution was lower than 0.1%. [Arg^8]-vasopressin was obtained from Peptide Institute, Inc. (Osaka, Japan). Prostaglandin J_2 was obtained from Sigma (St. Louis, MO, U.S.A.).

Determination of cytosolic free Ca^{2+} concentration

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined using the fura-2 fluorescence method as described previously (Grynkiewicz *et al*., 1985; Asano *et al*., 1998). The Ca^{2+}-free bathing solution was the same as the normal Tyrode solution except that $CaCl_2$ was omitted and 0.5 mM EGTA was added to the solution (pH 7.4). Fura-2 acetoxymethyl ester (fura-2 AM) was obtained from Dojin Chemicals (Japan). Cells were trypsinized, washed twice in the standard solution, adjusted to a cell density of 10^6 cell ml^{-1} and loaded with 1 μM fura-2 AM for 60 min in a 20°C-shaking water bath. After incubation, the medium containing fura-2 AM was removed, and fluorescent cells in suspension were measured at 37°C while stirred continuously in a cuvette placed by a spectrofluorometer (CAF-100, JASCO Co, Ltd., Tokyo). The excitation wavelengths were 340 and 380 nm, and emission was 500 nm. Fluorescence signals were calibrated using 0.5% Triton W-100 for maximum fluorescence, 300 mM EGTA pH < 8.0 for minimum fluorescence.

Recording technique and data analysis

Membrane potentials and currents were recorded with glass pipettes under whole-cell clamp conditions (Hamill *et al*., 1981; Nakajima *et al*., 1992), using a patch-clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany). The heat-polished patch electrode, filled with the artificial internal solution (for composition, see above), had the tip resistance of 3–5 MΩ. The series resistance was compensated. Membrane potentials and currents were continuously monitored with a high-gain storage oscilloscope (COS 5020-ST, Kikusui Electronic, Tokyo, Japan). The data were stored on a videotape using the PCM converter system (RP-890, NF, Electronic circuit design, Tokyo, Japan). The data were reproduced, low-passed, filtered at 1 kHz (−3 dB) with a Bessel filter (FV-625, NF, 48 dB/octave slope attenuation), sampled at 5 kHz and analysed off-line on a computer using p-Clamp software (Axon Instruments, CA, U.S.A.).

Measurement of DNA synthesis

Cells were grown to subconfluence in 24-well tissue culture dishes and the growth was arrested for 48 h in a serum-free medium consisting of DMEM. The DMEM medium was employed to maintain the VSMC in quiescent, but not catabolic, conditions resembling that of healthy cells in the normal arterial wall *in vivo*. The medium was then removed, and fresh DMEM containing vasopressin (100 nM) or PDGF-BB (25 ng ml^{-1}) was added to the quiescent cells. The cells were subsequently incubated for 18 h in the absence or presence of troglitazone, pioglitazone, nifedipine, nicardipine or La^{3+}. In several experiments, 0.5 mM EGTA was added to the control solution to reduce extracellular Ca^{2+} concentration. The cells were then incubated with metyl-[^{3}H]-thymidine (0.5 μCi ml^{-1}, Amersham Pharmacia Biotech UK Ltd., Bucks,

U.K.) for 4 h in the absence or presence of these agents. The medium was then removed, the cells were washed twice in ice cold 5% trichloroacetic acid (TCA) and then incubated in 5% TCA on ice for 15 min. The cells were solubilized by adding 0.5 ml of 0.5 N NaOH. 0.2 ml aliquots were then neutralized and counted in scintillation fluid. Proteins of cells were measured by using Bradford method.

Statistical analysis

The data were expressed as a mean ± s.d. and ANOVA and Fisher's PLSD for multiple comparisons and the unpaired Student's *t*-test were performed. Differences with a value of $P<0.05$ were considered significant.

Results

Effects of troglitazone and pioglitazone on Ca^{2+} mobilization elicited by vasopressin and PDGF

Figures 1 and 3 show the effects of vasopressin and PDGF on intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in rat aortic smooth muscle cells (A7r5 cells). In the presence of extracellular Ca^{2+}, vasopressin (100 nM) or PDGF (30 ng ml^{-1}) induced a biphasic increase of $[Ca^{2+}]_i$ (Figures 1A,B and 3A). The first transient increase of $[Ca^{2+}]_i$ elicited by these agonists resulted mainly from Ca^{2+} release of intracellular store sites, and the persistent elevation of $[Ca^{2+}]_i$ resulted from the entry of extracellular Ca^{2+}. Figures 1A,B and 3A show the effects of nicardipine and La^{3+} on vasopressin- or PDGF-induced Ca^{2+} mobilization. After the $[Ca^{2+}]_i$ rise elicited by vasopressin (100 nM, Figure 1A,B) or PDGF (30 ng ml^{-1} Figure 3A) reached a steady-state, application of nicardipine (1 μM) or nifedipine (1 μM, data not shown) partly decreased the final sustained phase of $[Ca^{2+}]_i$ by about 10% of the control level (12 ± 10% ($n=5$) in vasopressin and 13 ± 8% ($n=4$) in PDGF). Alternatively, La^{3+} (1 mM) completely eliminated the sustained phase of $[Ca^{2+}]_i$, suggesting that vasopressin- or PDGF-induced Ca^{2+} entry occurred *via* a dihydropyridine-insensitive Ca^{2+} channel as well as the voltage-dependent L-type Ca^{2+} channel. Figure 1C–F show the effects of troglitazone (3–10 μM) and pioglitazone (10 μM) on the sustained rise of $[Ca^{2+}]_i$ induced by vasopressin. After $[Ca^{2+}]_i$ rise elicited by vasopressin reached to a steady state, troglitazone (3, 10 μM) decreased the sustained phase of $[Ca^{2+}]_i$ (Figure 1C,E). Similarly, troglitazone (10 μM) inhibited the sustained rise of $[Ca^{2+}]_i$ elicited by vasopressin in the presence of nicardipine (1 μM, Figure 1D). Similar results were obtained in four different cells. Pioglitazone (10 μM) also inhibited the vasopressin-induced sustained rise (Figure 1F). These results suggest that both troglitazone and pioglitazone inhibited vasopressin-induced sustained rise of $[Ca^{2+}]_i$ in aortic smooth muscle cells. Also, troglitazone (10 μM) inhibited the sustained rise induced by PDGF (Figure 3B).

