Attenuation by 4-Aminopyridine of Delayed Vasorelaxation by Troglitazone

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Troglitazone and other thiazolidinediones (TZDs) are thought to relax arterial smooth muscle by directly inhibiting calcium channels in smooth muscle cell membranes. However, until recently such inhibition was only examined acutely, ie, within only seconds or minutes after administration of these agents to arterial smooth muscle preparations. Recently, a novel experiment was reported in which troglitazone caused a 2-phase relaxation of perfused resistance arteries, namely, an acute relaxation (within the first 20 minutes of treatment), which was blocked by a nonselective calcium channel blocker and a delayed relaxation (after 2 hours), which was not. We sought to determine if any of the 4 major potassium (K) channels in vascular smooth muscle play a role in the delayed relaxation. We incubated vascular contractile rings prepared from ventral tail arteries of rats with physiological buffer containing either 0 or 4 μmol/L troglitazone for 3 hours (4 μmol/L is typical of plasma levels from diabetic patients). Different K channel inhibitors (1 mmol/L 4-aminopyridine [4AP]; 1 mmol/L tetraethylammonium [TEA]; 5 μmol/L glyburide; 20 μmol/L barium) were coadministered with each level of troglitazone in additional preparations. Then these arterial rings were contracted with either norepinephrine (NE), arginine vasopressin (AVP), or high-K buffer. All contractions were significantly relaxed by troglitazone ($P < .05$). Only 4AP significantly attenuated troglitazone's relaxation of NE and AVP contractions ($P < .05$), though not high-K–induced contractions. TEA, glyburide, and barium had no such influence. Thus, for both adrenergic (NE) and nonadrenergic (AVP) contractions, the delayed arterial vasorelaxation by troglitazone may be mediated at least in part by activation of 4AP-sensitive K channels. Furthermore, the specific subtype of the channels involved is most likely those bound in the outer cell membrane where their effectiveness in terms of mediating relaxation would depend on an intact transmembrane K ion gradient.

THIAZOLIDINEDIONES (TZDs) such as ciglitazone, troglitazone, pioglitazone, and rosiglitazone have been recognized for a number of years for their novel antihyperglycemic mechanisms.[1] Accordingly, some are now approved for use in the treatment of type 2 diabetic patients. However, these agents are also known to relax arterial vascular smooth muscle independent of their systemic glycemic effects.[2-4] This relaxation appears not to involve the endothelium.[2,5,6] It is hoped that such direct vascular activity might help offset development of systemic hypertension and possibly nephropathies in diabetic patients.[7,8] Yet, mechanisms underlying this activity are not yet fully understood. Various studies using patch clamp techniques have clearly shown evidence that TZDs can decrease availability of calcium to the contractile apparatus by directly inhibiting calcium influx channels in the smooth muscle cell membrane.[2,9-12] Unfortunately, all these studies were limited to acute observations, ie, measures made within seconds or minutes after the administration of these agents to the corresponding cell membrane preparations. Recently, another group of investigators reported that troglitazone at the concentration of 10 μmol/L caused an endothelium-independent relaxation of norepinephrine (NE)-contracted arterial vessels biphasically, ie, first acutely (within a few minutes) and then again after a delay of 2 hours.[5] They also found that, while the early (acute) relaxant effect of such troglitazone could be blocked entirely by the calcium channel blocker SKF9365, the later (delayed) relaxant effect could not.[5] This, together with an earlier report of more effective inhibition by troglitazone of receptor-operated versus voltage-operated calcium channels,[13] led them to conclude that inhibition of receptor-operated calcium channels mediates troglitazone's early-phase but not its late-phase relaxant action.[5] They further concluded that while a direct inhibitory action of troglitazone on voltage-operated calcium channels may possibly still be involved in the late phase, other yet unidentified mechanisms may also play a role.[5]

