Articles in PresS. Am J Physiol Renal Physiol (October 31, 2007). doi:10.1152/ajprenal.00244.2007

Angiotensin II – stimulated calcium entry mechanisms in afferent arterioles: role of transient receptor potential canonical channels and reverse Na^+/Ca^{2+} exchange.

Susan K. Fellner and William J. Arendshorst

Department of Cell and Molecular Physiology

University of North Carolina at Chapel Hill

Chapel Hill, North Carolina 27599-7545

Corresponding author: Susan K. Fellner

sfellner@med.unc.edu

Abstract

In afferent arterioles, the signaling events that lead to an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and initiation of vascular contraction are increasingly being delineated. We have recently studied angiotensin II (Ang II) – mediated effects on sarcoplasmic reticulum (SR) mobilization of Ca^{2+} and the role of superoxide and cyclic adenosine diphosphoribose (cADPR) in these processes. Now we investigate the participation of transient receptor potential canonical channels (TRPC) and a Na^+/Ca^{2+} exchanger (NCX) in Ca^{2+} entry mechanisms. Afferent arterioles, isolated with the magnetized polystyrene bead method, were loaded with fura-2 to measure $[Ca^{2+}]_i$ ratiometrically. We observed that the Cl_{Ca2+} channel blocker niflumic acid (NFA) (10 and 50 μ M) affects neither the peak nor plateau $[Ca^{2+}]_i$ response to Ang II. Arterioles were pretreated with ryanodine (100 μM) and TMB-8 to block SR mobilization via the ryanodine receptor (RyR) and IP_3 receptor (IP_3R), respectively. The peak $[Ca^{2+}]_i$ response to Ang II was reduced by 40%. Addition of 2-APB to block TRPC – mediated Ca^{2+} entry inhibited the peak $[Ca^{2+}]_i$ Ang II response by 80% and the plateau by 74%. Flufenamic acid (FFA) (50 μM), which stimulates TRPC6, caused a sustained increase of $[Ca^{2+}]_i$ of 146 nM. This response was unaffected by diltiazem or nifedipine. KB-R7943 (at the low concentration of 10 μM) inhibits reverse (but not forward) mode NCX. KB-R7943 decreased the peak $[Ca^{2+}]_i$ response to Ang II by 48% and to FFA by 38%. We conclude that TRPC6 and reverse mode NCX may be important Ca^{2+} entry pathways in afferent arterioles.

Key words: renal microcirculation; voltage-gated calcium entry; vascular smooth muscle cell.

A rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) typically initiates vascular contraction. Over the past several years, we have studied Ca^{2+} signaling in freshly isolated afferent arterioles to gain a better understanding of mechanisms that control renal microvascular function and that may be important in the development of hypertension. In particular, we have examined events that lead to mobilization of Ca^{2+} from the sarcoplasmic reticulum (SR) via the inositol trisphosphate receptor (IP_3R) and ryanodine receptor (RyR) and have demonstrated the importance of superoxide generation and adenine diphosphoribose cyclase (ADPR cyclase) activation (13) (14) (12). As well, we have shown that Ca^{2+} entry via L-type voltage-gated Ca^{2+} channels (VGCC) stimulates Ca^{2+} - induced Ca^{2+} release (CICR) (15). Less well understood are the mechanisms involved in the activation of Ca^{2+} entry pathways following stimulation of afferent arterioles with angiotensin II (Ang II), endothelin (ET-1) or norepinephrine (NE).

It is well accepted that following Ang II stimulation of preglomerular microvessels, Ca^{2+} entry via L-type VGCC is a major pathway. In contrast, cortical efferent arterioles have little or no L-type expression (23) or activity (33). The mechanism by which Ang II causes depolarization sufficient to activate VGCC has remained a puzzle, largely because of the lack of specific pharmacologic probes. Some investigators believe that a rapid increase in $[Ca^{2+}]_i$ (likely more from mobilization of Ca^{2+} from the SR than from entry) stimulates a Ca^{2+} - activated chloride channel (Cl_{Ca2+}) with subsequent chloride efflux and membrane depolarization (19; 30). Functional support for this premise was provided by experiments in which removal of chloride from

the bath abolished the contractile response to Ang II in microperfused rabbit arterioles (28). In studies of renal blood flow, intra-renal infusion of the non-selective chloride channel blockers 4,4-diiosthioyanostilbene-2,2' disulfonic acid (DIDS), but not IAA-94 or niflumic acid (NFA), inhibited the vasoconstrictor response to Ang II (50). These same investigators measured $[Ca^{2+}]_i$ in afferent arterioles in the presence of NFA or IAA- 94 and found no inhibition of the $[Ca^{2+}]_i$ response to Ang II (50). Both DIDS and diphenylamine-2-carboxylic acid (DPC), another non-specific blocker of chloride channels, suppressed the peak and plateau $[Ca^{2+}]_i$ responses to Ang II in fresh preglomerular vascular smooth muscle cells (19). The lack of specificity of each of these Cl_{Ca2+} blockers limits interpretation of their effects.

Although opening of VGCC may be a major pathway for Ca^{2+} entry in response to Ang II, other Ca^{2+} entry mechanisms occur as well. In particular, non-selective cation channels (NSCC) of the transient receptor potential (TRP) families of ion channels, the TRP canonical (TRPC) channels (see reviews (52) (1; 45)) have been suggested to play a role in Ca^{2+} entry in vascular smooth muscle cells (VSMC). TRPC3, TRPC6 and TRPC7 proteins share ~ 75% identity, are activated by diacylglycerol (DAG) but not by protein kinase C (PKC). The homo- or heterotetramers are 6 membrane spanning units. TRPC6 has been associated with receptor operated Ca^{2+} entry (ROC) in a several VSMC types and in response to such vasoconstrictor agonists as vasopressin, angiotensin II and phenylephrine. In A7r5 cells stimulated with vasopressin, Ca^{2+} entry is markedly suppressed by siRNA directed against TRPC6 (49). In another study of A7r5 cells in which expression of TRPC3 was not found, heteromultimeric TRPC6-TRPC7 channels contribute to a vasopressin – induced cation current (37). Both vasopressin and

endothelin -1 as well as flufenamic acid (FFA) likewise stimulate cation currents in A7r5 cells (29). Ang II at low concentrations activates a cation conductance in mesenteric artery VSMC with TRPC6 channel properties (46). TRPC6 was found to be an essential component of a Ca^{2+} - permeable non-selective cation channel in cultured rabbit portal vein VSMC stimulated with phenylephrine (26). Pharmacologic studies demonstrate TRPC6 stimulation by FFA and inhibition by Gd^{3+} and SK&F96365 (26). Similar results were reported in mesenteric artery VSMC stimulated with phenylephrine and FFA (24).

Our laboratory has characterized the expression and abundance of transient receptor TRPC in cells derived from preglomerular vessels (11). Quantitative RT-PCR showed the presence of TRPC1, TRPC3 and TRPC6 mRNA (11). Protein levels of TRPC6 were ~7 fold greater in preglomerular VSMC than in endothelium denuded aortic VSMC (11). Stimulation of fura 2 – loaded renal interlobular arteries with norepinephrine (NE) in the presence of nifedipine (to block VGCC) caused an increase in $[Ca^{2+}]_i$ that could be distinguished from SOC . The pharmacologic profile of the $[Ca^{2+}]_i$ response was consistent with a ROC mechanism (10).

Given that TRPCs exist in renal resistance vessels (10) and that nonselective cation entry (NSCC) occurs through some of these channels, we explored the possibility of a relationship between cation entry via ROC and activation of VGCC. Because of the substantial Na^+ as well as Ca^{2+} entry following activation of TRPC6 (1.5-5 Ca^{2+}: 1 Na^+), we considered that depolarization might occur, sufficient to activate VGCC (8; 26; 49). We also asked the question if entry of Na^+ might activate Na^+/Ca^{2+} exchange in the reverse direction, that is, with Ca^{2+} entering rather than exiting the cell, as has been

suggested in cardiomyocytes and aortic VSMC (9) (32) . We used pharmacologic agents to stimulate or inhibit Ca^{2+} entry channels and to block sarcoplasmic (SR) receptors.

METHODS

All studies were approved by and performed in compliance with the guidelines and practices of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Preparation of fresh afferent arterioles. We used the magnetized polystyrene microsphere-sieving technique as previously described in our laboratory to isolate afferent arterioles (< 20 μm in diameter) from 5 week old (90 – 125 g) male Sprague Dawley rats maintained in the Chapel Hill Colony (16). Phosphate buffered saline (PBS), with the following composition in mM: 137 NaCl, 4.1 KCl, 0.66 KH_2PO_4, 3.4 Na_2HPO_4, 2.5 $NaHCO_3$, 1.0 $MgCl_2$, and 5 glucose, was adjusted daily to pH 7.4 at 4^0 and 23^0 C. The vessel segments in PBS containing 0.1% bovine serum albumin (BSA) were treated with collagenase Type IV (Worthington, 374 units/mg, 4-5 μg/ml) for 18 min at 34^0 C. Arterioles were loaded with fura-2- AM (3 μ M) and 0.1% BSA for 55 min at 23^0 C in the dark. After washing arterioles with PBS, the suspension was kept in Ca^{2+} (1.1 mM) – containing buffer on ice.

