Iloprost Inhibits Inositol-1,4,5-Trisphosphate-Mediated Calcium Mobilization Stimulated by Angiotensin II in Cultured Preglomerular Vascular Smooth Muscle Cells

KIT E. PURDY and WILLIAM J. ARENDSHORST
Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina.

Abstract. In a previous study of cultured preglomerular vascular smooth muscle cells, it was demonstrated that, although the stable prostacyclin analog iloprost alone had no effect on the intracellular calcium concentration ([Ca²⁺]), it did significantly attenuate the increase in [Ca²⁺], stimulated by angiotensin II (AngII). In this study, the mechanisms by which iloprost interacts with calcium signaling pathways stimulated by AngII were examined. [Ca²⁺], was assessed using the calcium-sensitive fluorescent dye fura-2. Initial studies identified two major components of the [Ca²⁺] response to AngII in this homogenous preparation of vascular smooth muscle cells from renal resistance vessels. Mobilization of internal stores was evident as an immediate TMB-8-sensitive peak increase in [Ca²⁺], (52 ± 6 to 297 ± 26 nM, P < 0.001) in a calcium-free medium. After [Ca²⁺], had returned to baseline levels during continued AngII stimulation, a nifedipine-sensitive entry pathway was revealed by the sustained stimulatory effect of added external calcium, which increased [Ca²⁺], to 112 ± 13 nM (P < 0.001). Coadministration of iloprost with AngII attenuated both the immediate peak (154 ± 14 nM) and sustained plateau (61 ± 9 nM) phases. Increases in endogenous levels of cAMP induced by the phosphodiesterase inhibitor milrinone mirrored the actions of iloprost, suggesting that the prostacyclin analog exerted its actions via cAMP activation. Blockade of cAMP-dependent protein kinase with KT 5720 reversed the effects of both iloprost and milrinone. When iloprost or milrinone was introduced after the initial mobilization peak had dissipated, the plateau phase of calcium entry was unchanged (92 ± 9 nM). The concept that iloprost does not directly modulate calcium entry was further supported by data showing that the activation of L-type calcium channels by BAY-K 8644 was unchanged during iloprost treatment. On the basis of the observation that iloprost did not alter thapsigargin stimulation of Ca²⁺-ATPase activity, it is concluded that the actions of cAMP are distinct from increasing calcium uptake into the sarcoplasmic reticulum. This study provides new information on the ability of iloprost to primarily attenuate inositol-1,4,5-trisphosphate-mediated calcium mobilization via cAMP, with secondary inhibition of L-type calcium entry channels. These data clarify the mechanism by which prostaglandins buffer AngII constriction in resistance arterioles.

The afferent arteriole plays an important role in the regulation of renal blood flow, GFR, and renin release, thus contributing to the control of extracellular fluid volume and arterial pressure. Angiotensin II (AngII) is a potent vasoconstrictor and is thought to play an important role in the regulation of the renal microcirculation. For a balanced biologic effect, the actions of AngII must be modulated by local lipid mediators such as prostaglandin E₂ (PGE₂) and PGI₂. AngII stimulates the release of PGE₂ and PGI₂, which in turn oppose the AngII activity by promoting vasodilation. In a previous study, we demonstrated that PG buffer AngII-induced constriction by attenuating AngII-mediated increases in intracellular calcium concentrations ([Ca²⁺]) in preglomerular vascular smooth muscle cells (VSMC). RIA indicated that PGI₂ is the predominant cyclooxygenase product involved in this response (1). Although it is thought that AngII activates both calcium mobilization via inositol-1,4,5-trisphosphate (IP₃) activation and calcium entry via dihydropyridine-sensitive L-type channels (2,3), very little is known regarding the mechanisms of prostanooid action on [Ca²⁺], particularly in the physiologically relevant resistance vessels of the microcirculation.