In the present study, we chose to determine whether potassium (K) channels play a role in the late-phase relaxation by troglitazone. Increased activity (opening) of such channels in the cell membrane (resulting in increased K efflux) could potentially relax NE and other agonist-induced contractions indirectly, by limiting and/or reversing agonist induced membrane depolarizations with subsequent inhibition (closing) of voltage-operated calcium channels.[14,15] We reasoned that if troglitazone relaxes arterial smooth muscle in this manner then one or more known inhibitors of K channels should block (or at least attenuate) that relaxation if coadministered with troglitazone. We chose to conduct such coadministrations with intact vascular ring preparations isolated from ventral tail arteries of rats. Concentrations of troglitazone and other TZDs have been identified which have no detectable immediate relaxant effects (as seen at >10 μmol/L troglitazone),[2] but yet very significant delayed relaxant effects in contractile rings from this particular vessel.[2,4,6] In addition, these concentrations (eg, 3 μmol/L troglitazone)[6] are more therapeutically relevant than higher levels (≥10 μmol/L) when compared to plasma concentrations reported from TZD-treated diabetic patients.[16]

MATERIALS AND METHODS

Tissue Preparation

Adult male Sprague Dawley rats were anesthetized with ketamine/xylazine (80/8 mg/kg, intraperitoneally) to permit isolation of ventral

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tail arteries. Each artery was carefully sectioned into multiple 3-mm rings with a series of evenly spaced scalpel blades tightly bound together. Each ring was mounted between 2 tungsten wire stirrups[17] and then suspended from an isometric force (tension) transducer into a normal physiological buffer (with K = 5.9 mmol/L) warmed to 37°C and gassed to pH 7.4 with 95%/5% O_2/CO_2.[18] Each ring was equilibrated at a passive (resting) tension of 1 g before experimentation.[4] This level of resting tension was not influenced by any of the experimental agents described below.

Preliminary Tests

Preliminary tests were performed to determine concentration and time dependencies for troglitazone's relaxant effects on contractions produced by NE and arginine vasopressin (AVP) in normal buffer. First, various concentrations of troglitazone (2, 4, 8, 16, and 32 μmol/L) were tested for immediate relaxant effects in tail arterial rings precontracted with either NE or AVP. Only the 16 and 32 μmol/L levels were at all able to relax such contractions immediately (ie, within 5 to 10 minutes after administration) and then only consistently at the 32 μmol/L level. Subsequently, the lower 3 levels (2, 4, and 8 μmol/L) were examined for delayed relaxant effects. Similar numbers of tail arterial rings were pretreated with each of these levels for 3 hours in parallel with a fourth group of rings pretreated with only troglitazone vehicle (dimethyl sulfoxide [DMSO]; 0.05%). Then each ring was contracted with either NE or AVP. Both maximal and submaximal NE and AVP contractions were notably relaxed by each of these 3-hour pretreatments with troglitazone. For example, maximal NE contractions were relaxed 22% by the 2 μmol/L, 45% by the 4 μmol/L, and 67% by the 8 μmol/L levels of troglitazone. Results were nearly identical for contractions produced by AVP. Finally, the time dependence of troglitazone's delayed relaxant action was examined. Different arterial rings were pretreated with 4 μmol/L troglitazone for 0.5, 1, 2, and 4 hours, and then contracted with either NE or AVP. The onset of relaxation by this level of troglitazone was not apparent until 1 hour. Relaxations at 2 and 4 hours were greater than that seen at 1 hour but not different from each other.

Study I: Effects of K Channel Inhibitors on Troglitazone's Delayed Relaxation of NE and AVP Contractions

Equal numbers of arterial rings, prepared as described above, were incubated for 3 hours with normal physiological buffer (K = 5.9 mmol/L) containing either 0 or 4 μmol/L troglitazone (n = 16 rings each). Different K channel inhibitors were coadministered with each level of troglitazone in similar numbers of additional ring preparations. Then all rings were contracted with cumulatively administered, graded levels of either NE (10^{-10} to 10^{-4} μmol/L) or AVP (10^{-11} to 10^{-7} μmol/L) and their contractile responses examined for statistically meaningful interactions between effects of troglitazone and each of the different K channel inhibitors.