Measurement of cytosolic free calcium concentration. We measured $[Ca^{2+}]_i$ as previously described (13). Afferent arterioles were identified by their morphology and measured external diameter of 15 – 20 μm. As well, we required visualization of microspheres in the lumen of the afferent arteriole or in the proximal branch of an interlobular artery from which it arose, to exclude the possibility that the vessel was an

efferent arteriole. A random segment of an afferent arteriole was centered in a small window of the optical field that was free of glomeruli or tubular fragments.

The VSMC were excited alternately with light of 340 and 380 nm wavelength from a dual-excitation wavelength Delta-Scan equipped with dual monochronometers and a chopper (Photon Technology International, Birmingham, NJ) (PTI). After passing signals through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube**.** Signal intensity was acquired, stored and processed by an IBM-compatible Pentium computer and Felix software (PTI). Background subtraction was performed in all studies. There was no interruption in the recording during the addition of reagents to the chamber. A video camera projected images of afferent arterioles onto a video monitor permitting visualization of vascular contraction.

We have previously demonstrated that application of fura-2 and pharmacologic agents on the abluminal side of the afferent arteriole results in no detectable contribution to the $[Ca^{2+}]_i$ signal from endothelial cells (12).

Reagents. We purchased Ang II, nifedipine, diltiazem, flufenamic acid (FFA) niflumic acid (NFA), 8- (*N*, *N*- diethylamino) octyl 3,4,5 trimethoxybenzoate (TMB-8), and ryanodine from Sigma Aldrich (St. Louis, MO), KB-R7943 and 2-aminoethoxydiphenyl borane (2-APB) from CalBiochem (San Diego, CA), fura 2-AM from Molecular Probes (Eugene, OR) and magnetized microspheres from Spherotech (Libertyville, IL).

Concentrations of inhibitors were based upon previous data from the literature. Lower concentrations of NFA block the Cl_{Ca2+} channel whereas higher concentrations (= or >50 μM) also stimulate the K_{Ca2+} channel (31) (21) (42) (41). Ryanodine (100 μM) and TMB-8 (10 μM) block the RyR and IP_3R respectively (13). 2-APB (<100 μM) not

only blocks the TRPC, but is also an inhibitor of the IP_3R (34) (35). FFA stimulates TRPC6 and inhibits TRPC3 (29) (24) (2) (26) and at higher concentrations (>100 μM) stimulates the K_{Ca2+} channel (41) and inhibits L-type channels (48). Thus we chose a concentration of 50 μM to avoid the latter actions. KB-R7943 selectively inhibits reverse mode Na^+/Ca^{2+} exchange at a concentration of 10 μM; concentrations of 30 μM or greater inhibit forward mode (27).

Statistics. The data are presented as means ± SEM. Each data set was derived from afferent arterioles originating from at least 3 separate experiments, 2 rats (4 kidneys) per experiment. Individual arterioles were studied only once and then discarded. Paired data for arterioles before and after agonist stimulation were tested with Student's paired t-test. Unpaired t-tests were employed for comparisons of responses between two groups.

RESULTS

$[Ca^{2+}]_i$ response to Ang II. Afferent arterioles respond to Ang II (1 μM) with a sharp peak response followed by a sustained plateau (Fig. 1). Based upon the methods employed in this study, the measured mean increase in peak $[Ca^{2+}]_i$ is 135 ± 11 nM and that of plateau, 49 ± 6 nM (n = 30, P <0.01, peak and plateau vs. baseline, Fig. 2B).

Does niflumic acid alter the $[Ca^{2+}]_i$ *responses to Ang II in afferent arterioles*? NFA has been considered to be among the most potent blockers of Ca^{2+} - dependent chloride channels (Cl_{Ca2+}) and therefore has been widely used to assess their role in the control of vascular tone (22; 31). As noted above, NFA inhibits the channels at low concentrations (≤ 10 μM) and activates the channels at higher concentrations (≥ 50 μM) and following

prolonged (minutes) exposure to NFA (25) (31). We studied two concentrations of NFA, 10 and 50 μM.

NFA (10 μM) pretreatment causes a small, non-significant increase in $[Ca^{2+}]_i$ compared to baseline (14 ± 2 nM, n = 11, $P = 0.3$). In the presence of NFA, Ang II increases peak $[Ca^{2+}]_i$ by 103 ± 18 nM and plateau by 59 ± 5 nM ($P = 0.2$ and 0.4 respectively, vs. control). NFA (50 μM) likewise causes a small, non-significant increase in baseline $[Ca^{2+}]_i$ (14 ± 5 nM, n = 7). The increase in peak $[Ca^{2+}]_i$ after addition of Ang II is 122 ± 24 and plateau, 58 ± 7 nM ($P = 0.6$ and 0.4, respectively, vs. control, Fig. 3). These results confirm those of Steendahl et al. who showed a lack of effect of NFA (50 μM) on $[Ca^{2+}]_i$ responses to Ang II in isolated rat afferent arterioles (50).

$[Ca^{2+}]_i$ entry in the absence of mobilization. The combination of a high concentration of ryanodine to block the RyR and of TMB-8 to block the IP_3R prevents release of $[Ca^{2+}]_i$ from the SR (13). In the presence of these two agents, the $[Ca^{2+}]_i$ response to Ang II should represent Ca^{2+} that has entered the cell from the extracellular space. When we pretreated afferent arterioles with ryanodine (100 μM) and TMB-8 (10 μM), baseline $[Ca^{2+}]_i$ is unchanged. The peak $[Ca^{2+}]_i$ response to Ang II is reduced to 83 ± 12 nM (39% inhibition) (n = 11, $P < 0.02$ vs. control) whereas the plateau $[Ca^{2+}]_i$ is not different from control (59 ± 5 nM, $P = 0.6$, Fig. 3).

Pretreatment of afferent arterioles with both ryanodine (100 μM) and 2-APB (50 μM) will additionally diminish $[Ca^{2+}]_i$ entry through TRPC channels, thereby preventing Ca^{2+} entry via a ROC mechanism. Under these conditions, we find that the peak $[Ca^{2+}]_i$ response to Ang II is further reduced to 27 ± 4 nM (80% inhibition) (N = 10, $P < 0.01$

vs. ryanodine plus TMB-8, and the plateau to 13 ± 1 (P = 0.01, Fig. 3). To further document that ryanodine and 2-APB effectively prevent mobilization of Ca^{2+} from the SR, we prepared afferent arterioles in nominally Ca^{2+} - free buffer. In the absence of external Ca^{2+}, the peak $[Ca^{2+}]_i$ response to Ang II was 9 ± 2 nM and the plateau, 4 ± 3 nM (n = 5, P > 0.7 and 0.8, respectively, vs. baseline). These results suggest that opening of TRPC channel contribute to a substantial fraction of $[Ca^{2+}]_i$ entry in afferent arterioles stimulated with Ang II.

Flufenamic acid (FFA) is a putative stimulator of TRPC6. Based upon the results with ryanodine and 2-APB suggesting that there is a Ca^{2+} entry mechanism that is independent of mobilization but inhibited by 2-APB, we studied the drug FFA, which has been shown to stimulate TRPC6 and to inhibit TRPC3 in VSMC(29) (26) (2; 24) . Stimulation of afferent arterioles with FFA (50 μM) causes a prompt and sustained rise in $[Ca^{2+}]_i$ of 145 ± 18 nM (n = 12, P < 0.01). Addition of Ang II in the continued presence of FFA promotes a further increase in $[Ca^{2+}]_i$ (peak, 54 ±14 nM, P < 0.03 vs. FFA; plateau, 43 ± 13 nM, Fig. 4), demonstrating that Ang II subsequently causes Ca^{2+} entry by TRPC6 - independent mechanisms such as activation of the NADPH oxidase, superoxide, cADPR cyclase pathway (13) (14). To further support the premise that FFA activates TRPC6 in afferent arterioles, we pretreated the vascular segments with 2-APB (50 μM), to block TRPC channel activity. The $[Ca^{2+}]_i$ response to FFA is reduced by 74% and contrasts with the 33% inhibition caused by KB-R 7943 (*vide infra*) (n = 4, P < 0.01 vs. control).

Activation of TRPC6 channels in A7r5 VSMC with oleoyl-2-acetyl-*sn*-glycerol (OAG) not only causes Ca^{2+} entry via the NSCC itself, but also causes sufficient

depolarization to open VGCC that are inhibited by voltage-gated Ca^{2+} channel blockers (CCB) in A7r5 cells (49). In contrast to studies that show a response to OAG in fresh or cultured VSMC, we have been unable to get reproducible responses to this agent in fresh, intact afferent arterioles. Furthermore, because FFA inhibits TRPC3, we could narrow our focus to TRPC6. We do not know the extent to which OAG may inhibit TRPC6 by causing an increase in PKC. Thus, we studied the $[Ca^{2+}]_i$ response of afferent arterioles to FFA in the presence and absence of the calcium channel blocker (CCB) nifedipine (n = 6) or diltiazem (n = 5) (10 μM both). The findings for the two inhibitors are not different from each other and therefore the data are pooled. In the presence of CCB, the $[Ca^{2+}]_i$ response to FFA is 102 ± 24 nM (*P*, NS vs. control). Hence, we could not demonstrate a linkage between presumed TRPC6 activation with FFA and opening of L-type channels contributing to the $[Ca^{2+}]_i$ response under these experimental conditions. However, the fact that FFA may block Cl_{Ca2+} channels may confound our results.

The subsequent peak $[Ca^{2+}]_i$ response to Ang II in the continued presence of FFA and CCB is 52 ± 6 nM, the same as in the absence of CCB. As one would anticipate, the plateau $[Ca^{2+}]_i$ level (largely representing Ca^{2+} entry) is reduced (Δ 21 ± 5 nM, *P* vs. control < 0.01, Fig. 4).