Data from both isolated preglomerular VSMC and preglomerular arterioles indicate that PGI₂ stimulates adenylate cyclase activity (4,5). These findings place PGI₂ in a large class of cAMP-elevating vasodilators, including β-adrenergic receptor agonists such as isoproterenol, dopamine, vasoactive intestinal peptide, and adenosine. The cAMP produced by these agents is thought to elicit effects subsequent to activation of cAMP-dependent protein kinase (PKA). The specific primary intracellular targets of PKA in decreasing [Ca²⁺], are not clear. Early studies suggested that PKA acted primarily in VSMC as it does in cardiac muscle, stimulating calcium removal from the cytoplasm by increasing the activity of the sarcoplasmic
reticulum \(Ca^{2+}\)-ATPase (6,7). PKA was observed to phosphor-
ylate the regulatory protein phospholamban, thus alleviating its
inhibitory effect on \(Ca^{2+}\)-ATPase (8). More recently, the focus
has shifted to cAMP/PKA effects on ion channels in the plasma
membrane, but no clear understanding has been developed.
Data on the effects of cAMP on L-type channels in VSMC are
contradictory. Electrophysiologic studies on VSMC from the
portal vein (9) and the aorta (10) revealed that cAMP seems to
inhibit L-type calcium current. However, other investigators
reported that cAMP has an excitatory effect on VSMC prepa-
rations from the portal vein (11) and the mesenteric artery (12).
Dihydropyridine-sensitive \(45Ca^{2+}\) influx is reportedly inhibited by
cAMP activation in aortic VSMC (13). In addition, calcium-
activated potassium channels (\(K_{Ca}\)) may be PKA targets. Ac-
tivation of \(K_{Ca}\) in VSMC produces membrane hyperpolariz-
ation and thus closure of voltage-gated L-type calcium channels.
Both the PGI\(_2\) analog iloprost and the catalytic subunit of PKA
have been observed to activate \(K_{Ca}\) in rat tail artery VSMC
(14). Vasodilation elicited by cAMP analogs is inhibited by \(K_{Ca}\)
blockers in cerebral arterioles and aorta (15,16). Another
possible action of cAMP signals is interaction with signal
transduction mediated by IP\(_3\) and calcium release from internal
stores. Phosphorylation of the IP\(_3\) receptor in aortic VSMC
occurs in response to elevations in cAMP levels (17). Further-
more, cAMP-elevating agents are reported to inhibit IP\(_3\) for-
mation and decrease the sensitivity of IP\(_3\) receptors in cultured
aortic A10 VSMC (18).

The purpose of this study was to perform a comprehensive
analysis of the actions of the prostacyclin analog iloprost and
to define mechanisms involved in PGI\(_2\), buffering of intracel-
lular calcium in the physiologically relevant preglomerular
resistance vessels. Interactions of iloprost with calcium mobil-
ization and entry pathways stimulated by AngII were assessed
using pharmacologic inhibitors of IP\(_3\)-mediated calcium re-
lease from internal stores and calcium entry through voltage-
gated L-type calcium channels. To gain further support for
these findings, we also examined the effects of iloprost on the
same calcium signaling pathways stimulated by alternative
agents that act independently of cell surface receptors. The
involvement of cAMP and PKA in iloprost activity was tested
using the phosphodiesterase inhibitor milrinone and the PKA
inhibitor KT 5720.

Materials and Methods

**Isolation of Preglomerular Resistance Vessels**

Experiments were performed with male Sprague-Dawley rats (200
to 300 g) from our Chapel Hill breeding colony. Animal experimen-
tation was conducted in accordance with the National Institutes of
Health Guide for the Care and Use of Laboratory Animals. To isolate
VSMC from renal resistance vessels, we used a technique previously
described by Zhu and Arendshorst (19). Cells of the digested microves-
sels were collected by brief centrifugation and washed once with PBS,
to remove collagenase. Next, the cells were suspended in 36 ml of
culture medium [RPMI 1640 medium supplemented with 100 U/ml
penicillin, 100 \(\mu\)g/ml streptomycin, 0.6 mM L-glutamine, and 10%
fetal calf serum (Hyclone Laboratories, Salt Lake City, UT)]. The
microvascular suspension was divided into twelve 60-mm culture
dishes and incubated at 37°C in 5% CO\(_2\)/95% air at 98% humidity.
The medium was changed the next day and every 2 or 3 d thereafter
until the cells became confluent. After approximately 3 wk in primary
culture, the cells were passaged by collection with 0.05% trypsin and
subpassaged every 7 to 10 d thereafter. The cells were seeded at a
density of 3 to 5 \(\times\) 10\(^3\) cells/cm\(^2\). Monolayers were studied between
passes 5 and 9.