As previously reviewed, there are 4 major types of K channels present in the arterial smooth muscle cell membrane capable of modulating contractility through effects on the membrane's potential[14,15]: calcium-activated K channels (K_{CA}), inward rectifier K channels (K_{IR}), ATP-sensitive K channels (K_{ATP}), and voltage-dependent K channels (K_V). We used tetraethylammonium chloride (TEA) at 1 mmol/L as recommended to specifically inhibit K_{Ca} channels.[14,15] Accordingly, we also used barium at 20 μmol/L, which largely inhibits only K_{IR} channels, glyburide at 5 μmol/L, which specifically inhibits only K_{ATP}, channels and 4-aminopyridine (4AP) at 1 mmol/L, which inhibits K_V and, to a lesser degree, K_{ATP} channels.[14,15] 4AP can also inhibit K channels in the intracellular membrane associated with the sarcoplasmic reticulum (SR), where by doing so it inhibits sequestration of Ca into the SR.[19] None of the above concentrations of these K channel inhibitors are generally considered high enough to increase contractility of in vitro preparations of arterial smooth muscle.

Study II: Effect of 4AP on Troglitazone's Delayed Relaxation of High-K–Induced Contraction

Equal numbers of arterial rings, prepared as described above, were incubated for 3 hours with normal physiological buffer (K = 5.9 mmol/L) containing either 0 or 4 μmol/L troglitazone (n = 12 rings each). 4AP, 1 mmol/L, was coadministered with each level of troglitazone to additional ring preparations (n = 12 rings each). Then all rings were contracted by immersion in a high-K buffer (K = 90 mmol/L) and their contractile responses examined for statistically meaningful interactions between effects of troglitazone and 4AP.

Additional Tests

In half of the arterial rings described above in studies I and II the endothelium was removed (prior to experimentation) to determine if its absence influenced the results. In study I, some of the rings contracted with AVP were treated simultaneously with 1 μmol/L phentolamine to block any possible influence of endogenous NE released spontaneously from adrenergic nerve endings. In study II, all the rings contracted with high-K buffer were given 1 μmol/L phentolamine 10 minutes after the high K to block the known contribution of K-evoked release of NE on that contraction.[20]

Statistical Analyses

All data collected in the above-mentioned studies were subjected to analysis of variance (ANOVA), followed by mean comparisons with the Bonferroni method when appropriate. Probability of error values (*P*) less than .05 were considered significant. Results were typically expressed in the form of mean ± SEM.

RESULTS

Study I: Effects of K Channel Inhibitors on Troglitazone's Delayed Relaxation of NE and AVP Contractions

Pretreatment of rat tail arterial rings for 3 hours with troglitazone (4 μmol/L), in the absence of any K channel inhibitors, substantially reduced (relaxed) contractions produced by either NE or AVP (Fig 1). This relaxant effect was considerably attenuated in the presence of 1 mmol/L 4AP (coadministered with troglitazone), as apparent from comparison of the results in Fig 2 with those in Fig 1. Other K channel inhibitors (glyburide, barium, and TEA) failed to significantly influence troglitazone's relaxant actions (Figs 3, 4, and 5, respectively). Although TEA appeared to slightly attenuate troglitazone's ability to relax maximal NE and AVP contractions, this effect failed to achieve statistical significance.

Neither the relaxant effect of troglitazone nor its attenuation by 4AP were influenced by removal of the endothelium. In additional experiments, phentolamine (added only during contractions produced by AVP) failed to influence the effects of either troglitazone or 4AP (data not shown).