The Na^+ entry that accompanies Ca^{2+} entry following activation of TRPC3 or TRPC6 (nonselective cation entry) has been shown to activate reverse direction Na^+/Ca^{2+} exchange (NCX) in cardiomyocytes and aortic VSMC (9) (32). To investigate if activation of TRPC6 with FFA in afferent arterioles is associated with operation of reverse mode NCX, we pretreated the vessels with KB-R7943 (10 μM). In the presence of the inhibitor, the $[Ca^{2+}]_i$ response to FFA was 91 ± 12 nM (37% inhibition) (n = 9, *P*

<0.05 vs. control, Fig. 5). In a similar fashion, we studied the $[Ca^{2+}]_i$ response to Ang II in the presence of KB-R7943. The peak $[Ca^{2+}]_i$ response was reduced to 71 ± 12 nM and the plateau to 23 ± 7 (n = 9, peak $P = 0.01$, plateau $P < 0.05$ vs. control, Fig. 5). These results suggest that entry of Na^+ via TRPC6 activates reverse mode NCX following either FFA or Ang II.

DISCUSSION

Non-specificity of blockers of Cl_{Ca2+}. Interpretation of results obtained using pharmacologic agents to probe mechanisms of Ca^{2+} signaling in biologic tissues is often limited by lack of specificity of the agent. A major case in point is the attempt to block the Cl_{Ca2+} channel. It has traditionally been proposed that an increase in $[Ca^{2+}]_i$ activates this channel, causing membrane depolarization of sufficient magnitude to open VGCC. Because a major fraction of Ca^{2+} entry in afferent arterioles is believed to be the result of activation of L- type VGCC, documentation of the role of the role of Cl_{Ca2+} channels is an important issue. Experiments in chloride – free buffers tend to substantiate the relevance of this concept, but changes in intracellular pH (from lack of Cl^- / HCO_3^- exchange) may cloud the interpretation of the results (28).

Not one of the pharmacologic agents utilized to block Cl_{Ca2+} channels in VSMC has absolute specificity. The fenamates (NFA, FFA) variably stimulate K_{Ca2+} channels, stimulate TRPC6, and inhibit TRPC3 in a dose – dependent fashion (21; 43). DPC has been reported to block L –type VGCC, fast Na^+ channels, and NSCC in a variety of cells (39) (6). DIDS, which is membrane impermeant, can inhibit Ca^{2+} - Mg^{2+} - ATPase, inhibit Cl^- / HCO_3^- exchange and stimulate chloride conductance by elevating $[Ca^{2+}]_i$ (5). Because NFA, reportedly the most potent among the Cl_{Ca2+} blockers (21; 25), appears to

have minimal effects on K_{Ca2+} channels at a concentration of 10 μM, we studied the effect of both 10 and 50 μM on the $[Ca^{2+}]_i$ response to Ang II in afferent arterioles. At neither concentration was there a change in the peak or plateau $[Ca^{2+}]_i$ responses. These results agree with those of others obtained in afferent arterioles (50). In contrast, in single VSMC derived from preglomerular vessels, high concentrations of DPC (100 and 500 μM) are reported to inhibit both the peak and plateau responses to Ang II (19).

Further complicating the interpretation of experiments designed to test the magnitude of Ca^{2+} entry via VGCC is the fact that examples of all 3 classes of CCB (nifedipine, diltiazem and verapamil) inhibit the ability of nicotinic acid dinucleotide phosphate (NAADP) to affect lysosomal $[Ca^{2+}]_i$ release (20; 36; 56). Preliminary work in our laboratory suggests that Ang II as well as endothelin-1 stimulates the formation of NAADP to influence Ca^{2+} signaling in afferent arterioles.

TRPC channels in VSM. The recent explosion of interest in TRPC channels as mediators of Ca^{2+} entry in VSMC led our laboratory to study the distribution and expression of TRPC in VSMC of preglomerular vessels (11). As well, the role of TRPC in norepinephrine – induced ROC was studied in interlobular arteries (10). As noted above, TRPC3, 6 and 7 can form mon- or heterotetramers, are stimulated by diacylglycerol (DAG) and are considered to be NSCC (8). Thus agonist stimulation of G protein coupled receptors in VSMC that result in formation of phospholipase C (PLC) can initiate formation not only of IP_3 – mediated Ca^{2+} signaling events, but also DAG activation of this group of TRPC. TRPC6 and often TRPC3 have been shown to be present in some large arteries (coronary, aorta, main renal artery) (11; 24; 53; 55) and in some A7r5 cell lines (38). TRPC3 may be the predominant subtype in endothelial cells

(44) (55), whereas small arteries and arterioles appear to contain largely TPRC6 (54). Preglomerular VSMC have 6-8 fold more TRPC 6 protein than aortic VSMC (11). One cannot exclude the possibility that fresh cellular preparations of small vessels contain endothelial cells that contribute to the presence of TRPC3 (11).

Study of TRPC channel function in fresh, intact arterioles presents unique challenges that are not present in work done in cultured VSMC or in fresh single cells. Previous pharmacologic maneuvers have proven to provide reliable data regarding Ca^{2+} signaling events in our fresh preparation of the rat. The extensive literature supporting the effect of FFA to stimulate TRPC6 and to inhibit TRPC3, as well as the effect of low concentrations of KB-R7943 to block reverse mode NCX-1, substantiate use of these agents in studies of afferent arteriolar VSMC. Nonetheless, we appreciate the limitations that use of pharmacologic agents may impose. One approach would be the use gene targeting in mice to examine more specifically one TRPC sub-type compared to another. Such experiments were done in $TRPC6^{-/-}$ mice (7). Surprisingly, the $TRPC6^{-/-}$ mice are hypertensive and contractility in response to phenylephrine is increased in aortic and mesenteric artery rings. Analysis of mRNA with PCR shows more than doubling of TRPC3 in aortic and cerebral arteries. The authors conclude that in these larger arteries, TRPC3 and TRPC6 are functionally nonredundant. They postulate that TRPC6 may suppress a high basal activity of TRPC3, important for the tight regulation of the NSCC complex in the regulation of vascular tone (7). These important issues need to be addressed in future studies of resistance arterioles.

Another approach is the use of blocking or inhibitory antibodies. In studies of rabbit mesenteric VSMC, anti-TRPC6 antibodies raised against putative intracellular

epitopes, reverse the I_{cat1} (store depletion – independent cation current) activity of Ang II (1 nM) when applied to the cytoplasmic surface of inside-out patches (46). In this same study, FFA (100 μM), which activates TRPC6, stimulates an I_{cat1} that potentiates the current (46). To our knowledge, use of inhibitory antibodies has not been employed in fresh preparations of renal resistance vessels.

Ca^{2+} entry in afferent arterioles. To address the issue of Ca^{2+} entry pathways in afferent arterioles independent of mobilization events, we blocked the RyR and IP_3R with high concentrations of ryanodine and with TMB-8. The peak $[Ca^{2+}]_i$ response to Ang II is diminished by about 40% but the plateau is unchanged. We then sought to assess the contribution of TRPC in Ca^{2+} entry via NSCC. Although the cell membrane – permeant analogue of DAG, OAG, has been used to stimulate TRPC3 and 6 in a variety of isolated cell types, we were unable to obtain responses to OAG of sufficient magnitude or reproducibility in our preparation of afferent arterioles. As noted above, FFA has been reported to stimulate TRPC6 and to inhibit TRPC3 in aortic and mesenteric VSMC and in HEK cells (29) (26) (24) (46) (2). Thus, FFA (at concentrations of ~100 μM) is a useful tool to evaluate TRPC6 function in afferent arterioles.

We find that FFA causes a substantial (145 nM) increase in $[Ca^{2+}]_i$ and that addition of Ang II in the continued presence of FFA causes a second peak, presumably via a Ca^{2+} entry mechanism different from TRPC3 or 6. These other entry pathways could include SOC, activation of VGCC or of NCX operating in the reverse mode. Because some investigators have suggested that activation of TRPC3 or TRPC6 and thus entry of both Na^+ and Ca^{2+} might result in membrane depolarization (9) (32), we examined the $[Ca^{2+}]_i$ response to FFA in the presence of the CCB nifedipine or diltiazem

at concentrations known to block Ca^{2+} entry via L-type VGCC. The results with FFA plus CCB are not different from FFA control, but, as one would expect, the plateau phase of the subsequent Ang II response is diminished by CCB. Therefore, in our preparation of afferent arterioles utilizing pharmacologic probes, we did not find functional evidence that FFA causes depolarization sufficient to activate VGCC. These results are in agreement with findings in pig interlobular VSMC (51). A caveat is that if FFA simultaneously stimulates K_{Ca}^{2+} channels, the hyperpolarizing effect would act in the opposite direction (41). NFA and FFA have nearly identical stimulatory effects on K_{Ca}^{2+} in coronary VSMC (mean fractional increase in open probability of about 0.3 at a concentration of 100 μM). Neither tetraethylammonium (TEA) nor charybdotoxin, classic inhibitors of the channel, interfered with the change in open probability produced by NFA (41).