Figures 2A and 3C show the effects of troglitazone (10 μM) on Ca^{2+} mobilization induced by vasopressin or PDGF. $[Ca^{2+}]_i$ responses elicited by vasopressin or PDGF were compared in the absence and presence of troglitazone

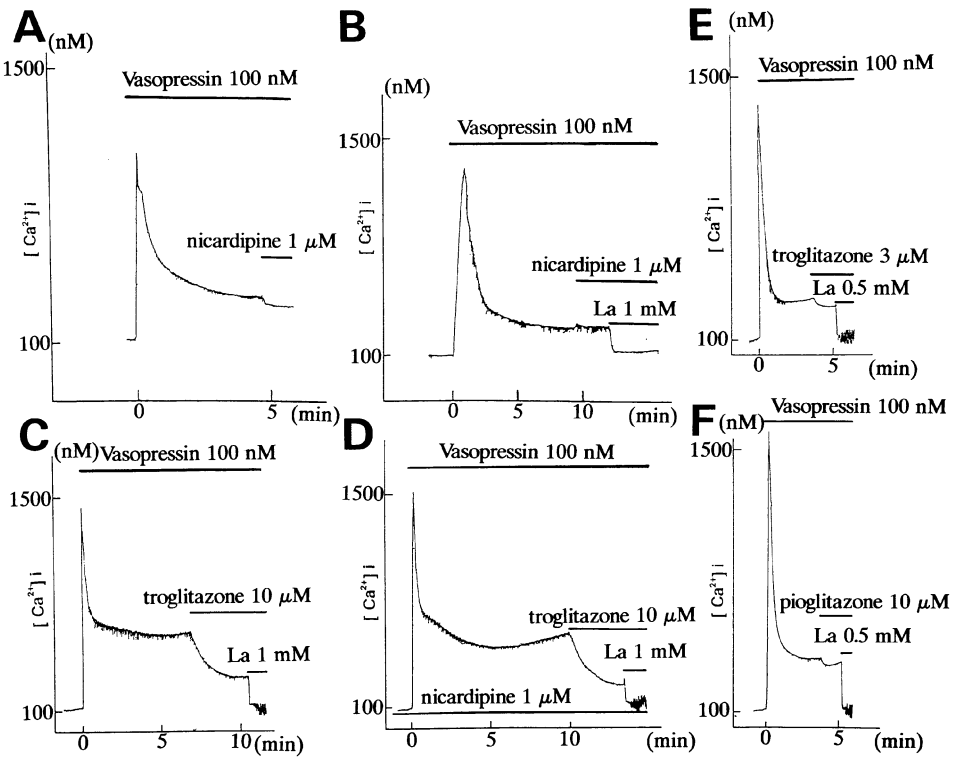

Figure 1 Effects of nicardipine, La^{3+}, troglitazone and pioglitazone on vasopressin-induced intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in aortic smooth muscle cells. (A,B) Effects of nicardipine (1 μM, A,B) and La^{3+} (1 mM, B) on vasopressin (100 nM)-induced sustained rise in $[Ca^{2+}]_i$. (C,D) Effects of troglitazone on vasopressin-induced sustained rise in $[Ca^{2+}]_i$ in the absence (C) and presence of nicardipine (1 μM, D). Results were representative of 4 similar experiments. $[Ca^{2+}]_i$ with calibration was obtained from the ratio signal of the emission light at 500 nm by excitation at 340 and 360 nm. Note that nicardipine (1 μM) slightly decreased the sustained rise in $[Ca^{2+}]_i$ induced by vasopressin, while La^{3+} and troglitazone (10 μM) inhibited it even in the presence of nicardipine. (E,F) Effects of troglitazone (3 μM, E) and pioglitazone (10 μM, F) on the sustained rise in $[Ca^{2+}]_i$ induced by vasopressin (100 nM).

(10 μM). There are no difference between control (130$\pm$29 nM, $n=5$) and troglitazone-treated cells (122$\pm$13 nM, $n=5$) on basal $[Ca^{2+}]_i$ (Figure 2Aa). The increased value of peak $[Ca^{2+}]_i$ elicited by vasopressin (100 nM, Figure 2Ab, left panel) or PDGF (30 ng ml^{-1}, Figure 3C, left part) tended to decrease in cells treated with troglitazone (10 μM), which was not significantly different. Similarly, the increased value of peak $[Ca^{2+}]_i$ elicited by vasopressin in the absence of extracellular Ca^{2+} was not altered (Figure 2Ac), suggesting that troglitazone did not significantly affect Ca^{2+} mobilization induced by Ca^{2+} release from intracellular storage sites. Alternatively, troglitazone reduced the increased value of the sustained phase of $[Ca^{2+}]_i$ elicited by vasopressin (Figure 2Ab, right part) or PDGF (Figure 3C, right part). Figure 2B also shows the effects of troglitazone on the Ca^{2+} entry elicited by vasopressin. Vasopressin (100 nM) transiently increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, but addition of Ca^{2+} into the bath solution rapidly increased $[Ca^{2+}]_i$ levels by promoting Ca^{2+} influx from the extracellular side (Figure 2B). In the presence of troglitazone (10 μM), the $[Ca^{2+}]_i$ rise elicited by Ca^{2+} influx was markedly decreased (Figure 2Bb), as compared with the control cell (Figure 2Ba). These observations also suggest that troglitazone inhibits vasopressin-induced Ca^{2+} entry in vascular smooth muscle cells.

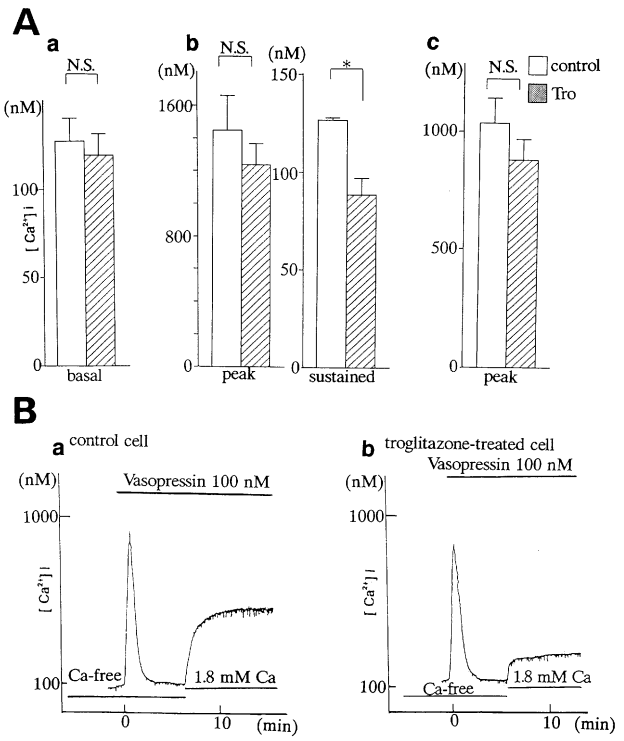

Figure 2 Effects of troglitazone and pioglitazone on $[Ca^{2+}]_i$. (A) Effects of troglitazone on $[Ca^{2+}]_i$. The bath was perfused with or without troglitazone (10 μM). The basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ response induced by vasopressin (100 nM) were compared. The resting $[Ca^{2+}]_i$ is shown in Aa. In the presence of extracellular Ca^{2+}, the increased $[Ca^{2+}]_i$ level at the peak and sustained phase (Ab), which was obtained by the subtraction from $[Ca^{2+}]_i$ level of these phases to the control level is plotted. In Ac, the effects of troglitazone (10 μM) on the transient $[Ca^{2+}]_i$ rise elicited by vasopressin in the absence of extracellular Ca^{2+} are illustrated. Data were obtained from five different experiments, and each column represents mean$\pm$s.d. $^*P<0.05$ vs controls. (B) Effects of troglitazone on $[Ca^{2+}]_i$ response elicited by vasopressin. Cells were perfused without (a) or with troglitazone (10 μM, b) in the absence of extracellular Ca^{2+}. After the transient $[Ca^{2+}]_i$ rise was elicited by vasopressin, Ca^{2+} (1.8 mM) was added to the bathing solution. Note that the $[Ca^{2+}]_i$ rise caused by Ca^{2+} entry was markedly decreased in the presence of troglitazone.

