Effects of Pioglitazone on Calcium Channels in Vascular Smooth Muscle

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Abstract Pioglitazone, an insulin-sensitizing, antidiabetic agent, has blood pressure-lowering effects in insulin-resistant hypertensive rats and attenuates growth factor–induced increases of intracellular Ca\(^{2+}\) in rat aortic vascular smooth muscle cells. To determine whether modulation of voltage-dependent Ca\(^{2+}\) channels plays a role in this association, we investigated the effects of pioglitazone on voltage-dependent current in cultured rat aortic (a7r5) and freshly dissociated rat tail artery vascular smooth muscle cells. Both cell types were studied with whole-cell patch-clamp techniques. Current through L-type Ca\(^{2+}\) channels was elicited with a voltage ramp in the presence of Ba\(^{2+}\) substituted for Ca\(^{2+}\). T-type Ca\(^{2+}\) current was studied using a two-pulse protocol that enabled the isolation of transient current. In a7r5 vascular smooth muscle cells, 2-minute application of pioglitazone (5 and 10 \(\mu\)mol/L) reduced L-type current by 7.9±1.0% (n=8) (mean±SEM, number of cells) and 14.5±3.0% (n=9) (P<0.01, two-tailed paired \(t\) test), respectively. In contrast, 2-minute application of pioglitazone had no significant effect on T-type Ca\(^{2+}\) current. In freshly dissociated tail artery vascular smooth muscle cells, 2-minute application of 10 \(\mu\)mol/L pioglitazone had an insignificant effect (4.8±5.6% reduction); however, 25 \(\mu\)mol/L pioglitazone reduced L-type current by 27.3±7.2% (n=5) (P<0.01). Two-minute application of 0.1% or 0.2% dimethyl sulfoxide (vehicle) alone had no significant effects on currents in either type of vascular smooth muscle cell. The blood pressure–lowering and growth-inhibiting effects of pioglitazone may be in part due to inhibition of inward Ca\(^{2+}\) current through L-type channels in vascular smooth muscle. (Hypertension. 1994;24:170-175.)

Key Words • insulin • calcium channels • muscle, smooth, vascular • thiazoles

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here is increasing evidence that insulin resistance exists in various genetic models of hypertension, such as Dahl salt-sensitive, Zucker obese, and spontaneously hypertensive rats.\(^1\)\(^-\)\(^5\) Several mechanisms have been proposed to explain this association, including resistance to a direct vasodilator effect of insulin.\(^5\) Recent work suggests that insulin may affect vascular tone through direct effects on the regulation of vascular smooth muscle cell (VSMC) intracellular calcium (\([Ca^{2+}]_i\)).\(^6\)\(^-\)\(^7\) Insulin attenuates vasopressin-\(^6\) and angiotensin-stimulated\(^6\) VSMC \([Ca^{2+}]_i\), transients, reduces transmembrane inward Ca\(^{2+}\) current,\(^6\) and shifts the voltage dependence of Ca\(^{2+}\) current to the right.\(^6\) Since \([Ca^{2+}]_i\) plays a major role in regulating vascular tone, the vasodepressor effects of insulin may be mediated through its effects on VSMC \([Ca^{2+}]_i\).

Recently, two classes of antidiabetic, insulin-sensitizing agents (biguanides and thiazolidinediones) were reported to lower blood pressure in genetic models of hypertension.\(^8\)\(^-\)\(^10\) Unlike sulfonylureas, which inhibit K\(^+\) channels, stimulate insulin secretion, and increase blood pressure, the thiazolidinediones are a novel group of compounds that increase the sensitivity of target tissues to insulin-stimulated glucose uptake without stimulating endogenous insulin secretion.\(^11\)\(^-\)\(^13\) However, the mechanisms by which these compounds lower blood pressure are currently unknown.