Na^+/Ca^{2+} exchange (NCX) in the renal microcirculation. NCX transports Ca^{2+} across the plasma membrane based on the transmembrane electrochemical gradient of Na^+ and Ca^{2+} (4). The exchangers may operate in the so-called forward mode (3 Na^+ entry and 1 Ca^{2+} exit) or the reverse mode (3 Na^+ exit and 1 Ca^{2+} entry). Because of this 3 Na^+: 1Ca^{2+} relationship, $[Ca^{2+}]_i$ is largely related to changes in cytosolic Na^+ concentration ($[Na^+]_i$) (4) (57) (58). The equation to describe this correlation is: $[Ca^{2+}]_i = [Ca^{2+}]_o \times ([Na^+]_i \div ([Na^+]_o)^3 \times e^{(Em FIRT)}$ (E_m is the membrane potential, F is the Faraday constant, T is absolute temperature and R is the gas constant) (57). Thus, when extracellular Na^+ and Ca^{2+} are constant, $[Ca^{2+}]_i$ entry via reverse mode NCX is proportional to the third power of ($[Na^+]_i$) (57) (4) (58).

Evidence for the presence of a NCX has been demonstrated in afferent and efferent arterioles of rabbit and rat (18; 40). Traditionally, the NCX has been thought to operate in the forward direction to facilitate Ca^{2+} exit from the VSMC following increases in $[Ca^{2+}]_i$. Studies performed in low extracellular Na^+ medium, while showing the presence of the exchanger, are examining the reverse mode of transport. When extracellular Na^+ is reduced, or when intracellular Na^+ is increased, Ca^{2+} enters the cell (reviewed in (3)). In rabbit afferent and efferent arterioles attached to a glomerulus, exposure to a nominally Na^+ - free bath caused an increase in $[Ca^{2+}]_i$ that was nearly twice as large in the afferent compared to the efferent arteriole. The change in $[Ca^{2+}]_i$ was not blocked by the CCB diltiazem, but was inhibited by the nonspecific inhibitor of NCX, Ni^{2+} (18). The functional role of NCX has been assessed in the isolated perfused rat kidney. Reduction of perfusate $[Na^+]$ in a graded manner caused increases in renal vascular resistance (RVR) presumably via reverse mode NCX (47). KB-R7943 inhibits NCX reverse mode at concentrations of 10 μM or less; higher concentrations (>30 μM) inhibit the forward mode as well (27). KB-R7943 (50 μM) caused an increase in RVR in the isolated rat kidney (47). It is likely that at this concentration, KB-R7943 is blocking Ca^{2+} exit when NCX is operating in forward mode.

In the current study, we employed KB-R7943 at a concentration (10 μM), reported to block only reverse mode NCX. The peak $[Ca^{2+}]_i$ response to Ang II is reduced by 50 % and that to FFA by 37 %. These data suggest that reverse mode NCX plays a part in the $[Ca^{2+}]_i$ response to Ang II. Furthermore, the data suggest that Ang II – induced activation of TRPC6 (or FFA stimulation of TRPC6) causes sufficient Na^+ entry to accomplish reverse mode NCX characteristics. Similar conclusions have been made in

a study of aortic VSMC in which KB-R7943 inhibited the $[Ca^{2+}]_i$ response to ATP (32) and in cardiomyocytes stimulated with Ang II (9). If KB-R7943 had been blocking NCX in the forward rather than the reverse mode in our preparation of afferent arterioles, one would expect an increase rather than a decrease in $[Ca^{2+}]_i$ following stimulation with Ang II.

In summary, we present new data showing the complex relationships among Ca^{2+} entry pathways in afferent arterioles. Investigation of any of these Ca^{2+} pathways must be viewed as a snapshot of a much larger picture in which intricate interactions, often occurring within milliseconds, work in concert to achieve a change in $[Ca^{2+}]_i$ that precedes vascular contraction. The pharmacologic agents used to explore the mechanisms by which Ang II stimulation of afferent arterioles causes depolarization and opening of VGGC may also affect TRPC and K_{Ca2+}. Thus, it has been difficult to unravel the way in which L- and T-type channel opening occurs, utilizing pharmacologic maneuvers. We present evidence that stimulation of TRPC6 with Ang II or with FFA causes Ca^{2+} entry that is in part the consequence of NCX operating in the reverse mode. Because TRPC channels have been implicated in the pathophysiology of hypertension (17), our findings in afferent arteriolar VSMC should open new avenues of exploration in the causes of genetic hypertension and potential involvement of the renal microcirculation.

GRANTS

This work was supported in part by an award from the Thomas H. Maren Foundation and from National Heart, Lung and Blood Institute Grant HL-02334.

FIGURE LEGENDS

Fig. 1. Representative tracing of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) response of an isolated afferent arteriole to angiotensin – II (Ang II).

Fig. 2. $[Ca^{2+}]_i$ responses of afferent arterioles to Ang II in controls and in the presence of niflumic acid (NFA). *A*: representative tracing showing the lack of inhibition by NFA (10 μM). *B*: Summary data showing that there is a non-significant increase in baseline ($[Ca^{2+}]_i$ (gray bars) and that neither 10 μM or 50 μM NFA affects the peak (black bars) or plateau (white bars) $[Ca^{2+}]_i$ responses.

Fig. 3. $[Ca^{2+}]_i$ responses of afferent arterioles to Ang II in the presence of ryanodine and TMB-8 to prevent $[Ca^{2+}]_i$ mobilization via the ryanodine receptor (RyR) and the IP_3 receptor (IP_3R) and in the presence of ryanodine and 2-APB to block the RyR, IP_3R and non-selective cation entry of TRPC. *A*: representative tracing of the inhibitory effect of ryanodine and TMB-8 on the Ang II – mediated $[Ca^{2+}]_i$ response.. *B*: typical tracing of the additional inhibitory effect of 2-APB on the Ang II response. *C*: summary figure of the inhibitory effect on $[Ca^{2+}]_i$ peak (black bars) and plateau (white bars) $[Ca^{2+}]_i$ values (* $P < 0.01$ vs. control, # $P < 0.01$ vs. ryanodine plus TMB-8).

Fig. 4. Afferent arteriolar $[Ca^{2+}]_i$ responses to flufenamic acid (FFA) followed by Ang II in the continued presence of FFA. *A*: representative tracing showing that Ang II causes a second $[Ca^{2+}]_i$ peak and plateau response following that of FFA. *B*: effects of the voltage-gated L-type Ca^{2+} channel (VGCC) blockers nifedipine and diltiazem. The VGCC blockers have no effect on the FFA (gray bars) or subsequent Ang II peak (black bars) $[Ca^{2+}]_i$ responses but do inhibit the Ang II plateau (white bars) ($P < 0.01$).

Fig. 5. KB-R7943 (10 μM), a blocker of reverse mode Na^+/Ca^{2+} exchange at this concentration, inhibits the peak (black bars) and plateau (white bars) $[Ca^{2+}]_i$ responses to Ang II (** $P< 0.01$, # $P <0.04$, respectively) and $[Ca^{2+}]_i$ responses to FFA (gray bars, * $P<0.05$).

Reference List

1. **Albert AP and Large WA**. Signal transduction pathways and gating mechanisms of native TRP-like cation channels in vascular myocytes. *J Physiol* 570: 45-51, 2006.

2. **Albert AP, Pucovsky V, Prestwich SA and Large WA**. TRPC3 properties of a native constitutively active Ca2+-permeable cation channel in rabbit ear artery myocytes. *J Physiol* 571: 361-369, 2006.

3. **Bell PD, Mashburn N and Unlap MT**. Renal sodium/calcium exchange; a vasodilator that is defective in salt-sensitive hypertension. *Acta Physiol Scand* 168: 209-214, 2000.

4. **Blaustein MP and Lederer WJ**. Sodium/calcium exchange: its physiological implications. *Physiol Rev* 79: 763-854, 1999.

5. **Brayden DJ, Krouse ME, Law T and Wine JJ**. Stilbenes stimulate T84 Cl- secretion by elevating Ca2+. *Am J Physiol* 264: G325-G333, 1993.

6. **Conforti L, Sumii K and Sperelakis N**. Diphenylamine-2-carboxylate blocks voltage-dependent Na+ and Ca2+ channels in rat ventricular cardiomyocytes. *Eur J Pharmacol* 259: 215-218, 1994.

7. **Dietrich A, Mederos YS, Gollasch M, Gross V, Storch U, Dubrovska G, Obst M, Yildirim E, Salanova B, Kalwa H, Essin K, Pinkenburg O, Luft FC, Gudermann T and Birnbaumer L**. Increased vascular smooth muscle contractility in TRPC6-/- mice. *Mol Cell Biol* 25: 6980-6989, 2005.

8. **Dietrich A, Schnitzler M, Kalwa H, Storch U and Gudermann T**. Functional characterization and physiological relevance of the TRPC3/6/7 subfamily of cation channels. *Naunyn Schmiedebergs Arch Pharmacol* 371: 257-265, 2005.

9. **Eder P, Probst D, Rosker C, Poteser M, Wolinski H, Kohlwein SD, Romanin C and Groschner K**. Phospholipase C-dependent control of cardiac calcium homeostasis involves a TRPC3-NCX1 signaling complex. *Cardiovasc Res* 73: 111-119, 2007.

10. **Facemire CS and Arendshorst WJ**. Calmodulin mediates norepinephrine-induced receptor-operated calcium entry in preglomerular resistance arteries. *Am J Physiol Renal Physiol* 289: F127-F136, 2005.

11. **Facemire CS, Mohler PJ and Arendshorst WJ**. Expression and relative abundance of short transient receptor potential channels in the rat renal microcirculation. *Am J Physiol Renal Physiol* 286: F546-F551, 2004.