Study II: Effect of 4AP on Troglitazone's Delayed Relaxation of High-K–Induced Contraction

In the absence of 4AP, 3-hour pretreatment of arterial rings with 4 versus 0 μmol/L troglitazone reduced contractions induced by high extracellular K (90 mmol/L) by 67% (from 2.7 ± 0.2 to 0.9 ± 0.1 g of contractile tension; $P < .05$). In the presence of 1 mmol/L 4AP (coadministered with troglitazone),

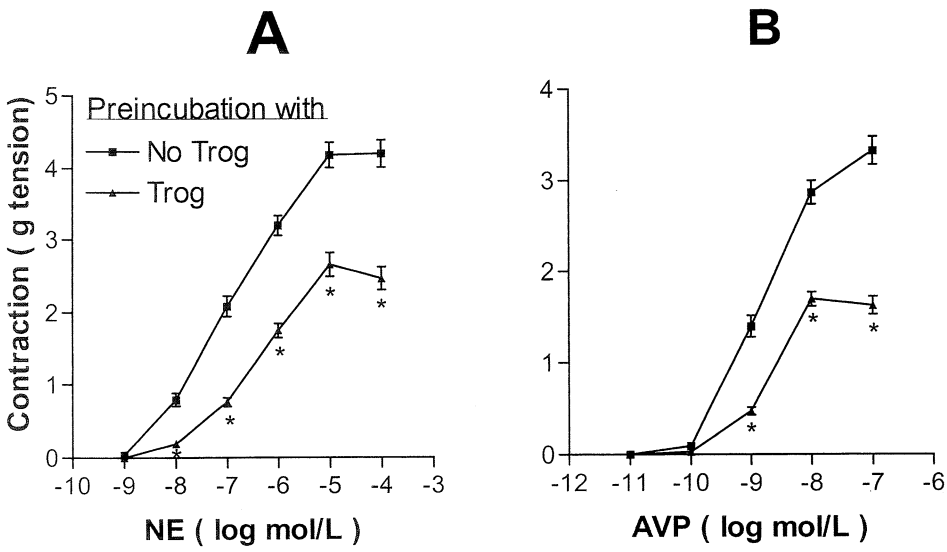

Fig 1. Contractions produced by NE (A) and AVP (B) in rat tail arterial rings after a 3-hour preincubation with either 0 or 4 μmol/L troglitazone (Trog) each in the absence of K channel inhibitors. Troglitazone significantly reduced (relaxed) several of these contractions (*$P < .05$ *v* No Trog).

this reduction in contractile tension was proportionately the same, namely, 67% (from 3.02 ± 0.2 to 1.0 ± 0.2 g tension; $P < .05$). Thus, 4AP did not significantly attenuate the delayed relaxant action of 4 μmol/L troglitazone on high-K–induced contractility.

The addition of phentolamine to high-K–contracted rings (10 minutes after their immersion in the high-K buffer) caused an immediate and marked reduction in the steady-state level of each contraction but in a manner that neither abolished the relaxant effect of the troglitazone nor the inability of 4AP to attenuate it. Thus, after phentolamine, troglitazone's reduction in high-K contractions in the absence of 4AP was 75% (from 1.2 ± 0.2 to 0.3 ± 0.04 g tension; $P < .05$) and in the presence of 4AP was 79% (from 1.9 ± 0.2 to 0.4 ± 0.1 g tension; $P < .05$). These data did not demonstrate an attenuating effect of 4AP on troglitazone's relaxation of high-K contractions.

Removal of the endothelium from half of the above-mentioned ring preparations did not influence results.

DISCUSSION

The preliminary results from this work support previous evidence that, while high concentrations of troglitazone (>10 μmol/L) can relax NE contractions in the rat tail artery immediately (within a few minutes), lower (more therapeutically-relevant) concentrations require a delay of almost an hour or more to do so.[2,6] The same results also extend such evidence to include delayed relaxations by troglitazone of nonadrenergic (AVP) as well as adrenergic (NE) contractions in this vessel. Similar delays have been reported for the vasorelaxant effects of pioglitazone on NE contractions in the same vessel.[4] The mechanism responsible for such delay in TZD vascular activ-