Recent observations\(^6\)\(^-\)\(^9\)\(^,\)\(^13\) suggested to us a possible mechanism by which these agents might affect vascular tone. First, these compounds appear to increase insulin sensitivity by exerting postbinding effects on facilitated glucose uptake.\(^13\) Second, glucose uptake can be modulated by insulin in VSMCs,\(^14\) and Draznin et al\(^13\) have demonstrated that the ability of insulin to stimulate glucose uptake is significantly determined by \([Ca^{2+}]_i\), in responsive tissue. Third, pioglitazone attenuates sustained increases in \([Ca^{2+}]_i\), induced by platelet-derived growth factor in rat aortic VSMCs.\(^8\) Finally, a major effect of insulin on \([Ca^{2+}]_i\), appears to be exerted by attenuation of Ca\(^{2+}\) movement through voltage-dependent Ca\(^{2+}\) channels. Therefore, we conducted the present study to test the hypothesis that pioglitazone, a thiazolidinedione, exerts an effect on VSMC \([Ca^{2+}]_i\) by modifying current through voltage-dependent Ca\(^{2+}\) channels.

These studies used a VSMC cell line (a7r5) and freshly dispersed rat tail artery VSMCs. A7r5 cells have previously been shown to respond to insulin, vasopressin, and other agents.\(^6\)\(^,\)\(^16\) Furthermore, ionic currents elicited by vasopressin and depolarization are readily recorded in a7r5 cells.\(^5\)\(^,\)\(^16\) Rat tail artery has also been used in previous vascular smooth muscle studies, and contractile responses of tail artery VSMCs in primary culture are reduced by insulin.\(^17\)

Methods

Cell Culture and Preparation

A7r5 VSMCs (derived from embryonic DB1X rat thoracic aorta) were obtained from American Type Culture Collection.
Maintenance of the cell line has been previously described. Cells were maintained in growth medium (Dulbecco's modified Eagle medium supplemented with 9% fetal bovine serum, 0.2% Tyrode, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) and placed in a 37°C water-jacketed incubator under 5% CO₂ and 100% humidity. Medium was replaced every 2 days. Confluent cells were released by treating cells with an enzyme solution containing 0.2% trypsin and 1 mmol/L EGTA in Ca²⁺/Mg²⁺-free Hanks' balanced salt solution (Sigma Chemical Co) for 4 minutes. Released cells were centrifuged for 5 minutes at 1000 rpm and then transferred in growth medium to other plates in a ratio of 1:4.

Freshly dissociated VSMCs were obtained from the tail artery of male Sprague-Dawley rats (250 to 300 g). Tail artery was removed with aseptic techniques and transferred to physiological saline solution (PSS) bicarbonate buffer containing (mmol/L) NaCl 140, KCl 5.4, NaHCO₃ 1.18, dextrose 5.5, MgSO₄ 1.17, CaCl₂ 1.6, and EDTA 0.03, with pH 7.4. Blood clots, fat, and connective tissue were removed. After several rinses with PSS, the tail artery was cut longitudinally, and endothelium was removed with a sterile swab. The artery was cut many times (0.5-mm interval) at the edges and then incubated in digestion solution for 45 to 60 minutes at 37°C in a water-bath incubator. Digestion solution was freshly made Hanks' solution (10 mL) supplemented with 5 mg collagenase, 3.5 mg papain, and 6 mg dithiothreitol. Freshly made Hanks' solution contained (mmol/L) NaCl 140, KCl 5.4, NaHCO₃ 4.17, KH₂PO₄ 0.44, NaH₂PO₄ 0.42, dextrose 5.5, CaCl₂ 0.1, and HEPES 5.0, with pH 7.4. After the digestion, tissue was transferred to a small Petri dish containing Hanks' solution and gently rinsed several times. Cells were then released by triturating the tissue gently with a glass transfer pipette. Cells were refrigerated in Hanks' solution at 4°C for several hours to 1 day before patch clamping.