**Measurement of \([Ca^{2+}]_i\)**

\([Ca^{2+}]_i\) measurements were performed using the acetoxymethyl
ester of the calcium-sensitive dye fura-2, as we described previously
(19,21). A monolayer of VSMC was grown on 22-mm\(^2\) glass covers-
slips, under the same conditions as described above. Confluent cells
were subjected to serum-free medium 24 h before experiments. Before
the study, the VSMC were washed twice with Hanks’ balanced salt
solution (HBSS) (135 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1 mM
MgCl\(_2\), 5 mM \(\alpha\)-glucose, 10 mM Hepes, pH 7.4) and incubated for 60
min in the dark at room temperature with 2 \(\mu\)M fura-2/acetoxymethyl
ester. After loading, the cells were washed three times with HBSS and
allowed to remain for 20 min. Immediately before testing, a coverslip
was mounted in a plastic chamber, creating a well for drug addition
directly over the center of the coverslip. The chamber was then
centered in the field of a \(\times\) 40 oil-immersion fluorescence objective of
an inverted microscope (IX-70; Olympus, Tokyo, Japan). Cells were
excited alternately with light of 340- and 380-nm wavelengths, from
dual monochromators of a dual-excitation wavelength Deltascan
(model RMD; Photon Technology International, Monmouth Junction,
NJ). Fluorescence was detected by a photometer after passage through
a barrier filter (510 nm). Fluorescence signal intensities were ac-
quired, stored, and processed using a Dell Pentium computer and
Felix software (Photon Technology International). To obtain autofluo-
rescence values, cells were treated in an identical manner with the single exception that they were not exposed to fura-2. The fluorescence counts at both wavelengths were typically <10% of those recorded from fura-2-loaded cells. After subtraction of these background readings from the recordings for stimulated preparations, [Ca^{2+}], was calculated from the 340/380-nm ratio, according to the formula [Ca^{2+}] = [(R - R_{\text{min}})/(R_{\text{max}} - R)] × (S_{b} × K_{p}) × K_{c} as described by Grynkiewicz et al. (22), using external calibration.

The first set of experiments was designed to determine the calcium pathways used by AngII to increase [Ca^{2+}]. In control experiments, solutions of either HBSS containing 1 mM calcium (Ca-HBSS) or calcium-free HBSS with 0.5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N″-tetraacetate (EGTA) (0 Ca-HBSS) were used to distinguish between mobilization from intracellular stores and calcium entry across the plasma membrane. To ensure that EGTA did not have deleterious effects on membrane stability or receptor function, several trials of each protocol were undertaken with nominally calcium-free HBSS (no EGTA was added; the measured calcium concentration was 30 nM). The results did not differ between the calcium-free buffers with or without EGTA. The mobilization and entry portions of the AngII response were confirmed with treatment with 10^{-7} M nifedipine, a dihydropyridine-sensitive L-type calcium channel inhibitor, and 10^{-6} M TMB-8, an inhibitor of IP_{3}-mediated calcium release from intracellular stores (23). Iloprost (10^{-7} M), a stable analog of PGI_{2}, was used to determine which of the calcium pathways activated by AngII is targeted by PG. The effect of increases in intracellular cAMP levels on AngII-stimulated calcium signaling mechanisms was assessed by coadministration of 10^{-6} M milrinone, an inhibitor of cAMP phosphodiesterase 3 (24). The PKA blocker KT 5720 (10^{-6} M) was used to examine the mechanisms of cAMP activity. The actions of iloprost on entry and mobilization were also tested using non-receptor-mediated activators, i.e., thapsigargin (10^{-6} M), which inhibits sarcoplasmic Ca^{2+}-ATPase, and BAY-K 8644 (10^{-7} M), which selectively activates L-type calcium channels. Recordings were interrupted for approximately 3 to 5 s for drug addition and 10 to 15 s for external buffer exchange. Control experiments established that no information was lost during these short periods.

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), with the exception of thapsigargin, which was obtained from Calbiochem (La Jolla, CA), and iloprost, which was a gift from Berlex Laboratories (Cedar Knolls, NJ). Each preparation was tested only once, to avoid possible receptor desensitization or tachyphylaxis.