Effects of troglitazone and pioglitazone on voltage-dependent L-type Ca^{2+} currents ($I_{Ca.L}$) and vasopressin-activated nonselective cation channels (I_{cat})

Troglitazone has been reported to modulate a certain type of K^+ channels, the ATP-sensitive K^+ channels, in pancreatic β-cells (Lee *et al*., 1996). Therefore, we first investigated the effects of troglitazone on membrane potentials in A7r5 cells. Under current clamp conditions with K^+-internal solution, the membrane potential was -39 ± 5 mV ($n=13$) in controls, and -40 ± 6 mV in the presence of troglitazone (30 μM, $n=10$, $P=$n.s.). Thus, troglitazone did not significantly affect the membrane potential (Figure 4A). On the other hand, tetraethylammonium (TEA, 30 mM, Figure 4B) depolarized the membrane potential. Glibenclamide (10 μM) did not affect the membrane potential. These observations suggest that ATP-sensitive K^+ channels do not contribute to the formation of resting membrane potential in these cells, but the basic mechanism of troglitazone on vasopressin-induced Ca^{2+} mobilization is not due to the influence of the membrane potential.

In vascular smooth muscle cells, vasoactive agents such as vasopressin induce Ca^{2+} influx through voltage-dependent Ca^{2+} channels and/or receptor-operated Ca^{2+} channels. So, we examined the effects of thiazolidinediones (troglitazone and pioglitazone) on the voltage-dependent L-type Ca^{2+} current ($I_{Ca.L}$) as shown in Figures 5 and 6. The patch pipette was filled with the Cs^+-internal solution, and the bathing solution contained 5 mM Ba^{2+} in the place of Ca^{2+}. The cell was held at -40 mV, and the command voltage steps to $+0$ mV were applied at 0.2 Hz. DMSO at concentrations less than 0.1% did not affect I_{Ba} significantly, but nifedipine (0.1$-$1 μM, Figure 5Aa), nicardipine (1 μM, data not shown) or La^{3+} (1 mM, Figure 5Ab) almost completely blocked I_{Ba}. Troglitazone (10 μM, Figures 5B and 6A) also reduced the amplitude of I_{Ba} by 67$\pm$11% ($n=5$). The inhibitory effect of troglitazone on I_{Ba} was partly reversible. Figure 6B,C show the effects of troglitazone (10 μM) on current-voltage relationships of I_{Ba}. I_{Ba} was elicited by depolarizing command steps from a holding potential of -40 mV. The amplitude of the peak inward current was plotted at each command potential (Figure 6C). Troglitazone consistently reduced the amplitude of I_{Ba} at each command potential without affecting the shape of the current-voltage relationships of I_{Ba}. Figures 5B and 8A indicate the relationships between concentration of troglitazone and percent inhibition of I_{Ba}. Troglitazone suppressed I_{Ba} in a concentration-dependent manner. The half maximal inhibitory concentration of troglitazone was 4.6 μM (Figure 8A). Figure 5C and D also illustrate the effects of pioglitazone on I_{Ba}. Pioglitazone reduced I_{Ba}, but the potency of pioglitazone on I_{Ba} was less than that of troglitazone (Figures 5D and 8A). These results indicate that thiazolidinediones (troglitazone and pioglitazone) can inhibit voltage-dependent L-type Ca^{2+} channels in vascular smooth muscle cells as previously described (Zhang *et al*., 1994; Song *et al*., 1997; Nakamura *et al*., 1998).

In addition, the contractile agonists such as vasopressin and endothelin-1 induce Ca^{2+} influx through receptor-operated Ca^{2+} channels, in which Ca^{2+}-permeable nonselective cation channels (I_{cat}) are involved (Byrne & Large, 1988; Van Renterghem *et al*., 1988; Amedee *et al*., 1990; Wang & Large, 1991; Nakajima *et al*., 1996; Iwasawa *et al*., 1997; Minowa *et al*., 1997). Therefore, we next examined the effects of

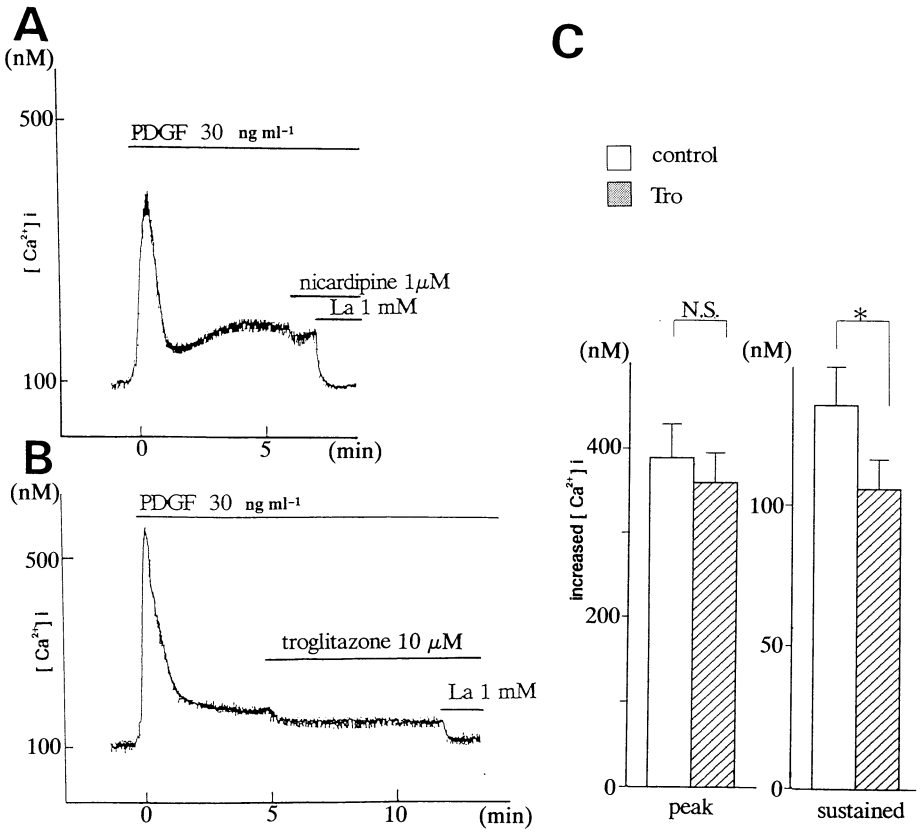

Figure 3 Effects of troglitazone on PDGF-induced intracellular Ca^{2+} concentration. (A) Effects of nicardipine (1 μM) and La^{3+} (1 mM) on PDGF (30 ng ml^{-1})-induced sustained rise in $[Ca^{2+}]_i$. (B) Effects of troglitazone (10 μM) on PDGF-induced sustained rise in $[Ca^{2+}]_i$. (C) Effects of troglitazone on PDGF-induced $[Ca^{2+}]_i$ mobilization. The bath was perfused with or without troglitazone (10 μM). The $[Ca^{2+}]_i$ responses induced by PDGF (30 ng ml^{-1}) were compared in the presence of extracellular Ca^{2+}. The increased $[Ca^{2+}]_i$ level at the peak and sustained rise was obtained by the subtraction from $[Ca^{2+}]_i$ level of these phases to the control level. Data were obtained from five different experiments, and each column represents mean $\pm$ s.d. $^*P<0.05$ vs controls.