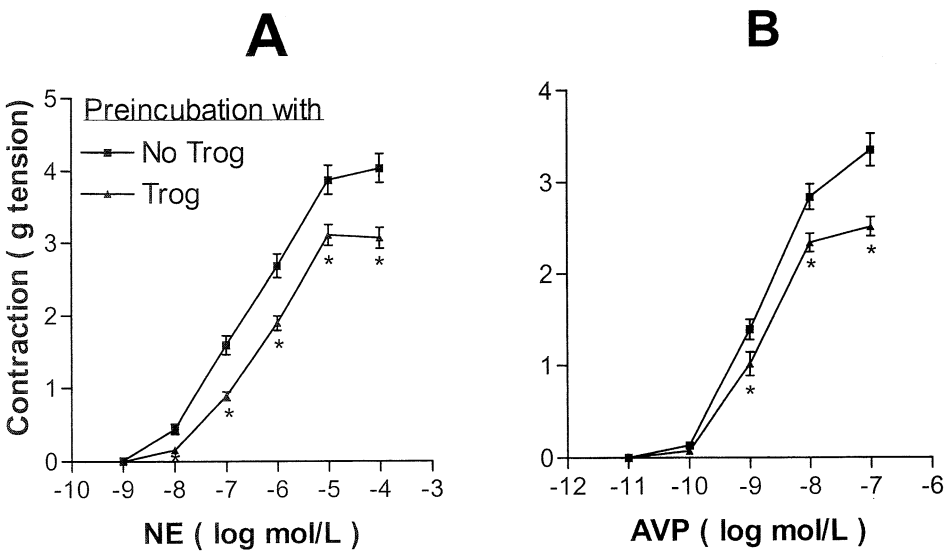

Fig 2. Contractions produced by NE (A) and AVP (B) in rat tail arterial rings after a 3-hour preincubation with either 0 or 4 μmol/L troglitazone (Trog) each in the presence of 1 mmol/L 4AP. Troglitazone significantly reduced (relaxed) several of these contractions (*$P < .05$ *v* No Trog). When compared by 2-factor ANOVA to contractions in Fig 1, 4AP (factor 2) significantly interacted with troglitazone (factor 1) to attenuate its relaxant effects on these contractions ($P < .05$).

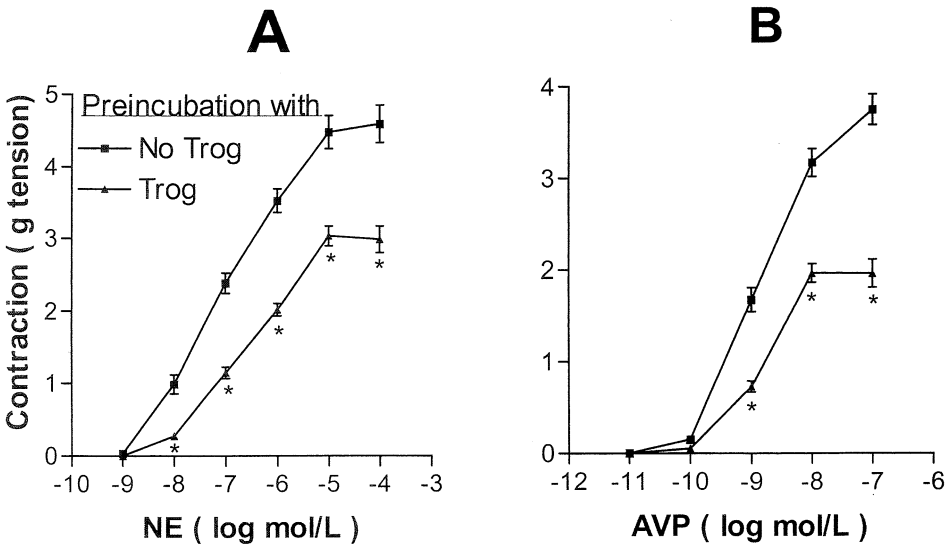

Fig 3. Contractions produced by NE (A) and AVP (B) in rat tail arterial rings after a 3-hour preincubation with either 0 or 4 μmol/L troglitazone (Trog) each in the presence of 5 μmol/L glyburide. Troglitazone significantly reduced (relaxed) several of these contractions (*$P < .05$ *v* No Trog). When compared by 2-factor ANOVA to contractions in Fig 1, glyburide (factor 2) failed to significantly interact with these effects of troglitazone (factor 1).