**Statistical Analyses**

Data are presented as means ± SEM. Data sets were analyzed statistically with ANOVA, followed by post hoc testing with the Student-Newman-Keuls test. P < 0.05 was considered statistically significant.

**Results**

To systematically examine the effects of iloprost on calcium signaling mechanisms activated by AngII in our preparation of cultured VSMC obtained from preglomerular arterioles, we first sought to identify distinct temporal components of the AngII response and define their involvement using specific inhibitors. The first set of control studies was conducted with 1 mM calcium in the extracellular fluid. Figure 1A shows that AngII caused a rapid peak increase in [Ca^{2+}], to 307 ± 22 nM from a baseline value of 45 ± 3 nM (P < 0.001), followed by a decrease to a sustained plateau phase, during which [Ca^{2+}] remained relatively stable at 94 ± 6 nM (P < 0.001 versus baseline values). This general response to AngII agrees well with earlier results from our laboratory on AngII effects on similarly prepared renal VSMC (1). Coadministration of the L-type calcium channel inhibitor nifedipine with AngII nearly completely abolished the plateau phase, returning [Ca^{2+}] to baseline levels of 62 ± 6 nM at 200 s (P > 0.19), but had no significant effect on the immediate peak increase (293 ± 34 nM). Other experiments were conducted to further discriminate between calcium release into the cytosol from intracellular stores and calcium entry from the extracellular compartment via channels in the plasma membrane. Figure 1B presents the stimulatory action of AngII when the extracellular solution was alternated between Ca-HBSS and 0 Ca-HBSS. Initially, control studies were performed in which VSMC were exposed to alternating solutions of Ca-HBSS and 0 Ca-HBSS every 50 s for 200 s. The first change from Ca-HBSS to 0 Ca-HBSS caused a nonsignificant fluctuation (52 ± 6 to 44 ± 5 nM, P > 0.30), as did all subsequent changes in calcium buffer solutions. Therefore, the plasma membranes of unstimulated VSMC are relatively insensitive to large changes in the extracellular calcium concentration. AngII (10^{-7} M) in 0 Ca-HBSS produced a peak response of 297 ± 26 nM at 200 s, similar to the increase recorded with calcium in the bath. After [Ca^{2+}]; returned to baseline levels, addition of calcium to the bathing solution at 350 s resulted in an immediate sustained increase in [Ca^{2+}], to 112 ± 13 nM. This entry response was not statistically different from the sustained phase observed during AngII stimulation in control experiments. Figure 1B shows that the addition of nifedipine had little effect on the early portion of the AngII-stimulated calcium response (255 ± 26 nM), whereas it nearly abolished the sustained phase, returning [Ca^{2+}], to 56 ± 3 nM (P > 0.09 versus baseline values), indicating that calcium entry is predominantly mediated by L-type calcium channels.

To further investigate the involvement of calcium mobilization, TMB-8 (10^{-6} M), a blocker of IP_{3}-mediated release of calcium from internal stores, was administered simultaneously with AngII in the presence of 1 mM calcium in the extracellular compartment (Figure 1C). TMB-8 attenuated the AngII-induced peak response to 103 ± 9 nM (P < 0.001 versus control values). When coadministered with AngII in the alternating Ca-HBSS protocol, TMB-8 in 0 Ca-HBSS produced a similar decrease in the immediate peak, with [Ca^{2+}], increasing from a baseline value of 38 ± 6 to 70 ± 5 nM (Figure 1D). It is noteworthy that the entry portion of the response to AngII was also markedly reduced with TMB-8 treatment. ([Ca^{2+}], returned to 42 ± 6 nM, not different from the baseline values).

Having established the two phases and mechanisms of AngII-induced changes in [Ca^{2+}], in preglomerular VSMC, we conducted experiments to determine whether the stable PGI_{2} analog iloprost affects calcium mobilization, calcium entry, or both mechanisms. As shown in Figure 2A, iloprost (10^{-7} M) partially attenuated the initial mobilization response from 297 ± 26 to 154 ± 14 nM (P < 0.001) and completely inhibited the sustained entry response from 112 ± 13 to 61 ± 9 nM (P > 0.17) after coadministration with AngII in the alternating
Ca-HBSS protocol. When iloprost was added at 300 s, after AngII-induced mobilization had occurred, \([\text{Ca}^{2+}]_{i}\) increased to 92 ± 6 nM, a value not different from that observed with AngII alone (Figure 2B). Therefore, iloprost appears primarily to blunt calcium mobilization elicited by AngII and thus inhibit calcium entry, which is tightly coupled to initial mobilization events. To extend this conclusion, we sought to exclude the possibility that TMB-8 affected entry independently of its effects on mobilization. Using a similar protocol, TMB-8 was administered at 300 s, after AngII-induced mobilization had occurred, and external calcium was added back to the preparation at 350 s. We observed that TMB-8 had no effect on calcium entry induced by AngII under these conditions, ruling out nonspecific effects of TMB-8 on calcium entry.