12. **Fellner SK and Arendshorst W**. Endothelin-A and -B receptors, superoxide, and Ca2+ signaling in afferent arterioles. *Am J Physiol Renal Physiol* 292: F175-F184, 2007.

13. **Fellner SK and Arendshorst WJ**. Angiotensin II Ca2+ signaling in rat afferent arterioles: Stimulation of cyclic ADP ribose and IP3 pathways. *Am J Physiol Renal Physiol* 288: F785-F791, 2004.

14. **Fellner SK and Arendshorst WJ**. Angiotensin II, reactive oxygen species and Ca2+ signaling in afferent arterioles. *Am J Physiol Renal Physiol* 2005.

15. **Fellner SK and Arendshorst WJ**. Voltage-gated Ca2+ entry and ryanodine receptor Ca2+-induced Ca2+ release in preglomerular arterioles. *Am J Physiol Renal Physiol* 292: F1568-F1572, 2007.

16. **Fellner SK and Parker L**. Endothelin-1, superoxide and adeninediphosphate ribose cyclase in shark vascular smooth muscle. *J Exp Biol* 208: 1045-1052, 2005.

17. **Firth AL, Remillard CV and Yuan JX**. TRP channels in hypertension. *Biochim Biophys Acta* 2007.

18. **Fowler BC, Carmines PK, Nelson LD and Bell PD**. Characterization of sodium-calcium exchange in rabbit renal arterioles. *Kidney Int* 50: 1856-1862, 1996.

19. **Fuller AJ, Hauschild BC, Gonzalez-Villalobos R, Awayda MS, Imig JD, Inscho EW and Navar LG**. Calcium and chloride channel activation by angiotensin II-AT1 receptors in preglomerular vascular smooth muscle cells. *Am J Physiol Renal Physiol* 289: F760-F767, 2005.

20. **Genazzani AA, Empson RM and Galione A**. Unique inactivation properties of NAADP-sensitive Ca2+ release. *J Biol Chem* 271: 11599-11602, 1996.

21. **Greenwood IA and Large WA**. Comparison of the effects of fenamates on Ca-activated chloride and potassium currents in rabbit portal vein smooth muscle cells. *Br J Pharmacol* 116: 2939-2948, 1995.

22. **Greenwood IA and Leblanc N**. Overlapping pharmacology of Ca2+-activated Cl- and K+ channels. *Trends Pharmacol Sci* 28: 1-5, 2007.

23. **Hansen PB, Jensen BL, Andreasen D and Skott O**. Differential expression of T- and L-type voltage-dependent calcium channels in renal resistance vessels. *Circ Res* 89: 630-638, 2001.

24. **Hill AJ, Hinton JM, Cheng H, Gao Z, Bates DO, Hancox JC, Langton PD and James AF**. A TRPC-like non-selective cation current activated by alpha 1-adrenoceptors in rat mesenteric artery smooth muscle cells. *Cell Calcium* 40: 29-40, 2006.

25. **Hogg RC, Wang Q and Large WA**. Action of niflumic acid on evoked and spontaneous calcium-activated chloride and potassium currents in smooth muscle cells from rabbit portal vein. *Br J Pharmacol* 112: 977-984, 1994.

26. **Inoue R, Okada T, Onoue H, Hara Y, Shimizu S, Naitoh S, Ito Y and Mori Y**. The transient receptor potential protein homologue TRP6 is the essential component of vascular alpha(1)-adrenoceptor-activated Ca(2+)-permeable cation channel. *Circ Res* 88: 325-332, 2001.

27. **Iwamoto T, Watano T and Shigekawa M**. A novel isothiourea derivative selectively inhibits the reverse mode of Na+/Ca2+ exchange in cells expressing NCX1. *J Biol Chem* 271: 22391-22397, 1996.

28. **Jensen BL, Ellekvist P and Skott O**. Chloride is essential for contraction of afferent arterioles after agonists and potassium. *Am J Physiol* 272: F389-F396, 1997.

29. **Jung S, Strotmann R, Schultz G and Plant TD**. TRPC6 is a candidate channel involved in receptor-stimulated cation currents in A7r5 smooth muscle cells. *Am J Physiol Cell Physiol* 282: C347-C359, 2002.

30. **Large WA and Wang Q**. Characteristics and physiological role of the Ca(2+)-activated Cl- conductance in smooth muscle. *Am J Physiol* 271: C435-C454, 1996.

31. **Ledoux J, Greenwood IA and Leblanc N**. Dynamics of Ca2+-dependent Cl- channel modulation by niflumic acid in rabbit coronary arterial myocytes. *Mol Pharmacol* 67: 163-173, 2005.

32. **Lemos VS, Poburko D, Liao CH, Cole WC and van Breemen C.** Na+ entry via TRPC6 causes Ca2+ entry via NCX reversal in ATP stimulated smooth muscle cells. *Biochem Biophys Res Commun* 352: 130-134, 2007.

33. **Loutzenhiser K and Loutzenhiser R**. Angiotensin II-induced Ca(2+) influx in renal afferent and efferent arterioles: differing roles of voltage-gated and store-operated Ca(2+) entry. *Circ Res* 87: 551-557, 2000.

34. **Luo D, Broad LM, Bird GS and Putney JW, Jr.** Signaling pathways underlying muscarinic receptor-induced [Ca2+]i oscillations in HEK293 cells. *J Biol Chem* 276: 5613-5621, 2001.

35. **Ma HT, Venkatachalam K, Li HS, Montell C, Kurosaki T, Patterson RL and Gill DL**. Assessment of the role of the inositol 1,4,5-trisphosphate receptor in the activation of transient receptor potential channels and store-operated Ca2+ entry channels. *J Biol Chem* 276: 18888-18896, 2001.

36. **Mandi M, Toth B, Timar G and Bak J**. Ca2+ release triggered by NAADP in hepatocyte microsomes. *Biochem J* 395: 233-238, 2006.

37. **Maruyama Y, Nakanishi Y, Walsh EJ, Wilson DP, Welsh DG and Cole WC**. Heteromultimeric TRPC6-TRPC7 channels contribute to arginine vasopressin-induced cation current of A7r5 vascular smooth muscle cells. *Circ Res* 98: 1520-1527, 2006.

38. **Moneer Z, Pino I, Taylor EJ, Broad LM, Liu Y, Tovey SC, Staali L and Taylor CW**. Different phospholipase-C-coupled receptors differentially regulate capacitative and non-capacitative Ca2+ entry in A7r5 cells. *Biochem J* 389: 821-829, 2005.

39. **Nakahara T, Mitani A, Saito M, Sakamoto K and Ishii K**. Diphenylamine-2-carboxylic acid potentiates the cyclic nucleotides-mediated relaxation of porcine coronary artery: possible involvement of the inhibitory effect on the efflux of cyclic nucleotides. *Vascul Pharmacol* 41: 21-25, 2004.

40. **Nelson LD, Mashburn NA and Bell PD**. Altered sodium-calcium exchange in afferent arterioles of the spontaneously hypertensive rat. *Kidney Int* 50: 1889-1896, 1996.

41. **Ottolia M and Toro L**. Potentiation of large conductance KCa channels by niflumic, flufenamic, and mefenamic acids. *Biophys J* 67: 2272-2279, 1994.

42. **Pallone TL, Cao C and Zhang Z**. Inhibition of K+ conductance in descending vasa recta pericytes by ANG II. *Am J Physiol Renal Physiol* 287: F1213-F1222, 2004.

43. **Piper AS, Greenwood IA and Large WA**. Dual effect of blocking agents on Ca2+-activated Cl(-) currents in rabbit pulmonary artery smooth muscle cells. *J Physiol* 539: 119-131, 2002.

44. **Poteser M, Graziani A, Rosker C, Eder P, Derler I, Kahr H, Zhu MX, Romanin C and Groschner K**. TRPC3 and TRPC4 associate to form a redox-sensitive cation channel. Evidence for expression of native TRPC3-TRPC4 heteromeric channels in endothelial cells. *J Biol Chem* 281: 13588-13595, 2006.

45. **Ramsey IS, Delling M and Clapham DE**. An introduction to TRP channels. *Annu Rev Physiol* 68: 619-647, 2006.

46. **Saleh SN, Albert AP, Peppiatt CM and Large WA**. Angiotensin II activates two cation conductances with distinct TRPC1 and TRPC6 channel properties in rabbit mesenteric artery myocytes. *J Physiol* 577: 479-495, 2006.

47. **Schweda F, Seebauer H, Kramer BK and Kurtz A**. Functional role of sodium-calcium exchange in the regulation of renal vascular resistance. *Am J Physiol Renal Physiol* 280: F155-F161, 2001.

48. **Shimamura K, Zhou M, Ito Y, Kimura S, Zou LB, Sekiguchi F, Kitramura K and Sunano S**. Effects of flufenamic acid on smooth muscle of the carotid artery isolated from spontaneously hypertensive rats. *J Smooth Muscle Res* 38: 39-50, 2002.

49. **Soboloff J, Spassova M, Xu W, He LP, Cuesta N and Gill DL**. Role of endogenous TRPC6 channels in Ca2+ signal generation in A7r5 smooth muscle cells. *J Biol Chem* 280: 39786-39794, 2005.

50. **Steendahl J, Holstein-Rathlou NH, Sorensen CM and Salomonsson M**. Effects of chloride channel blockers on rat renal vascular responses to angiotensin II and norepinephrine. *Am J Physiol Renal Physiol* 286: F323-F330, 2004.

