Augmented Contributions of Voltage-Gated Ca\(^{2+}\) Channels to Contractile Responses in Spontaneously Hypertensive Rat Mesenteric Arteries

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The observation that organic Ca\(^{2+}\) channel blockers are more effective in lowering blood pressure and peripheral resistance in hypertensive compared to normotensive subjects suggests that there is a greater contribution from voltage-gated Ca\(^{2+}\) channels (Ca\(_L\)) to vascular force maintenance in hypertensive arteries. This study tests this hypothesis by comparing the effects of Bay k 8644 and nisoldipine on basal force development, contractile responses to norepinephrine and serotonin, and Ca\(^{2+}\) currents (I\(_{Ca}\)) in mesenteric artery (MA) from Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). MA rings were used to record isometric contractions at L\(_{max}\). Single cells were isolated by collagenase plus elastase for measurement of Ca\(_L\) properties by patch-clamp methods. Contractile responses to Bay k 8644 were larger and more sensitive in SHR than WKY, and were larger in endothelium-denuded compared to intact rings. In SHR, the addition of 10 nmol/L Bay k 8644 increased contractile sensitivity to norepinephrine (NE) and serotonin (5HT), and increased maximum response to 5HT. In WKY, 10 nmol/L Bay k 8644 produced a small increase in 5HT sensitivity with no effect on maximum response, and had no effect on NE responses. In the presence of 1 \(\mu\)mol/L nisoldipine, the maximum response and the sensitivity to both NE and 5HT were decreased in both WKY and SHR with the inhibitory effects of nisoldipine being larger in SHR than WKY. Peak I\(_{Ca}\) was larger in SHR, and current-voltage curves were shifted toward more negative voltages compared to WKY. Bay k 8644 increased I\(_{Ca}\) in both WKY and SHR myocytes with no apparent difference in the magnitude of its effect when expressed as a percent of control I\(_{Ca}\). These results suggest that Ca\(_L\) contribute significantly to tonic force maintenance as well as to agonist responses in MA from both WKY and SHR, but with a much larger contribution in SHR. Differences in the sensitivity of Ca\(_L\) to Bay k 8644 were not responsible for the differences in contractile responses to this agonist. Am J Hypertens 1997;10:1231–1239 © 1997 American Journal of Hypertension, Ltd.

KEY WORDS: Hypertension, arterial smooth muscle contractions, Ca\(^{2+}\) currents, norepinephrine, serotonin, endothelium, nisoldipine, Bay k 8644.
An increase in intracellular Ca$^{2+}$ is generally regarded to be the primary mechanism by which activation of smooth muscle is coupled to contraction. The increase in cytoplasmic free Ca$^{2+}$ that activates vascular smooth muscle occurs as a result of both intracellular Ca$^{2+}$ release and extracellular Ca$^{2+}$ influx. Agonist stimulation of smooth muscle results in activation of phospholipase C, which increases the production of the second messenger inositol 1,4,5-trisphosphate (IP$_3$). IP$_3$ promotes the release of Ca$^{2+}$ stores in the sarcoplasmic reticulum with the subsequent increase in intracellular free Ca$^{2+}$, phosphorylation of myosin light chains, and activation of contraction. However, the production of IP$_3$, the Ca$^{2+}$ transient, and the myosin light chain phosphorylation associated with agonist activation are all transient. In the absence of extracellular Ca$^{2+}$ influx the steady, tonic component of smooth muscle contraction (ie, force maintenance) cannot be sustained. Also, the tonic level of Ca$^{2+}$ and inhibition of voltage-dependent K$^+$ channels14 are larger and their voltage-dependence shifted in the negative direction that activates vascular smooth muscle. The extent to which Ca$^{2+}$L are involved in hypertensive arterial smooth muscle is known to be effective antihypertensive agents. These results suggest that under normal conditions Ca$^{2+}$L may be active in hypertensive smooth muscle, and may provide a significant contribution to force maintenance and peripheral resistance in hypertensive smooth muscle. The extent to which Ca$^{2+}$L are involved differentially in agonist-mediated contractions in WKY and SHR, however, is not clear. It was the purpose of this study to test the hypothesis that Ca$^{2+}$L provides a greater contribution to agonist-mediated contraction in hypertensive compared to normal arterial smooth muscle.