Whole-Cell Patch Clamp

Confluent a7r5 cells were replated onto 35 x 10-mm Petri dishes and incubated for 2 hours under 5% CO₂ and 100% humidity at 37°C in growth medium. Before recording, a plastic ring was inserted into the dish, forming a sub chamber approximately 0.4 mL in volume. Cells were superfused with extracellular medium at room temperature (approximately 23°C) at 3 mL/min. Whole-cell voltage-clamp experiments were performed with an Axopatch 200 patch-clamp amplifier (Axon Instruments Inc) controlled by a Labmaster TL-1 DMA A/D digital-to-analog interface using the pClamp (Axon Instruments) suite of programs.

For study of the effect of pioglitazone on L-type Ca²⁺ channels, the extracellular medium contained (mmol/L) BaCl₂ 20, NaCl 113, KCl 4, MgCl₂ 2, and HEPES 10, pH 7.4, and the electrode solution contained (mmol/L) CsCl 120, Cs₂EGTA 10, MgCl₂ 1.4, Mg-ATP 3.6, Na-GTP 1, and HEPES 10, pH 7.2. The current was elicited by a ramp protocol with a holding potential of −80 mV and a ramp going from −100 to 50 mV in 300 milliseconds with a digital resolution of 1 mV. Linear leakage and holding current were removed from the entire current response by subtracting the linear current trend and average holding current between −80 and −60 mV. The response to the voltage ramp protocol from −80 to 50 mV was analyzed.

For study of the effect of pioglitazone on T-type Ca²⁺ channels, the extracellular medium was the same except that Ca²⁺ was substituted for Ba²⁺; the electrode solution remained unchanged. T-type Ca²⁺ current was analyzed using a two-pulse voltage protocol. The initial holding potential was −80 mV. The first pulse depolarized the cell from −80 to −15 mV for 75 milliseconds and then repolarized the cell to −30 mV. After 150 milliseconds at −30 mV, the cell was depolarized again to −15 mV for 75 milliseconds and then repolarized to −80 mV. Linear leakage was removed automatically by the pClamp clampex program during data acquisition. The current elicited by the second pulse was subtracted from the first pulse, and the difference between the two pulses is T-type current.

Pioglitazone was dissolved in dimethyl sulfoxide (DMSO) and later diluted for use in extracellular medium. Control medium was the extracellular medium containing the same final concentration of DMSO as the pioglitazone solutions. In experiments with a7r5 cells, pioglitazone concentrations were 5 and 10 μmol/L with corresponding DMSO concentrations of 0.1% and 0.2% in volume, respectively. In experiments with tail artery VSMCs, pioglitazone concentrations were 10 and 25 μmol/L with corresponding DMSO concentrations of 0.04% and 0.1%, respectively. Protocols for either T- or L-type channels were run throughout the experiments at 20-second intervals. Currents were elicited under control conditions for 2 minutes before application of pioglitazone. Pioglitazone was perfused constantly for 2 minutes and then washed out with control medium.

Statistical comparisons used paired and unpaired t tests, with the criterion for significance adjusted by the Bonferroni correction for number of preplanned comparisons. In all cases, a value of P<.01 was therefore more than sufficient to achieve statistical significance. In the preplanned comparison of the effect of two pioglitazone concentrations (as in Figs 3 and 6), the criterion for significance was P<.05.