To verify that iloprost exerts its effects by stimulating adenylate cyclase, the intracellular cAMP concentration was increased using milrinone, which is an inhibitor of phosphodiesterase 3, the major phosphodiesterase isoform present in preglomerular VSMC (24). Milrinone (10^{-6} M) mirrored the effects of iloprost on the AngII response. Figure 3A shows that the peak response to AngII stimulation was attenuated to 149 ± 12 nM and the sustained phase decreased to 68 ± 8 nM. Both of these values are similar to those observed with concurrent administration of iloprost and AngII. In the experiment presented in Figure 3B, milrinone was administered at 300 s, after the peak response had waned, and the compound no longer had an effect on the late portion of the AngII response. \([\text{Ca}^{2+}]_{i}\) reached a value of 124 ± 14 nM \((P > 0.6)\) after exposure to external calcium. In another series of experiments, the involvement of PKA was assessed using the specific PKA inhibitor KT 5720. As evident in Figure 4, the inhibitory effects of both iloprost and milrinone on the initial peak calcium response to AngII were reversed with blockade of PKA \((P < 0.01\) for iloprost and \(P < 0.03\) for milrinone). These

---

**Figure 1.** Angiotensin II (AngII) (10^{-7} M) stimulation of the intracellular calcium concentration (\([\text{Ca}^{2+}]_{i}\)) in cultured renal preglomerular vascular smooth muscle cells (VSMC), characterized by an immediate peak increase in \([\text{Ca}^{2+}]_{i}\), followed by a sustained plateau phase. Coadministration of nifedipine (10^{-7} M), an L-type calcium channel antagonist, blocks the sustained portion of the AngII response (A and B). TMB-8 (10^{-6} M), an inhibitor of intracellular mobilization, blocks both the peak and sustained portions of the \([\text{Ca}^{2+}]_{i}\) response to AngII (C and D). (A and C) Cells were bathed in 1 mM calcium-containing Hanks’ balanced salt solution (HBSS) throughout the observation period. (B and D) VSMC were exposed to alternating solutions of calcium-free HBSS (0) and calcium-containing HBSS (Ca), under control conditions as well as during AngII stimulation. Values are means ± SEM for nine preparations in each group.
results reinforce the view that iloprost exerts its actions on calcium signaling via stimulation of cAMP production and subsequent activation of PKA.

The potential effects of iloprost on these calcium mobilization and entry pathways were further assessed using non-receptor-mediated activators. To determine whether iloprost attenuates mobilization by increasing the rates of calcium uptake into the sarcoplasmic reticulum, the cells were treated with thapsigargin, an inhibitor of the sarcoplasmic reticulum Ca$^{2+}$-ATPase. Figure 5 shows that thapsigargin (10$^{-6}$ M) alone produced an increase from 54 ± 3 to 144 ± 13 nM in 0 Ca-HBSS. After [Ca$^{2+}$]$_i$ returned to baseline levels, the external buffer was changed to Ca-HBSS at 1000 s, and [Ca$^{2+}$]$_i$ immediately increased to 250 ± 18 nM. These data suggest that thapsigargin stimulates store-operated capacitative entry after emptying of internal stores. When iloprost (10$^{-7}$ M) was administered with thapsigargin at 200 s, the peak [Ca$^{2+}$]$_i$ response reached 139 ± 13 nM, a value similar to that observed for thapsigargin alone ($P > 0.8$). Therefore, iloprost does not appear to affect the activity of the Ca$^{2+}$-ATPase to increase sequestration. Furthermore, iloprost had little effect on the late phase after external calcium addition; [Ca$^{2+}$]$_i$ increased to 238 ± 38 nM. Coadministration of nifedipine (10$^{-7}$ M) at 200 s had no effect on the peak response but did attenuate the sustained phase, with [Ca$^{2+}$]$_i$ increasing to 158 ± 23 nM ($P < 0.002$). Approximately one-half of the calcium entry stimulated by thapsigargin seems to be mediated by nifedipine-sensitive L-type calcium channels; the remaining fraction probably enters through store-operated capacitative entry channels. Of particular note, iloprost had no inhibitory effect on the L-type-sensitive portion of the sustained response to AngII.