51. **Utz J, Eckert R and Trautwein W**. Changes of intracellular calcium concentrations by phenylephrine in renal arterial smooth muscle cells. *Pflugers Arch* 438: 725-731, 1999.

52. **Vazquez G, Wedel BJ, Aziz O, Trebak M and Putney JW, Jr.** The mammalian TRPC cation channels. *Biochim Biophys Acta* 1742: 21-36, 2004.

53. **Walker RL, Hume JR and Horowitz B**. Differential expression and alternative splicing of TRP channel genes in smooth muscles. *Am J Physiol Cell Physiol* 280: C1184-C1192, 2001.

54. **Wang J, Shimoda LA and Sylvester JT**. Capacitative calcium entry and TRPC channel proteins are expressed in rat distal pulmonary arterial smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 286: L848-L858, 2004.

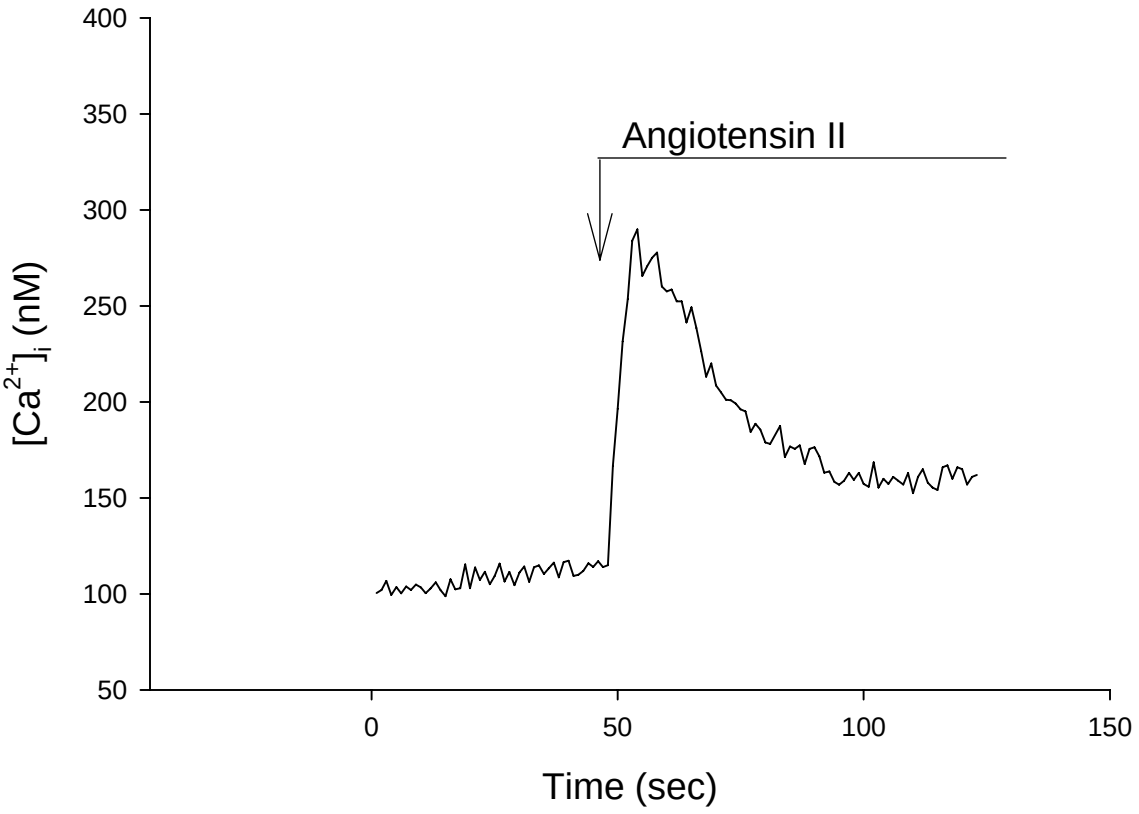
55. **Yip H, Chan WY, Leung PC, Kwan HY, Liu C, Huang Y, Michel V, Yew DT and Yao X**. Expression of TRPC homologs in endothelial cells and smooth muscle layers of human arteries. *Histochem Cell Biol* 122: 553-561, 2004.

56. **Yusufi AN, Cheng J, Thompson MA, Burnett JC and Grande JP**. Differential mechanisms of Ca(2+) release from vascular smooth muscle cell microsomes. *Exp Biol Med (Maywood)* 227: 36-44, 2002.

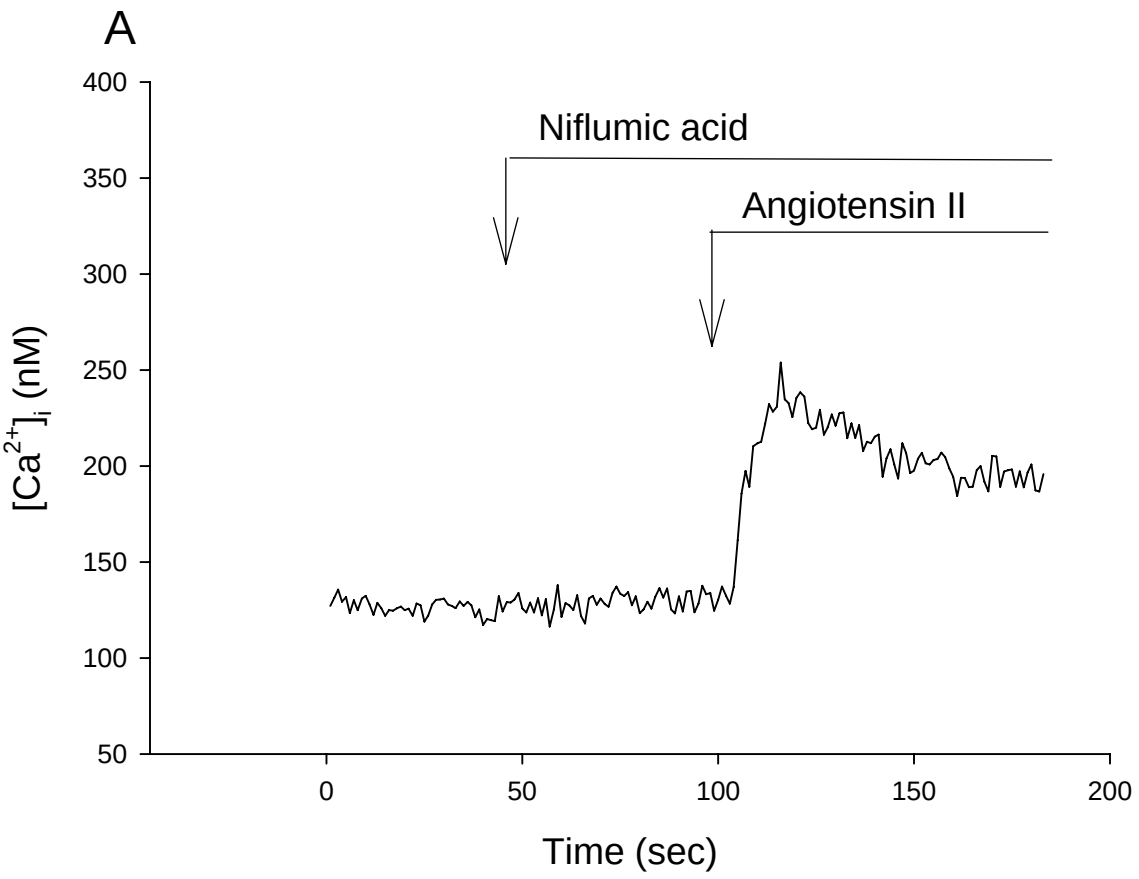
57. **Zhang S, Dong H, Rubin LJ and Yuan JX**. Upregulation of Na+/Ca2+ exchanger contributes to the enhanced Ca2+ entry in pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension. *Am J Physiol Cell Physiol* 292: C2297-C2305, 2007.

58. **Zhang S, Yuan JX, Barrett KE and Dong H**. Role of Na+/Ca2+ exchange in regulating cytosolic Ca2+ in cultured human pulmonary artery smooth muscle cells. *Am J Physiol Cell Physiol* 288: C245-C252, 2005.