Results

Two types of inward currents were observed in a7r5 cells when cells were depolarized from −80 to 50 mV with the ramp protocol (Fig 1A). As described in our previous work, the first component of the current corresponds to T-type current, and the second component corresponds to L-type current. Previous studies have shown that current through L-type Ca²⁺ channels is enhanced by Ba²⁺, whereas current through T-type Ca²⁺ channels is reduced (Fig 1A). Although the T- and L-type currents overlap each other, T-type current does not affect the peak L-type current. In preliminary experiments to determine the optimal pulse potential to elicit peak T-type current with minimal L-type contamination, currents were elicited by pulses from −80 mV to various potentials with a 10-mV step (Fig 1B). Peak transient current was elicited by depolarization to −11.25±2.2 mV (n=24 cells). At −20 mV, the current averaged 88.1±3.5% (n=24) of the peak current. Therefore −15 mV was chosen as the voltage at which to analyze effects on peak T-type current. In response to the pulse protocol used to study T-type current, a transient current was observed when the cell was depolarized from −80 to −15 mV by the first pulse, and it inactivated within 75 milliseconds (Fig 1C). After an additional 150 milliseconds at −30 mV, in most cases very little current was elicited by a second pulse to −15 mV. Regardless, any non-inactivating current present in the response was removed by the subtraction protocol, leaving isolated T-type current. These currents were mediated by Ca²⁺ channels: K⁺ current was blocked by tetraethylammonium in the medium and Cs⁺ in the electrode, and both T- and L-type currents were abolished in extracellular solution containing neither Ba²⁺ nor Ca²⁺ (Fig 1). Similarly, both T- and L-type currents were observed in freshly dissociated rat tail artery VSMCs (see below) and were also abolished in 0 mmol/L Ca²⁺/Ba²⁺ solution. T-type current was not analyzed in tail artery cells because T-type current was usually very small in these cells.
Pioglitazone reduced L-type current in both a7r5 and rat tail artery VSMCs. Fig 2 shows the time course of pioglitazone and DMSO effects in a typical a7r5 cell.

Fig 3 summarizes the results of several experiments. Two-minute application of 5 μmol/L pioglitazone significantly reduced peak L-type current by 7.9±1.0% (n=8) (mean±SEM, number of cells) below the immediately preceding current measured in 0.1% DMSO (P<.01, two-tailedpaired t test). During 2-minute application of 0.1% DMSO alone, no significant change (−0.6±1.0%, n=7) in L-type current occurred (P>.75). Correspondingly, 10 μmol/L pioglitazone significantly reduced peak L-type current by 14.5±3.0% (n=9) (P<.01), whereas 0.2% DMSO alone had no significant effect (2.4±1.6%, n=7, P>.18). The effects of 5 and 10 μmol/L pioglitazone, compared with the effects of 0.1% and 0.2% DMSO, respectively, were significant (P<.01, two-tailed unpaired t test). Pioglitazone at 10 μmol/L had a greater inhibitory effect on L-type current than at 5 μmol/L (P<.01, two-tailed unpaired t test).
In contrast, pioglitazone had no significant effect on T-type current in a7r5 cells. Fig 4 shows the results of a typical experiment. Applications of 10 μmol/L pioglitazone and 0.1% DMSO did not significantly reduce peak T-type current (5.6±2.5%, n=9 and 3.6±2.0%, n=9, respectively, P>.1, paired t test).

Freshly dispersed tail artery VSMCs were spindle shaped and approximately 5 μm in diameter by 50 μm long. This was much smaller than a7r5 cells, which were approximately 40×150 μm. Correspondingly, the peak L-type Ca$^{2+}$ current in tail artery VSMCs was only 39.3±6.5 pA (n=7), which was much smaller than the peak L-type Ca$^{2+}$ current in a7r5 cells, which averaged 1265±89 pA (n=86) (data from previous experiments). Tail artery VSMCs contracted in response to depolarization and application of 10^{-7} mol/L vasopressin. Similar results were obtained from tail artery VSMCs with the same protocol as in experiments on L-type current in a7r5 cells. Fig 5 shows the time course of pioglitazone effects and representative L-type currents in a typical tail artery VSMC. Fig 6 summarizes the results of several experiments. Application of 25 μmol/L pioglitazone for 2 minutes significantly reduced peak L-type current by 27.3±7.2% (n=5) below the immediately preceding current measured in 0.1% DMSO (P<.01, two-tailed paired t test). However, 10 μmol/L pioglitazone did not significantly reduce peak L-type current (4.8±5.6%, n=4). Application of 0.04% and 0.1% DMSO alone had no significant effect on L-type current (4.4±4.9% reduction, n=4 and 4.7±5.1% reduction, n=5, respectively). The effect of 25 μmol/L pioglitazone, compared with the effects of 10 μmol/L pioglitazone and 0.1% DMSO, was significant (P<.05, two-tailed unpaired t test).