**METHODS**

The mesenteric artery and its branches were obtained from 12-week-old male WKY and SHR rats. The main MA was cut into 3-mm long ring segments for use in contractile studies. Small MAs were used for the isolation of single cells by collagenase and elastase dissociation. Ca$^{2+}$ transient currents (I$_{Ca}$) were measured in these cells using whole cell patch clamp methods.

**Contractile Studies** For these studies, the rings were mounted in vitro at their optimum length for force development in a temperature-controlled bath containing a physiologic salt solution (PSS) at 37°C and continuously aerated with 95% O$_2$/5% CO$_2$. Some preparations were gently rubbed to remove the endothelium before mounting. After a 2- to 3-h equilibration, duplicate responses to a 120 mmol/L K$^+$–PSS solution were determined, where K$^+$ was substituted for Na$^+$ on an equimolar basis. This response was used as a test of tissue viability and for normalization of agonist contractile responses. In one set of experiments, responses to the cumulative addition of the L-type Ca$^{2+}$ channel agonist Bay k 8644 were obtained in segments either with or without endothelium. In a separate set of experiments, cumulative concentration–response studies were performed using norepinephrine (NE) or serotonin (5HT) in different tissues. After the initial control responses to NE or 5HT, the baths were drained and rinsed twice. Either Bay k 8644 (10 nmol/L) or nisoldipine (1 μmol/L) was added to the bath for 30 min, then the dose–response test was repeated. The tests were always performed using three rings per animal. One ring was not exposed to either dihydropyridine and served as a control. Recovery periods of at least 1 h between drug tests were used.

Force was divided by wall cross-sectional area and expressed as active stress as previously described. Peak values of stress were determined at each drug concentration and also expressed as percentage of the maximal response. Values of active stress and percent response were averaged at each drug concentration for the two groups (WKY and SHR).
Cell Isolation  Myocytes were isolated enzymatically from small MAs using methods previously described by us. Individual branches were removed, cut open longitudinally, and incubated in a Ca\(^{2+}\)-free solution at 37°C for about 40 min. The branches were then cut into small pieces and collagenase (250 U/mL) plus elastase (25 U/mL) were added to the incubation solution for about 30 min at 37°C. The tissue and enzyme solution were separated by filtering through 210-μm mesh, washed with 1 mL of enzyme-free buffer, and triturated gently using a Pasteur pipette. Released cells were separated from the tissue by filtering through the 210-μm mesh.

Electrophysiological Methods  An aliquot of cells was transferred to a chamber placed on the stage of an inverted microscope (Nikon Diaphot, Melville, NJ) and allowed to adhere to the chamber’s glass bottom. Perfusion with a Ca\(^{2+}\)-free solution was initiated to remove debris and loosely attached cells from the chamber. The [Ca\(^{2+}\)] of the perfusate was slowly increased to 2 mmol/L to avoid cell damage. Membrane currents were recorded using the whole cell, patch-clamp configuration at room temperature (about 22 to 24°C). Micropipettes (2 to 3 MΩ resistance) were made from capillary tubing (WPI Kwik-fil, Sarasota, FL) using a programmable puller (P-80/PC; Sutter Instruments, San Rafael, CA) and fire polished. Series resistance and capacitance compensation were adjusted maximally using a patch-clamp amplifier with a 100 MΩ head stage (model 8900; Dagan, Minneapolis, MN). Experimental protocols were controlled using a computer (466/L; Dell, Austin, TX) and PCLAMP software (version 5.5.1; Axon Instruments, Foster City, CA). Current signals were converted from analog to digital form at a sampling rate of 10 kHz using a Labmaster A/D board (Axon Instruments) and stored in the computer for analysis. Multiple responses (n = 5) to hyperpolarizing voltage-clamp steps (20 mV) were obtained for each protocol, averaged, and used to provide capacitance and leak compensation of the raw data. Experimental current records were analyzed using PCLAMP 5.5.1 software.

Procedures  Cell break-in was accomplished by gentle suction at a holding potential of −60 mV. Membrane potential was stepped at 10-sec intervals from −60 to 0 mV for 3 to 5 min during cell dialysis with the pipette solution until \(I_{\text{Ca}}\) stabilized. Current-voltage measurements were then performed from a holding potentials of −90 mV. Voltage-clamp steps 75 msec long were applied from −60 to +40 mV in 10-mV increments every 10 sec. After control measurements, cells were exposed to 10 mmol/L Bay k 8644 added to the perfusate sequentially, and the same measurements were repeated at each concentration. The response to the agonist was monitored using a voltage-clamp step from −60 to 0 mV every 15 sec until a steady response was achieved.