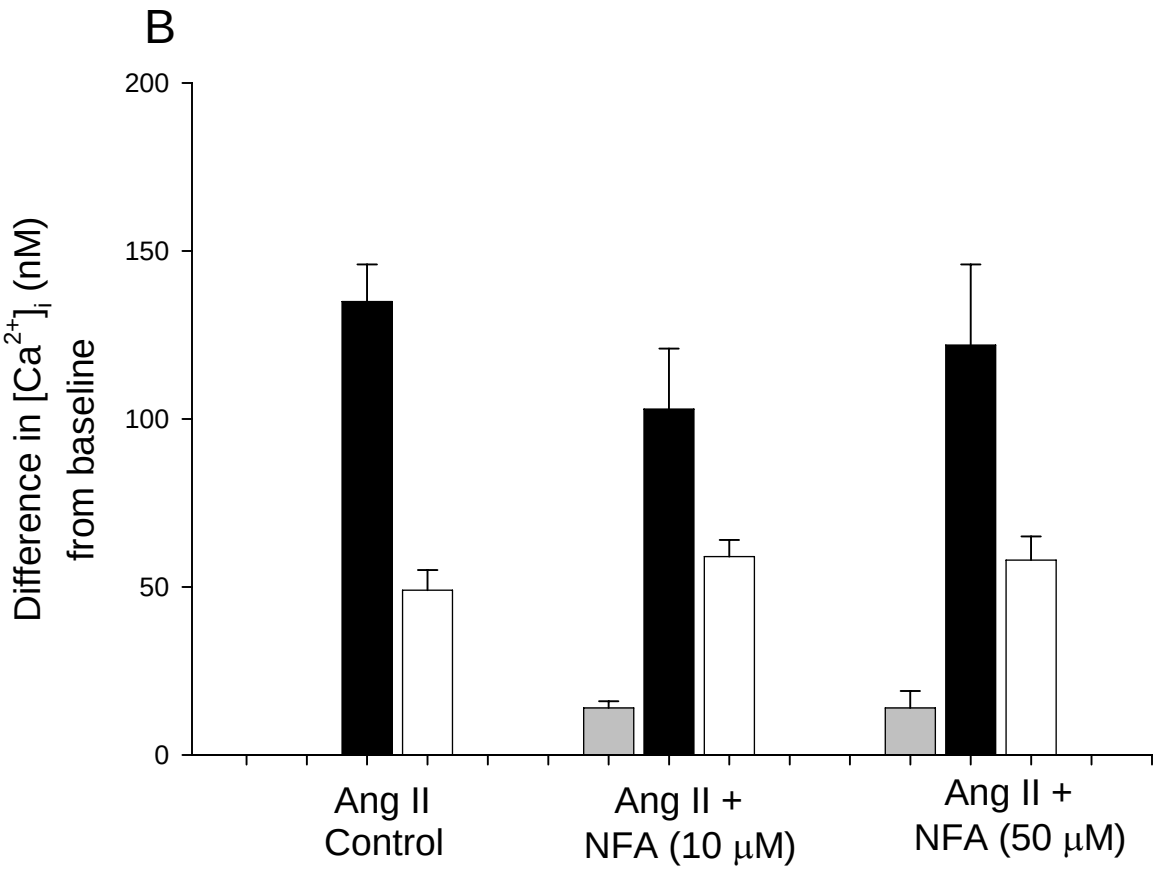
Fellner
Fig. 1



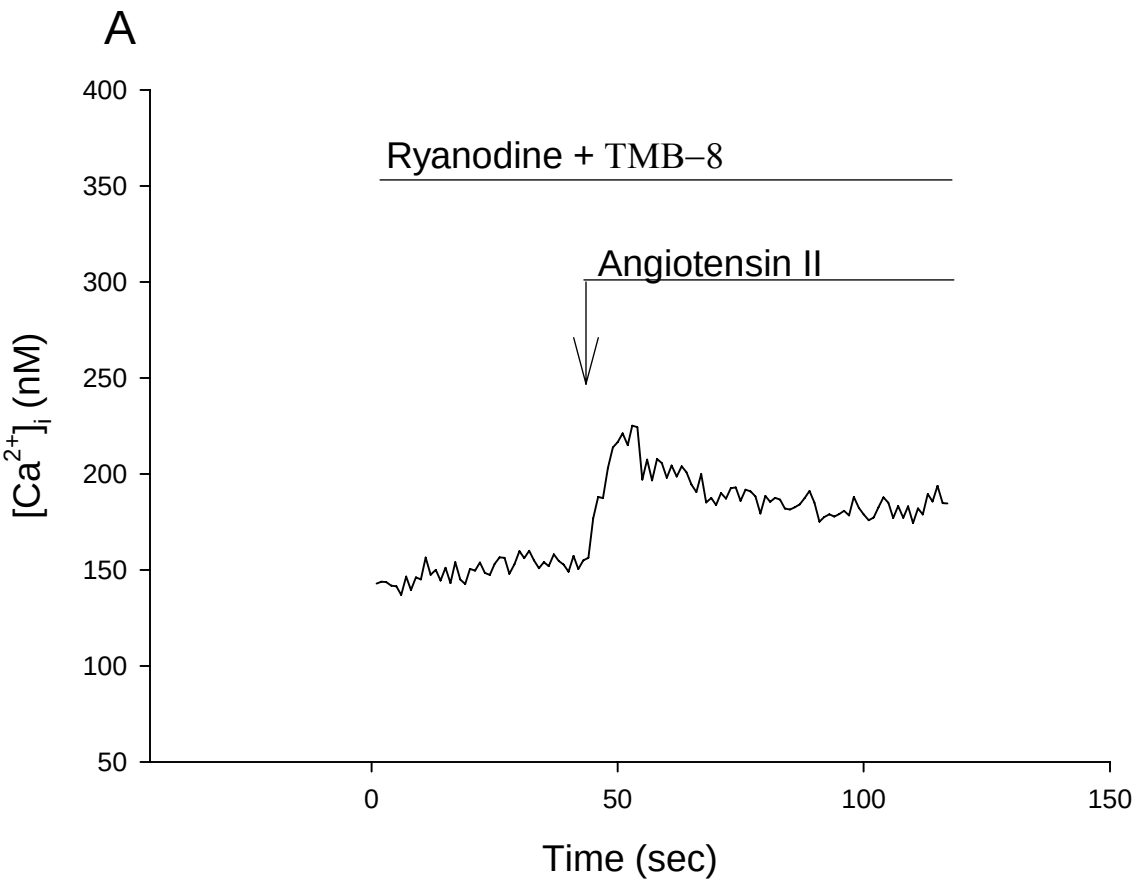
Fellner
Fig. 2 A



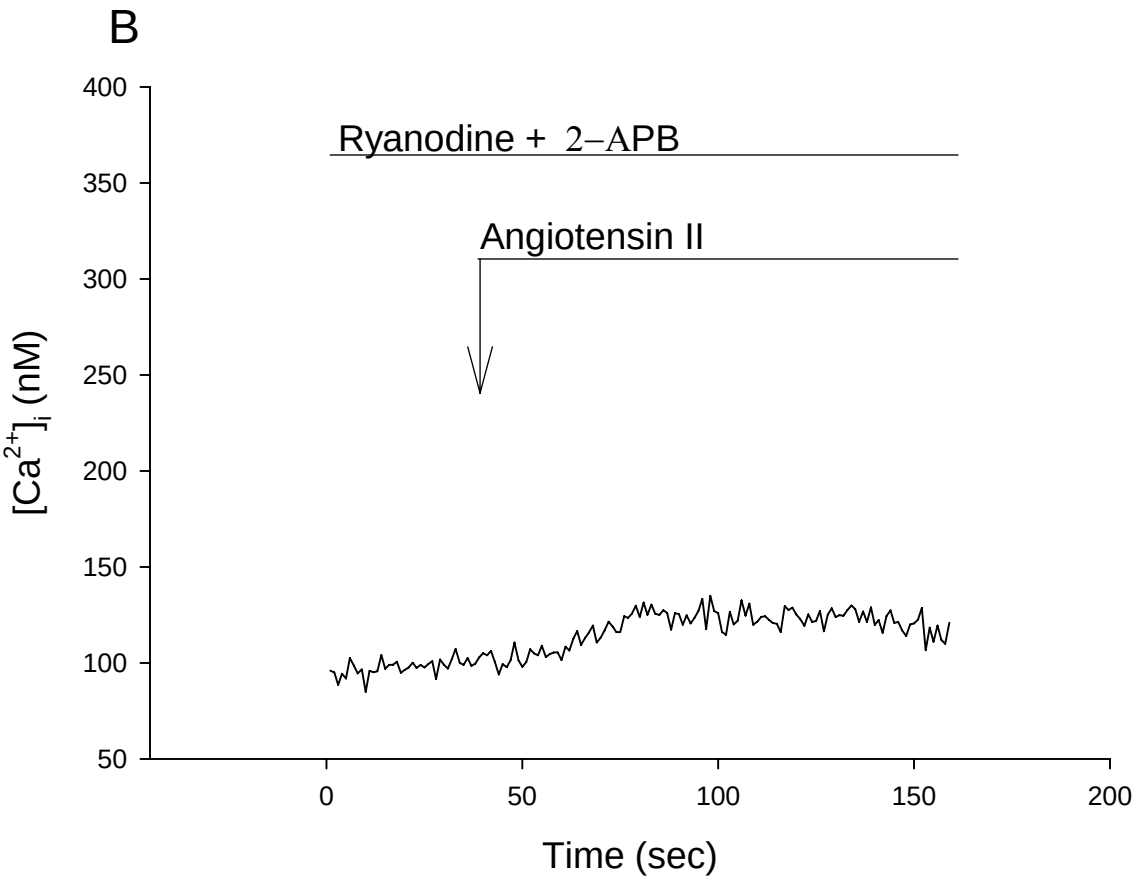
Fellner
Fig. 2B



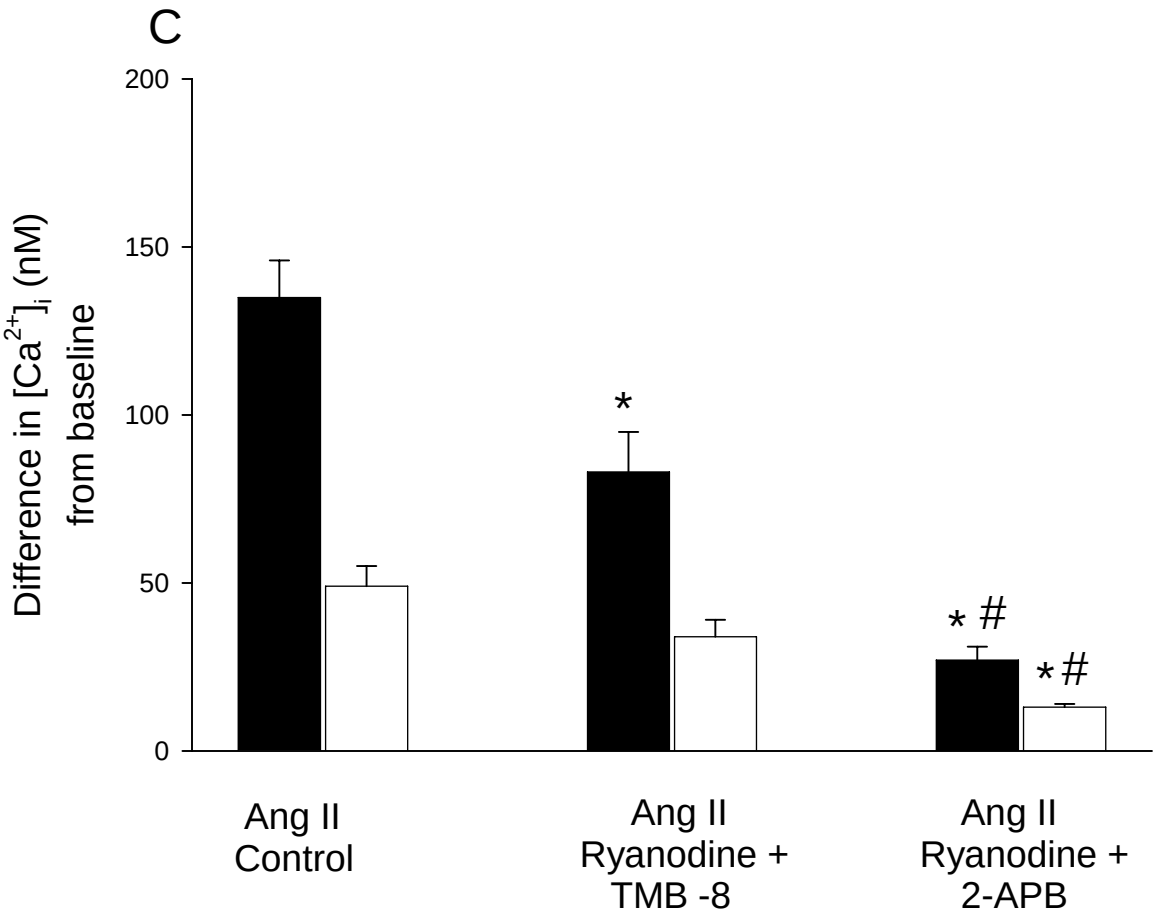