To further examine the possible direct effects of iloprost on L-type calcium channel activity, renal VSMC were stimulated with BAY-K 8644, yielding a progressive increase in [Ca$^{2+}$]$_i$.
from 57 ± 7 to 94 ± 11 nM (P < 0.003) (Figure 6). Simultaneous treatment with nifedipine and BAY-K 8644 completely abolished the response, confirming that BAY-K 8644 specifically stimulated L-type channels and that nifedipine exerted a selective inhibitory action. When iloprost was administered simultaneously with the calcium channel agonist, the PGI₂ analog had no effect on calcium entry; [Ca²⁺]Ca eventually increased to 106 ± 7 nM (P > 0.6 versus BAY-K 8644 alone). These data reinforce the notion that iloprost has no direct effect on L-type calcium channel activity.

**Discussion**

We present new evidence that the vasodilatory PGI₂ analog iloprost primarily attenuates the initial IP₃-mediated mobilization phase of the AngII response in cultured VSMC isolated from preglomerular resistance vessels. Linked to this initial event is a secondary inhibitory action on calcium entry through L-type channels. Studies using the cAMP-elevating agent milrinone and the PKA blocker KT 5720 strongly suggest that these actions of iloprost are mediated via the cAMP/PKA second messenger system. Our comprehensive assessment of the interactions of iloprost and AngII-stimulated calcium signaling help to unify previous observations by other investigators that indicated that cAMP-elevating vasodilators modulate a single mechanism, i.e., either IP₃ receptor or L-type channel activation, but not both (9,10,17,18). Our results do not support the previously held view that the cAMP/PKA signaling pathway acts by stimulating calcium uptake into the sarcoplasmic reticulum via Ca²⁺-ATPase (6,7). We observed that iloprost has no effect on [Ca²⁺]Ca during thapsigargin blockade of sarcoplasmic reticulum Ca²⁺-ATPase. To our knowledge, these observations are novel for a homogeneous population of VSMC originating from resistance arterioles in general and the renal afferent arterioles in particular. They extend previous studies on the mechanism of action of cAMP-elevating vasodilators in nonvascular cells and large conduit vessels, such as the aorta, to regulatory cells in the microcirculation.

The modulatory effect of iloprost on calcium mobilization provides a mechanism for the well known action of vasodilatory PG to buffer resistance vessel contraction stimulated by...
hormonal and paracrine agents such as AngII. The physiologic relevance of PG actions in the microvasculature of the kidney has been demonstrated in both laboratory and clinical studies. Previous experiments performed in our laboratory demonstrated that PGE2 and PGI2 and their synthetic analogs dilate the renal vasculature and effectively attenuate the vasoconstriction produced by AngII and norepinephrine (25). These prostanoids can counteract agonist-induced vasoconstriction at concentrations lower than those required to elicit vasodilatation. A defect in this prostanoid-initiated buffering system is associated with excessive renal vasoconstriction in young, genetically hypertensive rats (26,27). Furthermore, patients with high AngII levels may develop acute renal failure when nonsteroidal anti-inflammatory drugs are administered to block PG production, because of the unopposed AngII-mediated vasoconstriction and marked reductions in GFR (28).