Chemicals and Solutions  The PSS used for contractile studies had the following composition (in mmol/L): 114 NaCl, 4.5 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 24 NaHCO\(_3\), 1.2 NaH\(_2\)PO\(_4\), 2.4 Na\(_2\)HPO\(_4\), and 11 dextrose with pH of 7.4 (KOH) and osmolality of 301 ± 2 mOsm/L. All drugs were freshly made in double distilled water and added to the baths with a volume dilution that never exceeded 1/1000. The incubation buffer for enzymatic cell isolation and the external solution for patch-clamp studies had the following composition (in mmol/L): 100 CsCl, 20 tetraethylammonium chloride (TEACl), 5 NaCl, 5 MgATP, 10 HEPES, and 10 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) at pH 7.2 (with CsOH), and had an osmolality of 306 ± 3 mOsm/L. Collagenase was purchased from Worthington Biochemical (CLS3, Freehold, NJ). Elastase was purchased from ICN Pharmaceuticals (porcine pancreas, Cleveland, OH). Bay k 8644 was obtained from Research Biochemicals Int. (Natick, MA), and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Nisoldipine was a gift from Miles Laboratories Inc. (West Haven, CT).

Statistical Analysis  Statistical comparisons of membrane currents were performed using a two-way analysis of variance with repeated measures for unpaired data using the StatWorks (Cricket Software, Philadelphia, PA) application on a Macintosh computer (PowerMac 7100; Apple, Cupertino, CA). Probability values < 0.05 were considered to be significant. Average values for the two groups are given for each procedure as mean ± 1 SEM.

RESULTS  The cumulative addition of Bay k 8644 caused a large dose-dependent contractile response in MAs from SHR that was significantly greater than that in WKY as shown in Figure 1. This response was augmented after endothelium removal in terms of threshold, sensitivity, and maximum response. In the presence of the endothelium, the maximum response to Bay k 8644 in SHR was about 55% of the response to 120 mmol/L KCl and occurred at 1 μmol/L. When the endothelium was removed, the maximum response was equal to that of 120 mmol/L KCl and occurred at 0.3 μmol/L. In contrast, responses to Bay k 8644 in WKY tissues were smaller in both endothelium-intact and endothelium-denuded preparations. As in SHR mesenteric arteries, removal of the endothelium enhanced responses to Bay k 8644 in WKY. Contractions to 120
mmol/L KCl were not significantly different with or without endothelium between WKY and SHR. The effects of augmenting Ca_{l} activity on NE dose–response relations were determined by pretreating tissues with 10 nmol/L Bay k 8644. These responses were performed after removal of the endothelium in the presence of 0.1 μmol/L propranolol (to block β-adrenoceptors) and 0.1 μmol/L desipramine (to block neuronal norepinephrine uptake). Tissues were pretreated with Bay k 8644 for 30 min before the initiation of the cumulative NE responses. As shown in Figure 2, Bay k 8644 had no significant effect on NE dose–responses relations in the WKY, but produced a significant augmentation of responses in the SHR. As the maximum response to NE was not affected by Bay k 8644, the augmentation in SHR amounted to a leftward shift in the dose–response relation.

The effects of augmenting Ca_{l} activity on serotonin dose–response relations were also determined using endothelium-denuded preparations preexposed to 10 nmol/L Bay k 8644. As shown in Figure 3, Bay k 8644 increased the sensitivity to 5HT in both WKY and SHR, but with a larger effect in SHR. The effect of Bay k 8644 in WKY was significant only at low concentrations of 5HT, whereas the effect in SHR appeared as a parallel shift in the dose–response relation to the left.
The maximum response to 5-HT was significantly increased in SHR, but not in WKY, as indicated in Figure 3C.

The contribution of the normal level of Ca_L activity to NE dose–response relations was assessed by pre-treating endothelium-denuded preparations with 1 μmol/L nisoldipine. NE responses were then obtained in the presence of 0.1 μmol/L propranolol and 0.1 μmol/L desipramine. As shown in Figure 4, the NE dose–response curves were significantly shifted to the right in both groups by nisoldipine but with a much larger effect in the SHR. Nisoldipine also decreased the maximum response to NE in both WKY and SHR, also with a larger effect in SHR.