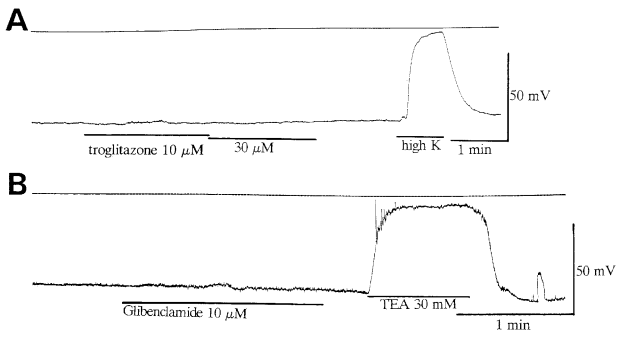

Figure 4 Effects of troglitazone on membrane potential in A7r5 cells. (A) Effects of troglitazone (10 and 30 μM) on membrane potential. A high K^+ solution contained 140 mM KCl. (B) Effects of glibenclamide (10 μM) and tetraetylammonium (TEA, 30 mM) on membrane potential. The patch pipette contained the K^+-internal solution. The zero current level denotes dotted lines.

thiazolidinediones (troglitazone and pioglitazone) on Ca^{2+}-permeable nonselective cation channels (Figure 7). With CsCl in the patch pipettes, the cells were held at -60 mV. When vasopressin (100 nM) was added to the normal Tyrode solution, an inward current was elicited (Figure 7A). The current-voltage relationships of the vasopressin-induced current were examined using the ramp voltage steps. With 140 mM Na^+ in the extracellular solution, the current-voltage relationships of the current reversed at -2 ± 3 mV ($n=12$, Figure 7B). The reversal potential of the current was unaffected by changing $[Cl^-]_i$ or $[Cl^-]_o$ (data not shown), suggesting that vasopressin activates I_{cat}, but not a Cl^- current in these conditions, which was compatible with the previous papers (Van Renterghem *et al.*, 1988; Krautwurst *et al.*, 1994; Nakajima *et al.*, 1996; Iwasawa *et al.*, 1997; Asano *et al.*, 1997). Figure 7C shows the effects of nifedipine, La^{2+}, Cd^{2+}, troglitazone and pioglitazone on the vasopressin-activated I_{cat}. The current was not significantly affected by nifedipine (10 μM, Figure 7Ca) and nicardipine (10 μM, data not shown), but completely blocked by Cd^{2+} (Figure 7Cb), or La^{3+} (1 mM, Figure 7Cc). Troglitazone (5–30 μM) also inhibited vasopressin-activated I_{cat} in a concentration-dependent manner (Figures 7Cd and 8B). The half maximal inhibitory concentration of troglitazone on vasopressin-activated I_{cat} was 5.7 μM as indicated in Figure 8B. Pioglitazone (30 μM) also decreased the vasopressin-activated I_{cat} (Figures 7Ce and 8B), but the inhibitory effects of pioglitazone on I_{cat} were less than that of troglitazone. Thus, thiazolidinediones (troglitazone and pioglitazone) inhibited vasopressin-mediated Ca^{2+}-permeable I_{cat} as well as $I_{Ca.L}$ in vascular smooth muscle cells.

Effects of troglitazone and pioglitazone on vasopressin- and PDGF-induced mitogenesis

Figures 9 and 10 show the effects of vasopressin (Figure 9A) or PDGF (Figure 10A) on cell proliferation. Vasopressin

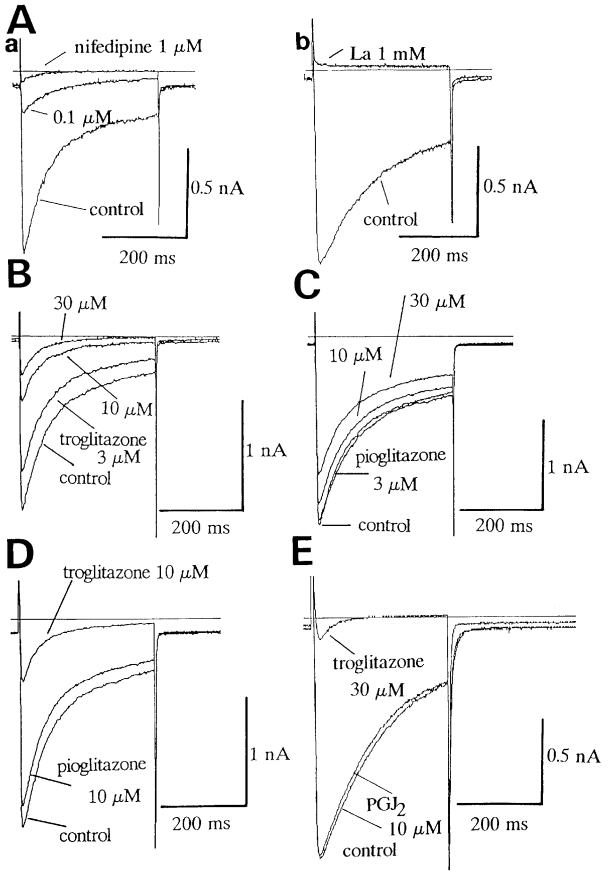

Figure 5 Effects of nifedipine, La^{3+}, thiazolidinediones (troglitazone and pioglitazone) and prostaglandin J_2 on the voltage-dependent L-type Ca^{2+} currents ($I_{Ca.L}$) in aortic smooth muscle cells. The cells were held at -40 mV, and the command voltage pulses to $+0$ mV were applied at 0.2 Hz. The zero current levels denote as lines. (A) Effects of nifedipine (0.1, 1 μM, a) and La^{3+} (1 mM, b) on $I_{Ca.L}$. (B,C) Effects of troglitazone (3–30 μM, B) and pioglitazone (3–30 μM, C) on $I_{Ca.L}$. (D,E) Comparative effects of troglitazone, pioglitazone and prostaglandin J_2 (10 μM) on $I_{Ca.L}$.