Our results indicate that the primary cellular target of vasodilator agents that stimulate cAMP is the TMB-8-sensitive IP3 signaling pathway in VSMC from renal resistance arterioles. This new finding unifies and extends to peripheral VSMC previous studies performed with nonvascular smooth muscle cells. In cell preparations such as platelets, cerebellar slices, and pancreatic acinar cells, PKA phosphorylates the IP3 receptor (29–31). Phosphorylation of the IP3 receptor most likely reduces its sensitivity to IP3, so that less calcium is released from intracellular stores upon stimulation or, alternatively, stronger stimulation is required to release the same amount of calcium. PKA has been observed to inhibit IP3-dependent calcium release in gastric smooth muscle (32). Tertyshnikova and Fein (33) used photolysis of caged IP3 in megakaryocytes and observed that the prostacyclin analog carbacyclin inhibited IP3-induced calcium release via PKA, without affecting the rate of calcium removal from the cytoplasm. We cannot exclude the possibility that cAMP affects the rate of IP3 production. Isoproterenol-induced increases in cAMP levels have been reported to reduce both IP3 formation and the sensitivity of the IP3 receptor during endothelin stimulation in cultured aortic A10 VSMC (18).

Alternatively, iloprost could attenuate [Ca2+]i by increasing the activity of calcium extrusion pumps. However, our results argue against this possibility. We find that iloprost has no effect on thapsigargin-mediated blockade of the sarcoplasmic reticulum Ca2+-ATPase. PGI2 and PKA do not affect the activity of Ca2+-ATPases in platelets (29,33). Further evidence against this possibility, at least concerning the sarcoplasmic reticulum, is provided by a recent report on phospholamban-deficient mice. Although phospholamban is the hypothesized target of PKA in the modulation of Ca2+-ATPase activity, the absence of phospholamban did not affect cAMP-mediated relaxation in aortic VSMC (34).

Another possibility, which is supported primarily by electrophysiologic data on VSMC from conduit vessels (9,10,13), is that cAMP-elevating agents can regulate calcium entry across the plasma membrane. Consistent with these observations, iloprost blocks entry when coadministered with AngII in our preparation of resistance arteriolar VSMC. However, we observed that iloprost has no effect on the sustained entry response when it is administered after the initial mobilization event has occurred. This is also the case when milrinone is used to elevate cAMP levels. Collectively, these data indicate that the effect of iloprost on calcium entry in preglomerular VSMC is secondary to the primary triggering event of mobilization. Stated differently, iloprost influences calcium entry through L-type channels only after it has inhibited mobilization from internal stores. Further support for an indirect effect on calcium entry is derived from our findings showing that iloprost does not affect [Ca2+]i, by direct modulation of L-type channels stimulated by the channel agonist BAY-K 8644.

These results also indicate the presence of another calcium entry channel, i.e., a store-operated channel that is activated by the depletion of intracellular calcium storage pools by thapsigargin. It is noteworthy that iloprost does not appear to directly affect entry through either store-operated or L-type channels. Neither nifedipine-sensitive nor nifedipine-insensitive portions of thapsigargin-stimulated entry were affected by iloprost. These findings highlight the relative importance of the inhibitory effect of iloprost on IP3-mediated calcium signaling.

Heterogeneity in calcium entry mechanisms is evidenced by the distinct actions of cAMP in different types of muscle cells. In contrast to our observations with VSMC, PKA phosphorylates and activates L-type calcium channels, resulting in enhanced calcium influx, in skeletal muscle cells (35). To our knowledge, there are no reports of PKA-induced phosphorylation of the L-type channel in VSMC from any vascular bed. PKA has been reported to enhance the activity of Kca in conduit vessels such as the aorta, tail artery, and middle cerebral artery (14,36,37). If this mechanism is operative in our VSMC, hyperpolarization would be expected to alter L-type channel activity. We observed no effect of iloprost on the direct activation of voltage-dependent L-type channels with BAY-K 8644. Therefore, our data indicate that it is unlikely that PKA modulates Kca channels in renal preglomerular VSMC.

Our results strongly suggest that AngII-induced calcium mobilization itself acts as a trigger for calcium entry, so that cAMP-induced blockade of IP3-mediated calcium mobilization necessarily reduces calcium entry. In all cases, entry was attenuated when inhibitory agents, such as TMB-8, iloprost, and milrinone, were coadministered with AngII. In marked contrast, each of these agents was without effect when it was introduced late in the response to AngII, after mobilization events had taken place. The signals linking initial calcium release from internal stores to subsequent stimulation of calcium entry are not clear. It is likely that released calcium triggers a change in ion permeability, leading to depolarization of the plasma membrane and activation of voltage-sensitive L-type calcium entry channels. Electrophysiologic studies indicate that AngII can reduce outward, ATP-sensitive, potassium currents in rat mesenteric arterial smooth muscle cells (38), as well as calcium-activated potassium currents (39). Another possibility is that AngII stimulates chloride currents to depolarize the plasma membrane. Cultured mesangial cells express chloride channels that are activated by AngII-induced increases in [Ca2+]i after calcium release from intracellular
stores (40). Blockade of chloride channels has been observed to abolish AngII constriction of afferent arterioles in microperfused rabbit afferent arterioles and isolated perfused hydronephrotic kidneys (41,42).