The normal contribution of Ca_L to serotonin dose–response relations was also determined in endothelium-denuded preparations. In the presence of 1 μmol/L nisoldipine, the dose–response for 5HT was shifted to the right in both WKY and SHR, with a much larger effect in the SHR as shown in Figure 5. The effect of nisoldipine in SHR was largest at lower 5HT concentrations. In addition, nisoldipine reduced the maximum stress responses to 5HT in both WKY and SHR, also with a larger effect in the SHR (WKY = $-26 \pm 5\%$ vs SHR = $-72 \pm 8\%$).

The effects of Bay k 8644 on I_Ca were determined using myocytes isolated from mesenteric arteries of the same animals in which the contractile responses were performed. The effects of Bay k 8644 were determined in endothelium-denuded preparations. A and B: Responses were normalized to the maximum stress before averaging. C: Summary of maximum responses to 5HT. Bay k 8644 increased the sensitivity to 5HT with a larger response in SHR than WKY. Maximum response was also augmented in SHR preparations. *Significant differences ($P < .05$). Values are the mean ± SEM for 6 to 12 preparations.
were determined. Under control conditions, I_{Ca} current density (I_{Ca} divided by cell capacitance to normalize for differences in cell size) was significantly larger in myocytes from SHR compared to WKY (3.0 ± 0.2 vs. 3.8 ± 0.2 pA/pF) as previously reported. As shown in Figure 6, both 10 nmol/L and 1 μmol/L Bay k 8644 increased I_{Ca} in WKY and SHR, and shifted the current-voltage relations to the left (in the hyperpolarizing voltage direction). At each concentration of Bay k 8644, I_{Ca} remained significantly larger in SHR compared to WKY. However, no differences existed in I_{Ca} responses to Bay k 8644 between WKY and SHR at either concentration when represented as percentage changes from initial values. The percentage increases in maximum I_{Ca} were as follows: at 10 nmol/L: WKY = 58 ± 13% (n = 6) and SHR = 48 ± 7% (n = 7); at 1 μmol/L: WKY = 114 ± 15% (n = 6) and SHR = 112 ± 6% (n = 7).

The hyperpolarizing voltage shift produced by Bay k 8644, shown in Figure 7, was quantitated by determining the voltage on the increasing limb of the current-voltage curve (in the negative voltage range between −60 and 0 mV) where current was 50% of the maximum value. Changes in the average value of this voltage from control for the various groups was as follows: at 10 nmol/L: WKY = −5.5 ± 0.3% (n = 6) and SHR = −6.8 ± 0.4% (n = 7); at 1 μmol/L: WKY = −7.5 ± 0.3% (n = 6) and SHR = −8.4 ± 0.3% (n = 7). The differences in values of voltage for half maximal current between WKY and SHR were statistically significant for both 10 nmol/L and 1 μmol/L Bay k 8644.

FIGURE 5. Effects of nisoldipine on serotonin (5HT) responses. A and B: Responses were normalized to the maximum response before averaging. C: Summary of maximum active stress responses to 5HT with 1 μmol/L nisoldipine. Nisoldipine produced a larger decrease in the maximum response and in sensitivity to 5HT in preparations from SHR. *Significant differences (P < .05). Values are the mean ± SEM for 7 to 9 preparations.

FIGURE 6. Effects of Bay k 8644 on calcium currents. No differences in the percentage increase in I_{Ca} were found for mesenteric artery myocytes from WKY (n = 11) and SHR (n = 10). Responses were as follows: at 10 nmol/L: WKY = 58 ± 13% and SHR = 48 ± 7%; at 1 μmol/L: WKY = 114 ± 15% and SHR = 112 ± 6%. I_{Ca} was measured in freshly isolated myocytes at voltages from −60 to +40 mV in 10 mV, 100-msec steps from a holding potential of −90 mV with [Ca^{2+}]_o = 2 mmol/L and [BAPTA]_i = 10 mmol/L at room temperature. After control measurements, cells superfused with Bay k 8644 and voltage steps were repeated. Peak currents were normalized by cell capacitance before averaging. Symbols are means and are defined in the figure with vertical bars ±1 SEM.
DISCUSSION