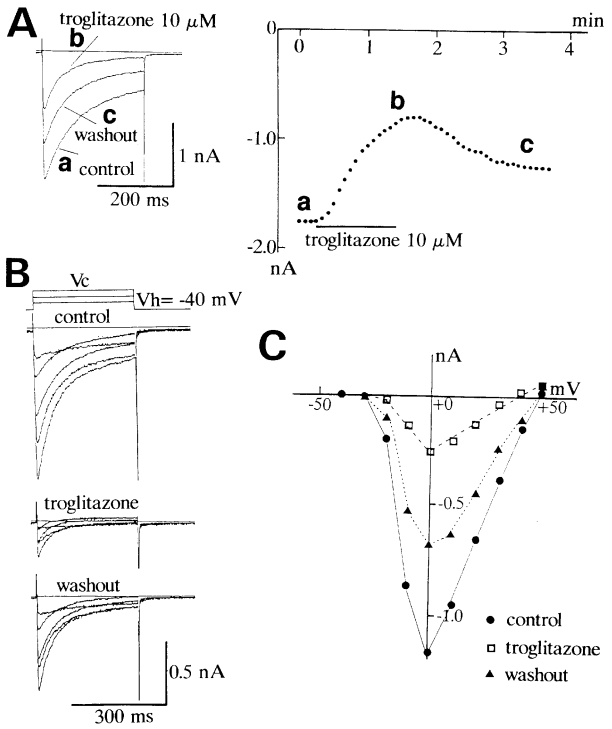

Figure 6 Effects of troglitazone on voltage-dependent L-type Ca^{2+} current ($I_{Ca.L}$). (A) Effects of troglitazone (10 μM) on $I_{Ca.L}$. The cell was held at -40 mV, and command voltage pulses to $+0$ mV were applied at 0.2 Hz. The patch pipette contained CsCl-internal solution. The time courses of the changes in the amplitude of $I_{Ca.L}$ measured from the zero current level are shown in the right part of A. The current traces shown in A are indicated in control, in the presence of troglitazone and after washout. (B,C) Effects of troglitazone on the current-voltage relationships of $I_{Ca.L}$. The cell was held at -40 mV, and command voltage pulses to various membrane potentials were applied. The current traces in B are shown in control, in the presence of troglitazone (10 μM) and after washout. The current-voltage relationships of $I_{Ca.L}$ measured at the peak are shown in C.

(100 nM) elicited an increase in the incorporation of [^{3}H]-thymidine into cells (Figure 9A). To investigate the involvement of extracellular Ca^{2+} on vasopressin-induced mitogenesis, the effects of extracellular Ca^{2+} and Ca^{2+} channel antagonistic agents were investigated as shown in Figure 9A. Removal of extracellular Ca^{2+} with EGTA suppressed the vasopressin-induced incorporation of [^{3}H]-thymidine. Nifedipine (1 μM) and nicardipine (1 μM) partly inhibited these vasopressin-induced thymidine incorporation by 32 ± 7 and $15\pm10\%$ ($n=4$), respectively. On the other hand, La^{3+} (1 mM) suppressed it by $85\pm7\%$ ($n=4$), which was more potent than the Ca^{2+} channel blocking agents. The effects of troglitazone and pioglitazone on vasopressin- or PDGF-induced mitosis were shown in Figures 9 and 10. Troglitazone (3–20 μM, Figures 9B and 10A) and pioglitazone (3–10 μM, Figures 9C and 10B) significantly decreased the incorporation of [^{3}H]-thymidine elicited by vasopressin and PDGF in a concentration-dependent manner.

Comparative effects of thiazolidinediones and prostaglandin J_2

Troglitazone has been known to be a potent activator of PPAR-γ (Lehmann *et al.*, 1995). We compared the effects of troglitazone and prostaglandin J_2, another potent activator of PPAR-γ (Kliewer *et al.*, 1995) on $I_{Ca.L}$ and I_{cat}. As shown in Figures 5E and 7D, prostaglandin J_2 (10 μM) did not affect $I_{Ca.L}$ and I_{cat} significantly ($n=4$). Similarly, prostaglandin J_2 (10 μM) did not affect the sustained phase of $[Ca^{2+}]_i$ rise induced by vasopressin (100 nM, data not shown). The effects of thiazolidinediones and prostaglandin J_2 on PDGF-induced incorporation of [^{3}H]-thymidine were also compared (Figure 10). Prostaglandin J_2 (0.1–10 μM) decreased the incorporation in a concentration-dependent manner, but the inhibitory effects of prostaglandin J_2 (10 μM) were much less than that of troglitazone (10 μM, Figure 10A) and pioglitazone (10 μM, Figure 10B).

Discussion

The major findings of the present study are: (1) Thiazolidinediones (troglitazone and pioglitazone) inhibited Ca^{2+} entry elicited by vasopressin and PDGF in aortic smooth muscle cells; (2) These agents inhibited vasopressin-mediated Ca^{2+}-permeable nonselective cation channels (I_{cat}) as well as voltage-dependent L-type Ca^{2+} channel ($I_{Ca.L}$); (3) Vasopressin and PDGF stimulated mitosis by measuring the incorporation of [^{3}H]-thymidine. Nifedipine and nicardipine partly inhibited the vasoactive agent-induced thymidine incorporation, but re-

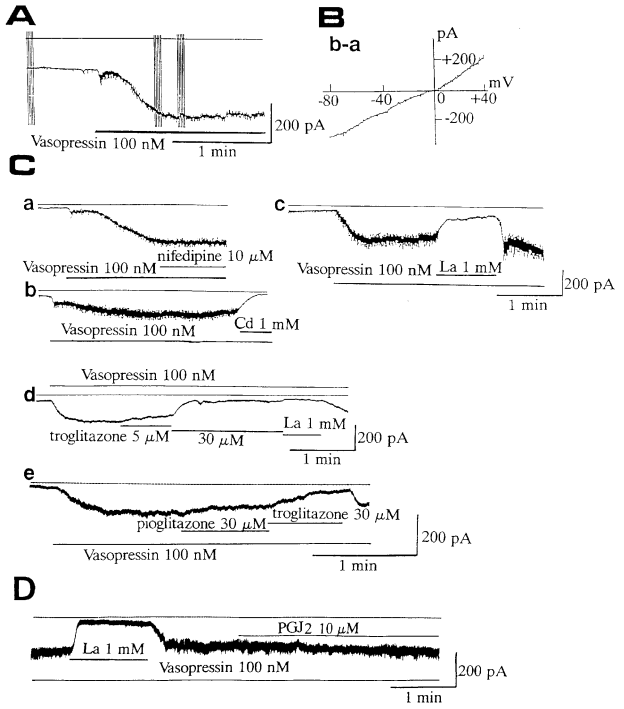

Figure 7 Effects of troglitazone and pioglitazone on vasopressin-activated nonselective cation currents. (A) Activation of nonselective cation currents by vasopressin. The cell was held at −60 mV. The patch pipette contained Cs-internal solution. Ramp voltage pulses from −80 mV to +40 mV (100 ms in duration) were applied before, during application of vasopressin (100 nM). The zero current level denotes dotted lines. The current-voltage relationships of the subtraction current from the current trace in the presence of vasopressin to control trace are shown in B. (C) Effects of nifedipine, La^{3+}, Cd^{2+}, troglitazone and pioglitazone on vasopressin-activated nonselective cation currents. Note that Cd^{2+} (1 mM, b), La^{3+} (1 mM, c, d) and troglitazone (5 and 30 μM, d, e) and pioglitazone (30 μM, e) inhibited the vasopressin-activated nonselective cation currents, while nifedipine (10 μM, a) did not inhibit it. (D) Effects of prostaglandin J_2 (10 μM) on vasopressin-activated nonselective cation currents. Results were representative of 4–6 similar experiments in each case.