ities is not known. All TZDs are highly lipid soluble and thus should reach any potential intracellular, cytosolic sites of action rather quickly. However, if like glyburide and other lipophilic sulfonylureas, TZDs must enter the space between the bilayers of cellular membranes and then move laterally within that space to finally reach delayed sites of action,[21] such delay becomes easier to envision. Regardless of what is responsible for the delay itself, it certainly can be argued that delayed actions (and related mechanisms) of TZDs are of greater relevance to their long-term use in diabetic patients than the acute (rapidly occurring) actions so often studied in the past. Thus, results of this and the few previous delayed-action studies are deserving of important consideration.

The principle new finding from the present work is that coadministration of 4AP with 4 μmol/L troglitazone significantly attenuated troglitazone's delayed relaxations of both NE and AVP, but not high-K–induced contractions in the isolated rat tail artery. 4AP is capable of inhibiting various types of K channels in both the outer membrane of the cell[14,15] and the intracellular membrane associated with the SR.[19] By inhibiting SR K channels, 4AP can inhibit SR calcium sequestration.[19] Conceivably, troglitazone could relax contractions produced specifically by NE and AVP by stimulating increased activity in (K transport through) either of these separately located populations of K channels. However, during contractions induced by a high concentration of extracellular K, such as the 90 mmol/L used in the present study (high enough to prevent K transport down its normal electrochemical gradient through cell membrane K channels),[14] only the intracellular SR K channels remain functionally intact. 4AP failed to attenuate troglitazone's relaxation of high K induced contractions in the present study. Obviously, we can conclude from this that troglitazone's relaxation of high-K contraction is not related to an action of troglitazone on 4AP-sensitive K channels in either the cell

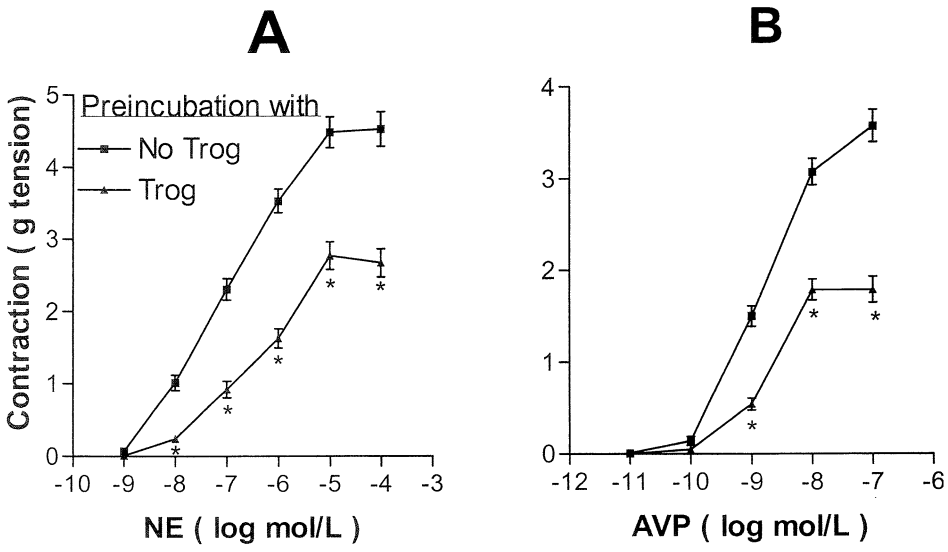

Fig 4. Contractions produced by NE (A) and AVP (B) in rat tail arterial rings after a 3-hour preincubation with either 0 or 4 μmol/L troglitazone (Trog) each in the presence of 20 μmol/L barium. Troglitazone significantly reduced (relaxed) several of these contractions (*$P < .05$ *v* No Trog). When compared by 2-factor ANOVA to contractions in Fig 1, barium (factor 2) failed to significantly interact with these effects of troglitazone (factor 1).