Fellner
Fig. 3A



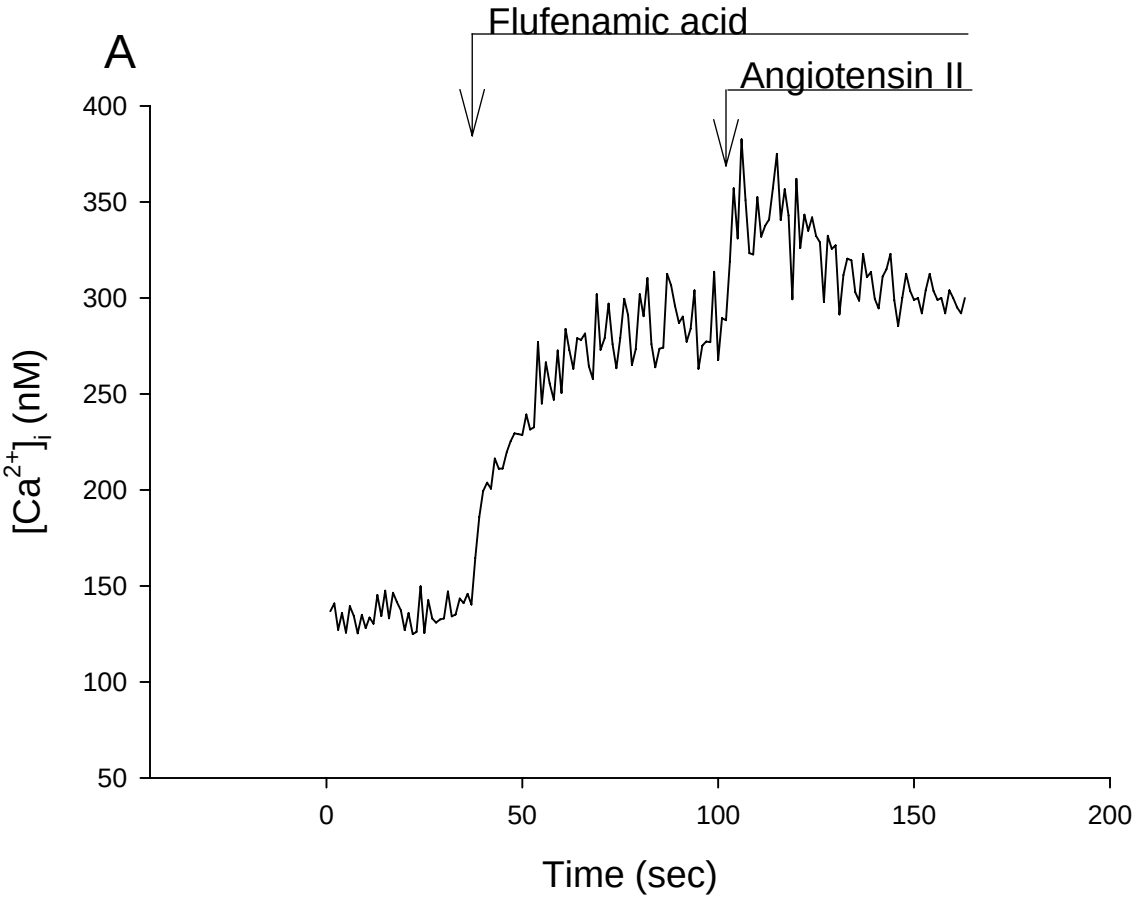
Fellner
Fig. 3B



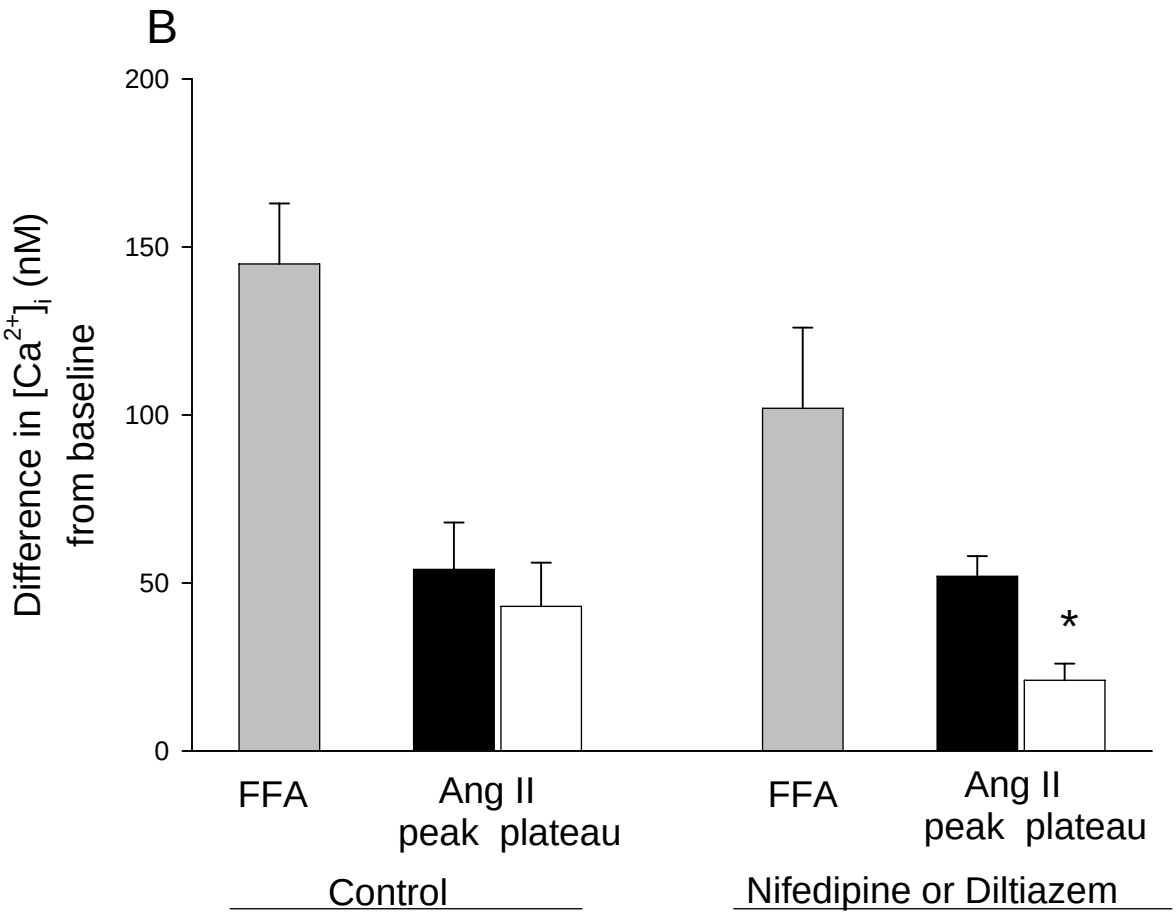
Fellner
Fig. 3C



Fellner
Fig. 4A



Fellner
Fig. 4B



Fellner
Fig 5