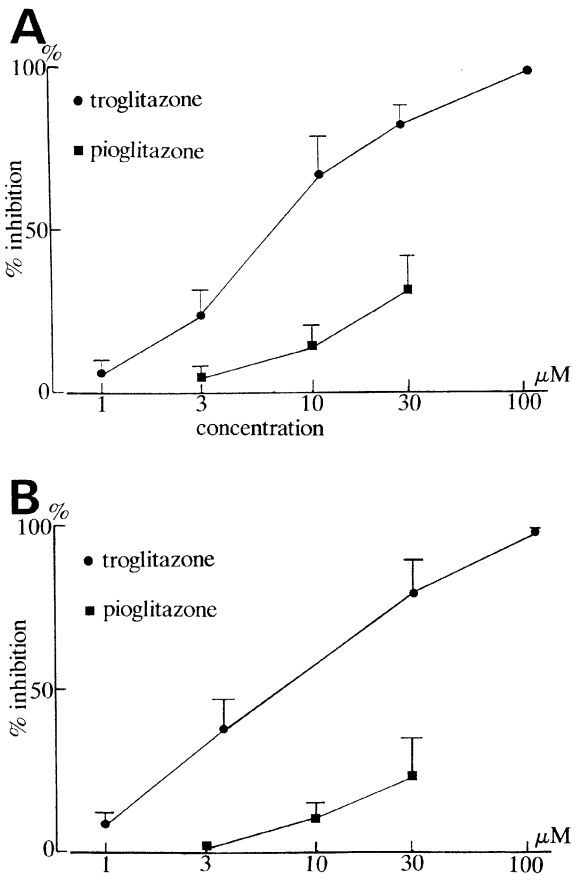

Figure 8 Comparative effects of troglitazone and pioglitazone on the voltage-dependent L-type Ca^{2+} currents ($I_{Ca.L}$) and vasopressin-activated nonselective cation currents (I_{cat}). (A) Concentration-dependent inhibitory effects of troglitazone and pioglitazone on $I_{Ca.L}$. The amplitude of the peak $I_{Ca.L}$ during application of thiazolidinediones was compared with the control value. The percent inhibition induced by thiazolidinediones on $I_{Ca.L}$ (man ± s.d. value) is shown. The data were obtained from six different cells including Figure 5. (B) Concentration-dependent inhibitory effects of troglitazone and pioglitazone on vasopressin-activated I_{cat}. The data were obtained from six different cells including Figure 7.

moval of extracellular Ca^{2+} with EGTA and La^{3+} markedly reduced it and (4) Troglitazone and pioglitazone also inhibited cell proliferation induced by vasopressin and PDGF in a concentration-dependent manner. These results suggest that thiazolidinediones (troglitazone and pioglitazone) preferentially inhibit agonist (vasopressin and PDGF)-induced Ca^{2+} entry, and proliferation of vascular smooth muscle cells, where the inhibitory effects of thiazolidinediones on I_{cat} as well as $I_{Ca.L}$ might be partly involved. Thus, it is likely that thiazolidinediones exert antihypertensive and antiatherosclerotic effects.

Several papers have shown that thiazolidinediones, such as troglitazone and pioglitazone, can reduce peripheral resistance and exert hypotensive effects (Dubey *et al*., 1993; Pershadsingh *et al*., 1993; Ogihara *et al*., 1995; Buchanan *et al*., 1995; Kotchen, 1996). One of the basic mechanisms may be due to the improvement of insulin resistance, because hypertension is attenuated by an agent that promotes insulin sensitivity (Landin *et al*., 1991; Ogihara *et al*., 1995) and insulin by itself has vasodilatory actions (Standley *et al*., 1991; Anderson & Mark, 1993; Ram *et al*., 1993; Kahn *et al*., 1993). On the other hand, the direct effects of thiazolidinediones on vascular smooth muscle cells have been reported. Troglitazone and pioglitazone have been shown to inhibit voltage-dependent L-type Ca^{2+} currents ($I_{Ca.L}$) in vascular smooth muscle cells (Zhang *et al*., 1994; Song *et al*., 1997; Nakamura *et al*., 1998). Also, in the present study, the half maximal inhibitory concentration of troglitazone was approximately 4.6 μM, and 10 μM pioglitazone decreased $I_{Ca.L}$ by approximately 20%. The clinical effective blood concentration is about 1–3 μM for troglitazone (Shibata *et al*., 1993), and 2–5 μM for pioglitazone, suggesting that these thiazolidinediones can inhibit $I_{Ca.L}$ at clinical settings.

The present study also provides evidence that troglitazone and pioglitazone inhibit intracellular Ca^{2+} rise through Ca^{2+} entry elicited by vasopressin and PDGF. In the absence of extracellular Ca^{2+}, troglitazone did not significantly inhibit $[Ca^{2+}]_i$ rise elicited by Ca^{2+} release from the storage sites, suggesting that it cannot inhibit the signalling pathways between receptors (vasopressin) and IP_3 production. Alternatively, it preferentially inhibited Ca^{2+} entry elicited by vasoactive agents (vasopressin and PDGF), possibly *via* direct inhibitory effects on the Ca^{2+} channels. Nicardipine or nifedipine, a potent dihydropyridine Ca^{2+} channel antagonist, partly inhibited the vasopressin and PDGF-induced sustained rise in $[Ca^{2+}]_i$, suggesting that thiazolidinediones may inhibit the vasoactive agents-induced sustained rise in $[Ca^{2+}]_i$ by inhibiting $I_{Ca.L}$. However, thiazolidinediones further inhibited