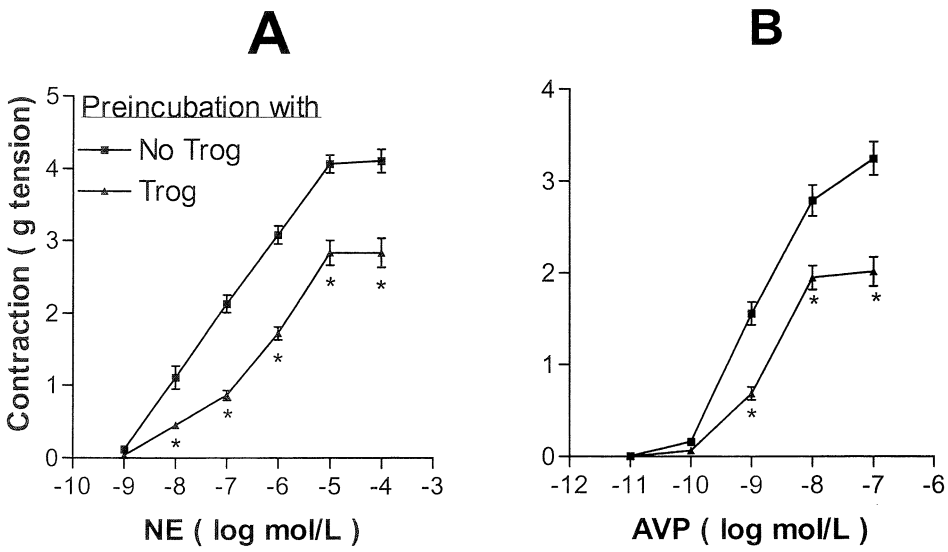

Fig 5. Contractions produced by NE (A) and AVP (B) in rat tail arterial rings after a 3-hour preincubation with either 0 or 4 μmol/L troglitazone (Trog) each in the presence of 1 mmol/L TEA. Troglitazone significantly reduced (relaxed) several of these contractions (*$P < .05$ *v* No Trog). When compared by 2-factor ANOVA to contractions in Fig 1, TEA (factor 2) failed to significantly interact with these effects of troglitazone (factor 1).

membrane or the SR. But, more importantly, we can conclude that 4AP most likely attenuates troglitazone's relaxation of specifically NE and AVP contractions (in the presence of an intact cell membrane K gradient) by interfering only with an action of troglitazone on those 4AP-sensitive K channels in the cell membrane and not the SR.

There are 2 populations of 4AP-inhibitable K channels in the cell membrane as described in Methods, the K_V and the K_{ATP}. The latter can also be inhibited by glyburide.[14,15] However, glyburide failed to attenuate the relaxant effects of troglitazone on NE and AVP contractions in the present study. Also, barium (which inhibits only K_{IR} channels) had no influence and TEA (which at 1 mmol/L inhibits only K_{Ca} channels) only slightly (but not significantly) inhibited troglitazone's relaxation of NE and AVP contractions. Thus, we can conclude that troglitazone may relax NE and AVP contractions at least in part by stimulating increased K efflux through K_V (though not K_{ATP}, K_{IR}, or K_{Ca}) in the cell membrane. Such a stimulating action would most likely mediate relaxation indirectly. As described earlier, the increased K efflux would limit (or reverse) agonist induced membrane depolarizations, with subsequent inhibition of calcium influx through voltage-operated calcium channels.