The results of this study provide evidence to support the hypothesis that L-type Ca\textsuperscript{2+} channels provide a greater contribution to agonist-stimulated contractile responses in mesenteric arteries of SHR compared to WKY. In agreement with results of previous investigations,\textsuperscript{18,19} we found larger contractile responses in SHR to the L-type Ca\textsuperscript{2+} channel activator Bay k 8644 in the presence as well as the absence of the endothelium. This finding is consistent with the differences in the properties of Ca\textsubscript{L} that we have reported in this tissue.\textsuperscript{20,24} We found that Ca\textsuperscript{2+} currents were larger and activated at more negative voltages in SHR compared to WKY. Both of these characteristics could contribute to the larger responses in SHR. In addition, a more depolarized resting membrane potential of arterial smooth muscle reported in SHR could contribute to the larger responses to Ca\textsubscript{L} activation in this group.\textsuperscript{25,26} Together, these results suggest that Ca\textsubscript{L} are more active at prevailing membrane potential in SHR compared to WKY.

Enhancing the activity of L-type Ca\textsuperscript{2+} channels with Bay k 8644 or inhibiting it with nisoldipine produced substantial effects on contractile responses to NE and 5HT that were quantitatively different between agonists and strains. In SHR, responses to NE were shifted to the left by Bay k 8644 in parallel to the control curve with no effect on maximum response. It has been shown that NE depolarizes the membrane potential in arterial smooth muscle.\textsuperscript{13,27} This effect in the presence of augmented Ca\textsubscript{L} activity could be responsible for the augmented contractile responses to NE in the SHR. It should be noted that the maximum response to NE was not altered. This is probably attributable to the depolarizing effect of NE on membrane potential saturates with increasing concentrations of NE.\textsuperscript{27} The lack of effect of Bay k 8644 on NE responses in WKY suggests that Ca\textsubscript{L} cannot be further activated by the agonist, membrane potential depolarization is smaller or is insufficient to activate Ca\textsubscript{L}, or the 5HT buffers the augmented Ca\textsuperscript{2+} influx to a greater extent,\textsuperscript{28} preventing a contractile effect.

Nisoldipine pretreatment depressed the contractile response to NE in both WKY and SHR with a larger effect in SHR. The effect of nisoldipine at low concentrations of NE in SHR mesenteric arteries was the most dramatic, and could be attributable to either a decrease in the contribution of Ca\textsubscript{L} to Ca\textsuperscript{2+} mobilization per se or to a reduction in the Ca\textsuperscript{2+} content of the 5HT.\textsuperscript{28} Because a contribution of Ca\textsubscript{L} could not be augmented in the WKY by Bay k 8644 it is likely that a reduction in 5HT Ca\textsuperscript{2+} content could be the primary reason for the decreased responses in WKY. In the SHR, however, because the Ca\textsubscript{L} effects on NE responses could be augmented and the effects of nisoldipine were larger than in WKY, it is likely that an inhibitory effect on both Ca\textsuperscript{2+} influx and 5HT release (ie, content) may play a role in the depressed NE responses in SHR.

The effects of modulation of Ca\textsubscript{L} on 5HT responses in WKY and SHR were generally similar to the effects on NE responses. The only differences were that the effects on 5HT responses were smaller than the effects on NE responses, and Bay k 8644 had a small but significant effect on 5HT responses in WKY. In addition, there was a much larger inhibitory effect of nisoldipine on the maximum response to serotonin in SHR compared to WKY than on maximum NE responses in the two groups. This suggests that 5HT may use Ca\textsubscript{L} influx for Ca\textsuperscript{2+} mobilization to a larger extent than NE.

The above discussion was based on the implicit assumption that the contribution of Ca\textsubscript{L} to NE and 5HT contractile responses was only the result of
changes in open probability secondary to changes in membrane potential. In fact, both NE and 5HT have been shown to augment CaL currents probably through a G-protein-mediated pathway. Differences in the magnitude of agonist augmentation of CaL current could contribute to the differences in the effects of the dihydropyridine on agonist-induced contractions between the WKY and SHR but this has not been studied to date.

In summary, L-type Ca\(^{2+}\) channels provide a larger contribution to contractile function in arterial smooth muscle under normal conditions in SHR compared to WKY. The larger effect of Bay k 8644 on contractile responses in SHR is not the result of a greater effect of the agonist on Ca\(^{2+}\) channels but may be attributable to differences in the voltage-dependence of the Ca\(^{2+}\) currents or the resting membrane potential. However, it remains to be determined whether the result of these experiments can be extrapolated to human hypertensio.

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