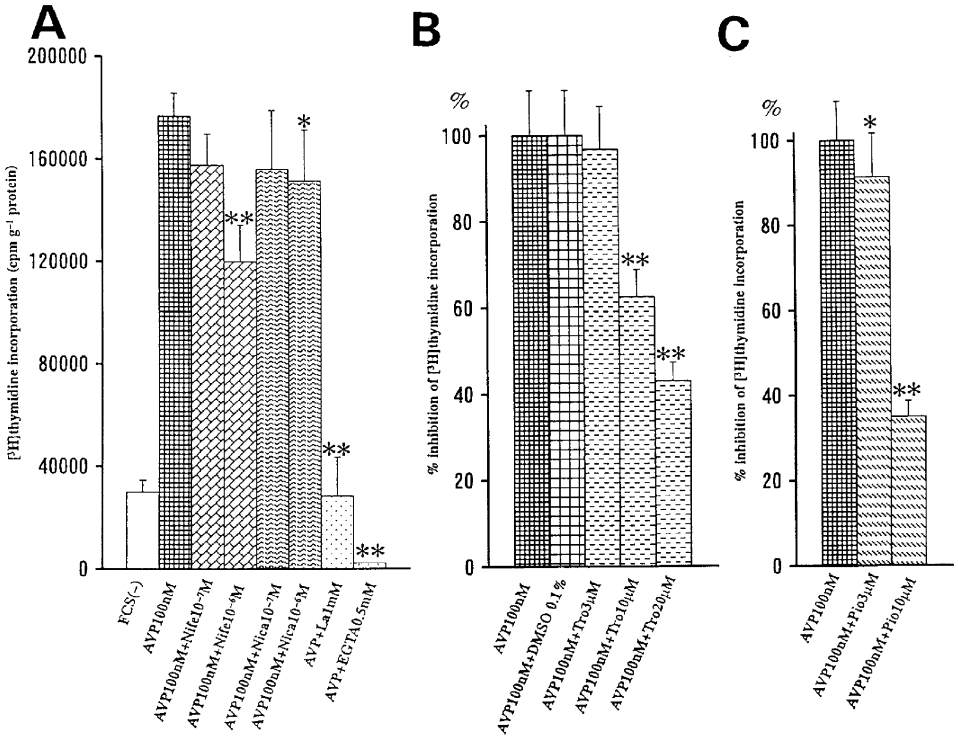

Figure 9 (A) Effects of removal of extracellular Ca^{2+} and Ca^{2+} channel antagonists on vasopressin-induced cell proliferation in A7r5 cells. The cell proliferation was measured by the incorporation of [^{3}H]-thymidine. Effects of nifedipine (Nife), nicardipine (Nica), La^{3+} and removal of extracellular Ca^{2+} with EGTA on vasopressin (AVP)-induced [^{3}H]-thymidine incorporation. Each data was obtained from four different experiments, and mean ± s.d. value is plotted. $*P<0.05$ vs controls $**P<0.01$ vs controls. (B,C) Effects of thiazolidinediones (troglitazone and pioglitazone) on vasopressin (AVP)-induced [^{3}H]-thymidine incorporation. Effects of troglitazone (3–20 μM) and pioglitazone (3–10 μM) on vasopressin-induced [^{3}H]-thymidine incorporation. Each data was obtained from four different experiments, and the mean ± s.d. value is shown. $*P<0.05$ vs controls $**P<0.01$ vs controls.

the sustained rise in $[Ca^{2+}]_i$ elicited by vasopressin even in the presence of nicardipine as in the case of La^{3+}. Thus, it is likely that vasopressin and PDGF induce Ca^{2+} entry *via* a dihydropyridine-insensitive pathway as well as a dihydropyridine-sensitive pathway as previously described (Ruegg *et al*., 1989; Thibonnier *et al*., 1991; Byron & Taylor, 1995; Nakajima *et al*., 1996), and thiazolidinediones may inhibit it. The dihydropyridine-insensitive Ca^{2+} entry pathways have been thought to be mediated through receptor-mediated Ca^{2+} channels such as capacitative Ca^{2+} entry (CRAC) and second messenger-operated channels (Pacaud *et al*., 1993; Putney & Bird, 1993; Fasolato *et al*., 1994; Byron & Taylor, 1995). The receptor-activated Ca^{2+} channel is also mediated partly by Ca^{2+}-permeable nonselective cation channels (I_{cat}) (Byrne & Large, 1988; Van Renterghem *et al*., 1988; Amedee *et al*., 1990; Wang & Large, 1991; Krautwurst *et al*., 1994; Nakajima *et al*., 1996; Minowa *et al*., 1997). As shown in Figure 7, vasopressin activated I_{cat}, which was completely blocked by La^{3+} or Cd^{2+}. Troglitazone and pioglitazone also inhibited I_{cat} in a concentration-dependent manner, but nifedipine and nicardipine failed to inhibit it. The half maximal inhibitory concentration of troglitazone was 5.7 μM, which was quite similar to that of troglitazone on $I_{Ca.L}$. These results suggest that troglitazone effectively inhibits vasopressin-activated I_{cat} as well as $I_{Ca.L}$ in vascular smooth muscle cells. These findings are somewhat compatible with the findings that thiazolidinediones inhibit vasopressin-induced sustained rise of $[Ca^{2+}]_i$. However, the effects of thiazolidinediones on the other Ca^{2+} entry pathways such as CRAC cannot be ruled out in the present study, and further studies are needed.

Thiazolidinediones such as troglitazone and pioglitazone have been reported to inhibit mitogenesis elicited by growth factors (epidermal growth factor, insulin and basic fibroblast growth factor) (Dubey *et al*., 1993; Law *et al*., 1996) or some pathophysiological conditions (Yasunari *et al*., 1997; Shinohara *et al*., 1998) in vascular smooth muscle cells. The present study also provides evidence that troglitazone and pioglitazone inhibit the incorporation of [^{3}H]-thymidine elicited by vasopressin and PDGF. Several mechanisms underlying the inhibitory effects of thiazolidinediones on vasoactive agent-induced mitogenesis may be proposed. Since troglitazone is a potent activator of PPAR-γ at the nuclear level (Lehmann *et al*., 1995), thiazolidinediones may inhibit the mitogenesis by activating PPAR-γ. Actually, prostaglandin J_2, a potent activator of PPAR-γ (Kliewer *et al*., 1995), has been shown to inhibit serum-stimulated cell proliferation in vascular smooth muscle cells (Sasaguri *et al*., 1992). The present study also showed that prostaglandin J_2 inhibited PDGF-induced incorporation of [^{3}H]-thymidine. However, the inhibitory effects of prostaglandin J_2 on cell proliferation were much less than that of thiazolidinediones (troglitazone and pioglitazone). Thus, the other mechanisms unrelated to PPAR-γ might be mainly involved in the inhibitory effects of thiazolidinediones on agonists-induced cell proliferation. In addition, prostaglandin J_2 did not affect the sustained rise in $[Ca^{2+}]_i$ induced by vasopressin (data not shown) and failed to inhibit $I_{Ca.L}$ and I_{cat} as shown in the present study. Also, the inhibitory effects of thiazolidinediones on I_{cat} and $I_{Ca.L}$ were observed immediately after application of thiazolidinediones. Thus, it is unlikely that thiazolidinediones inhibit Ca^{2+} mobilization and cell prolif-