4AP did not block all of troglitazone's delayed relaxation of NE and AVP contractions in the present study. It only blocked a significant portion of it. It is conceivable that troglitazone competes with 4AP for control of K efflux through K_V channels in such a manner that the concentration of 4AP as employed in the present study (1 mmol/L) was not sufficient to completely block all the stimulating action of troglitazone. Unlike the concentrations of the other K channel inhibitors used in this study, chosen specifically to exert much more than half-maximal channel inhibition,[14,15] 4AP was limited to 1 mmol/L which at best is only half-maximally effective at inhibiting the K_V channel.[14] Higher levels of 4AP (up to 10 mmol/L), which are clearly maximally effective at blocking the K_V,[22] were avoided in the present study. In preliminary tests, we found that they produced substantial and unpredictable contractions on their own which interfered with NE and AVP contractions, thus confounding interpretation of results.

Thus, we cannot rule out the possibility that all of troglitazone's delayed relaxation of NE and AVP contractions in the present study involved the 4AP-sensitive K_V channels. However, another possibility is that troglitazone is also able to relax agonist-induced contractions via mechanisms totally unrelated to K channels, the most likely being a more direct inhibition of voltage-operated calcium channels in the smooth muscle cell membrane. As previously suggested by other investigators,[6] this would certainly be consistent with troglitazone's ability to relax contractions produced under high-K depolarizing conditions, ie, by raising the concentration of extracellular K to levels high enough to not only stop all K movement across the cell membrane, but also to open voltage-operated calcium channels by depolarizing the cell membrane. Of course, such a depolarizing level of extracellular K would also stimulate release of endogenous NE from sympathetic nerve endings adjacent to the smooth muscle.[20] Furthermore, high extracellular K would stimulate uptake (sequestration) of intracellular free calcium into the smooth muscle cell SR.[23] However, in the present work neither phentolamine (a nonselective NE receptor blocker) nor 4AP (known to block SR calcium uptake both directly and indirectly)[19,24,25] were able to alter troglitazone's relaxation of high-K contraction.

It is also possible that prolonged exposure to troglitazone may relax smooth muscle contractions by inhibiting release of calcium from the SR. This would be consistent with a previous report that during calcium readdition experiments the initial contraction of rat tail arterial rings by NE in calcium-free buffer was lower in rings pretreated with 3 μmol/L troglitazone for 90 minutes.[6] We have seen similar effects after 4 hours of pretreatment with 8 μmol/L troglitazone[26] and with the NE contractions produced in the presence of EGTA to prevent any possible influx of residual extracellular calcium otherwise loosely bound to the outer surface of the cell membrane.[27]

Therefore, troglitazone's delayed relaxation of arterial

smooth muscle contractions as induced by important physiological agonists (eg, NE and AVP) is no doubt related to multiple mechanisms ranging from inhibited release of intracellularly stored calcium to both direct and indirect inhibition of calcium influx through membrane-bound voltage-operated calcium channels. The present work is the first indication of the existence of the latter (indirect inhibition of calcium influx) and suggests that it relates to a stimulating action of troglitazone on 4AP-sensitive K_V channels in the smooth muscle cell membrane. What remains to be determined is whether that stimulating action is the result of an actual binding of troglitazone to the K_V channel itself (altering its intrinsic activity) or a change in signal transduction pathways linked ultimately to opening those channels.

Finally, there appeared to be some alterations in control NE and AVP contractions (ie, contractions in the absence of troglitazone) associated with some of the K channel inhibitors. This was especially apparent in Fig 2A, where 4AP appeared to suppress NE control contractions compared to corresponding control contractions in Fig 1A (in the absence of any K channel inhibitors) and Figs 3A, 4A, and 5A (each in the presence of another K channel inhibitor). To a lesser degree this was also apparent when control AVP contractions in Fig 2B (in the presence of 4AP) were compared to such contractions in Fig 3B (in the presence of glyburide). Conversely, it appeared that glyburide and barium each slightly enhanced control NE and AVP contractions in Figs 3 and 4, respectively, compared to all such control contractions in other figures. We believe these differences were due to nonspecific factors associated with the time of experimentation which, out of necessity, was not the same for all figures. We do not believe they relate to any specific actions of the K channel inhibitors in question or their potential to interact with the effects of troglitazone.

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