We have observed quantitative differences in the calcium responses of freshly collected and cultured preglomerular VSMC. As with any cells grown in culture, changes in phenotype are possible. Cultured renal VMSC demonstrate a time-dependent, peak/sustained response to receptor agonists, as described in this study. This general pattern has been reported for cultured mesangial cells (43,44), renal arterial VSMC (45), and aortic VSMC (46,47), as well as freshly isolated renal VSMC (48). However, our laboratory consistently observes that freshly collected preglomerular VSMC respond to agonist stimulation with a stable, square-shaped increase in [Ca\(^{2+}\)](i) (20,21,49). This is the case whether VSMC are prepared by sieving in combination with iron oxide or microspheres or by microdissection without sieving (20,21,49) (B. Iversen, unpublished observations). The relative contributions of the mechanisms underlying these temporal differences in the calcium responses of fresh and cultured preparations are not clear and warrant further investigation.

Despite these quantitative variations, we have observed many noteworthy similarities in basic calcium signaling mechanisms activated in the two preparations. Calcium responses elicited by AngII in both fresh and cultured preglomerular VSMC are characterized by mobilization and entry pathways, when tested using similar buffers (Figure 1) (21). AngII induces a similar signaling pattern in other renal microvascular preparations, including renal blood flow measured in vivo (2), microdissected afferent arterioles (3), and juxtamedullary nephron preparations (50). L-type channels mediate the calcium entry phase in fresh and cultured renal VSMC (Figure 1) (20,21). Calcium responses to thapsigargin that indicated calcium release from the sarcoplasmic reticulum during Ca\(^{2+}\)-ATPase blockade are recorded in both preparations (Figure 5) (21). Furthermore, similarities have been noted, in that AT\(_1\) receptors sensitive to losartan and candesartan are expressed in fresh and cultured renal VSMC preparations (19,20). Also, both fresh and cultured renal VSMC have a poorly defined, PD 123319-sensitive AT receptor (perhaps unique to the renal vasculature), which increases [Ca\(^{2+}\)](i) in vitro and mediates renal vasoconstriction in vivo (19,51). In contrast, this PD 123319-responsive AT receptor is absent in cultured aortic VSMC (19,52). Importantly, we have observed that iloprost attenuates the calcium response to AngII in both fresh and cultured preparations of preglomerular VSMC (1). Another similarity is the presence of the EP\(_4\) receptor for PGE\(_2\) (53).

In summary, we demonstrate for the first time that the prostacyclin analog iloprost opposes AngII activity in cultured preglomerular VSMC primarily by inhibition of the IP\(_3\)-triggered release of calcium from intracellular pools. Iloprost also inhibits AngII-induced calcium entry via voltage-gated L-type channels, by a secondary effect that is dependent on initial calcium mobilization and release from internal stores. Our findings are consistent with iloprost activation of cAMP and PKA to exert these actions. This comprehensive assessment of both the AngII-stimulated mobilization and entry phases unifies data from previous reports that focused on the effects of cAMP-elevating agents on isolated steps in the calcium cascade. In addition, these findings provide new information on the mechanism of action of vasodilator prostanooids in calcium signaling in VSMC from peripheral resistance vessels.

Acknowledgments

This research was supported by National Institutes of Health Grant HL02334. Dr. Purdy was supported by a Howard Hughes Predoctoral Fellowship. We thank Dr. Gary Bird (National Institute of Environmental Health Sciences, Research Triangle Park, NC) for helpful advice and discussions. We are also grateful to Berlex Laboratories for the gift of iloprost.

References


50. Inscho EW, Imig JD, Cook AK: Afferent and efferent arteriolar vasoconstriction to angiotensin II and norepinephrine involves release of Ca$^{2+}$ from intrarenal stores. *Hypertension* 29: 222–227, 1997