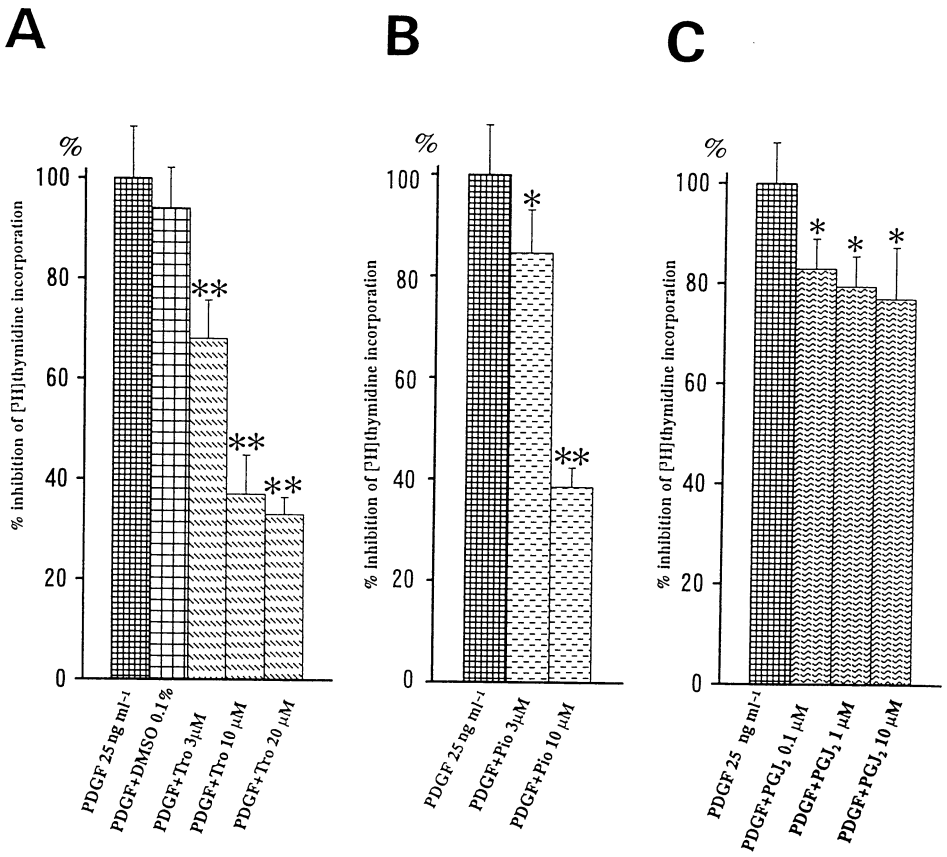

Figure 10 Comparative effects of troglitazone, pioglitazone and prostaglandin J_2 on PDGF-induced [^{3}H]-thymidine incorporation. Effects of troglitazone (3–20 μM), pioglitazone (3–10 μM) and prostaglandin J_2 (0.1–10 μM) on PDGF (25 ng ml^{-1})-induced [^{3}H]-thymidine incorporation. Each data was obtained from four different experiments, and the mean ± s.d. value is shown. *$P<0.05$ vs controls **$P<0.01$ vs controls.

eration *via* the intranuclear receptor (PPAR-γ). Alternatively, elevation of $[Ca^{2+}]_i$ may be an important intracellular signal stimulating mitogenesis (Mogami & Kojima, 1993; Berridge, 1995). The elevation of $[Ca^{2+}]_i$ elicited by agonists (vasopressin and PDGF) could occur through Ca^{2+} release from storage sites, and Ca^{2+} influx from the extracellular medium. The removal of extracellular Ca^{2+} markedly reduced the incorporation of [^{3}H]-thymidine, suggesting that extracellular Ca^{2+} plays a role in mediating mitogenic effects of these agents. Nifedipine or nicardipine, a voltage-dependent L-type Ca^{2+} channel blocker, partly decreased the incorporation of [^{3}H]-thymidine as previously reported (Block *et al*., 1989; Sperti & Colucci, 1991; Yang *et al*., 1993), but La^{3+} markedly depressed it. These results suggest that Ca^{2+} entry pathways other than $I_{Ca.L}$ may play essential roles in the mitogenic actions of vasopressin and PDGF. These results are somewhat compatible with the present study showing that nifedipine and nicardipine (1 μM) completely inhibited $I_{Ca.L}$, but only partly decreased the sustained rise in $[Ca^{2+}]_i$ elicited by vasopressin and PDGF. On the other hand, La^{3+} and thiazolidinediones (troglitazone and pioglitazone) markedly reduced $[Ca^{2+}]_i$ rise induced by these agents as well as $I_{Ca.L}$ and I_{cat}, and blocked the mitogenic effects of vasopressin and PDGF. Thus, it is suggested that thiazolidinediones inhibit Ca^{2+} entry and thereby cell proliferation induced by vasopressin and PDGF in vascular smooth muscle cells, in which the inhibitory effects of thiazolidinediones on I_{cat} as well as $I_{Ca.L}$ might be involved. In fact, it has been reported that blockers of PDGF-activated I_{cat} inhibit cell proliferation in mouse fibroblast (Jung *et al*., 1992). However, pioglitazone seemed to be as potent as troglitazone in reducing thymidine incorporation for several hours as shown in Figures 9 and 10, while it was less potent than troglitazone in blocking the agonist-induced sustained rise of $[Ca^{2+}]_i$ as well as $I_{Ca.L}$ and I_{cat}. Therefore, although chronic effects of thiazolidinediones on the Ca^{2+} mobilization and Ca^{2+} channels remain undetermined, the involvement of the mechanisms unrelated to $[Ca^{2+}]_i$, such as protein kinase C (Yasunari *et al*., 1997), tyrosine kinase and MAP kinase (Geissler *et al*., 1990; Law *et al*., 1996) could not be excluded in the present study, and further studies are needed to discriminate this possibility.

Insulin resistance is commonly observed in essential hypertension (Ferrannini *et al*., 1987; Sowers *et al*., 1994) and in type II diabetes mellitus (Warram *et al*., 1990; Lillioja *et al*., 1993), where enhanced vascular responsiveness to vasoconstrictor agents and blunted vasodilation are involved. Actually, insulin by itself has been reported to modulate $I_{Ca.L}$ and to have an inhibitory effect on vasopressin-induced I_{cat} in A7r5 cells (Standley *et al*., 1991; Ram *et al*., 1993; Kahn *et al*., 1993). Therefore, resistance to insulin action may be partly involved in hypertension. However, the present study also provides novel evidence that thiazolidinediones, insulin sensitizing agents, inhibit I_{cat} elicited by vasopressin as well as $I_{Ca.L}$. The inhibitory effects of thiazolidinediones on these channels may also contribute to reduce vascular contractility and lower blood pressure. The thiazolidinediones inhibit cell proliferation and migration induced by PDGF, insulin, basic fibroblast growth factor (Dubey *et al*., 1993; Law *et al*., 1996), and

vasopressin (in the present study), which stimulates cell hypertrophy of vascular smooth muscle cells (Geisterfer & Owens, 1989). In addition, we showed that thiazolidinediones had potent inhibitory effects on cell proliferation induced by vasoactive agents, as compared with L-type Ca^{2+} channel blockers (nifedipine and nicardipine) (Figure 9). Thus, thiazolidinediones may be promising agents to prevent the development of atherosclerosis and restenosis after percutaneous transluminal coronary angioplasty (PTCA).

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