Discussion

These results show that the insulin-sensitizing compound pioglitazone significantly attenuates inward current through voltage-dependent Ca$^{2+}$ channels in rat VSMCs in both an embryonic cell line and freshly dissociated tail artery cells. Furthermore, our data indicate that the effects of pioglitazone appear to be explained by action on L-type Ca$^{2+}$ channels. The ramp protocol we have used is appropriate for identifying the affected current as an L-type current, because the current activates in the appropriate voltage range, is selectively inhibited by isradipine, and activates fully but does not inactivate within the duration of the ramp (unpublished data, 1993). However, ramp protocols to study the L-type current preclude any speculation as to
**Mechanism, e.g., a voltage-dependent channel block that causes inactivation during the ramp versus a simple block of the channel. Tail current analysis would be necessary to quantify accurately the voltage dependence of the affected current.**

Because of the important role of \([Ca^{2+}]\) in the maintenance of vascular tone, inhibition of \(Ca^{2+}\) influx through voltage-dependent channels by pioglitazone is likely to be one mechanism by which this compound exerts its blood pressure-lowering effects in genetic models of hypertension.8,9 In long-term in vitro experiments, we9 have shown that pioglitazone (10 \(\mu M\)/L) reduced \(K^+\)-elicted contractions, possibly mediated by inhibition of \(Ca^{2+}\) channels such as demonstrated here. Recent observations in cultured VSMCs that pioglitazone inhibits VSMC proliferation,20 DNA synthesis,20 and platelet-derived growth factor-induced increase in \([Ca^{2+}]\) may be attributed in part to its ability to inhibit \(Ca^{2+}\) influx. Since increased peripheral vascular resistance associated with hypertension appears to be partially caused by accelerated growth (hyperplastic or hypertrophic) of VSMCs in small arteries and arterioles,21-23 antihypertensive effects of pioglitazone may also be related to these indirect effects of reduced VSMC \(Ca^{2+}\) influx.

Evidence is accumulating that vascular smooth muscle is an insulin-sensitive tissue.1,6,14,24-26 Insulin has been shown to attenuate the contractility of intact vessels24,25 and isolated VSMCs.6,26 Insulin attenuates agonist-mediated increases in VSMC \([Ca^{2+}]\) and causes a rightward shift of the voltage dependence of a voltage-dependent \(Ca^{2+}\) current in VSMCs.6 Insulin stimulates the activity of both cell membrane \(Ca^{2+}\)-ATPase and \(Na^+\),K\(^+\)-ATPase127-28 and increases the level of VSMC \(Na^+\),K\(^+\)-ATPase mRNA.27 Recent observations also suggest that insulin increased glucose transport in VSMCs.14 Thus, our observations that pioglitazone decreases VSMC voltage-mediated \(Ca^{2+}\) influx and prior observations that pioglitazone increases glucose transport in other tissues13 suggest that it has some of the same fundamental actions as insulin.

Sustained elevated levels of \([Ca^{2+}]\) in insulin-sensitive cells may render these cells relatively resistant to insulin-mediated glucose transport.29-34 Furthermore, insulin-resistant states are associated with elevations of \([Ca^{2+}]\) in several insulin-sensitive tissues, including VSMCs.1,32,33 Indeed, treatment of insulin-resistant, hypertensive individuals with a calcium channel blocker for 1 month34 resulted in improved insulin sensitivity, as
measured by insulin-stimulated glucose uptake in adipocytes. These observations suggest that inhibition of Ca²⁺ entry and lowering of [Ca²⁺], in states of [Ca²⁺], overload, i.e., insulin-resistant conditions, result in improved insulin sensitivity and decreased vascular contractility. Although we cannot relate in vivo concentrations of pioglitazone directly to our in vitro experiments, it is possible that this drug may have similar effects in vivo. The observations that a drug that both reduces blood pressure and increases insulin sensitivity has significant inhibitory effects on VSMC Ca²⁺ channel function suggest that altered VSMC [Ca²⁺], metabolism may be a fundamental abnormality linked to hypertension and insulin resistance.

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