Ca\textsuperscript{2+} Influx is Increased in 2-Kidney, 1-Clip Hypertensive Rat Aorta

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Abstract—Arteries from hypertensive rats show a greater contraction in response to Ca\textsuperscript{2+} channel activator and an increased sensitivity to Ca\textsuperscript{2+} entry blockers compared with those of normotensive rats. These facts suggest an altered Ca\textsuperscript{2+} influx through membrane channels. In this study, this hypothesis was tested by direct activation of voltage-gated Ca\textsuperscript{2+} channels using Bay K 8644, a dihydropyridine sensitive large conductance (L-type) Ca\textsuperscript{2+} channel opener in aortas from 2-kidney, 1-clip (2K1C) hypertensive rats. Because the membrane potential of smooth muscle cells is an important regulator of the conformational state of L-type Ca\textsuperscript{2+} channels and, consequently, dihydropyridine affinity, the effect of 10 mmol/L KCl on the responses to Bay K 8644 was also studied. Maximal contraction (ME) and sensitivity to Bay K 8644 were greater in 2K1C rats than in 2K normotensive rats (ME, 1.77±0.15 versus 1.25±0.19 g; negative log molar value [pD\textsubscript{2}], 8.27±0.07 versus 7.92±0.08). When the KCl concentration was increased from 4.7 to 10 mmol/L in the bathing medium, no differences were observed in the contractile effect of Bay K 8644 between 2K1C and 2K (ME, 1.28±0.13 versus 1.14±0.21 g; pD\textsubscript{2}, 8.56±0.08 versus 8.38±0.07). The cell resting membrane potential of 2K1C aorta smooth muscle cells were less negative than in 2K (−35.19±4.91 versus −48.32±1.88 mV). Basal intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) was greater in cultured vascular smooth muscle cells from 2K1C than from 2K (293.4±25.83 versus 205.40±12.83 nmol/L). In 2K1C, Bay K 8644 induced a larger increase in [Ca\textsuperscript{2+}], than in 2K (190.60±45.65 versus 92.57±14.67 nmol/L), and in 10 mmol/L KCl, this difference was abolished (134.90±45.12 versus 125.20±32.17 nmol/L). The main conclusion of the present work is that the increased contractile response to Bay K 8644 in 2K1C aortas is due to an increased Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels. (Hypertension. 2001; 38[part 2]:592-596.)

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An increased intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) is generally accepted as the primary signal by which the activation of vascular smooth muscle is coupled to the initiation of force development, and it occurs as a result of intracellular Ca\textsuperscript{2+} release, extracellular Ca\textsuperscript{2+} influx, or both, depending on the stimuli.\(^1\)

In general, contractile responses in blood vessels from hypertensive rats are more sensitive to changes in extracellular Ca\textsuperscript{2+} concentration and Ca\textsuperscript{2+} entry blockers than those from normotensive rats. Also, Ca\textsuperscript{2+} currents in vascular smooth muscle cells (VSMCs) from hypertensive rats are larger in hypertensive rats than in normotensive rats, suggesting an increased activity of this type of channel.\(^6\)–\(^8\) These observations support the idea that changes in transmembrane Ca\textsuperscript{2+} influx contribute to increased vascular reactivity in hypertension.

Conflicting observations have been reported with respect to the effectiveness of organic L-type Ca\textsuperscript{2+} channel blockers in inhibiting agonist-induced vascular contraction in hypertensive rats. Our previous report demonstrated that pretreatment with nifedipine depressed the contractile response to phenylephrine in both 2K and 2K1C aortas with a higher effect on 2K.\(^9\) These results suggested a decreased contribution of large conductance (L-type) Ca\textsuperscript{2+} channels to Ca\textsuperscript{2+} influx in agonist-stimulated contraction in aortas from 2K1C rats. Similarly, other authors observed that the L-type Ca\textsuperscript{2+} channel blocker was less effective in inhibiting agonist-induced intracellular Ca\textsuperscript{2+} increase in aorta from SHR.\(^10\) However, the action of several vasoactive agents involves a direct modulation of voltage-operated calcium channels, in addition to causing membrane depolarization.\(^11\) Further, not all of the vasoactive agents have the same effect on ionic conductances in vascular smooth muscle. Hence, our findings could be related to changes in the mechanisms linked to a particular receptor and a specific action of phenylephrine in the membrane.

A greater contractile response to Ca\textsuperscript{2+} channel activators has been observed in arterial smooth muscle from SHR, SHR stroke-prone and in Dahl salt-sensitive rats.\(^12\)–\(^14\) An enhanced activity of Ca\textsuperscript{2+} channels could contribute to the effects on...
contractile responses in hypertension. Concerning the differences in the vascular reactivity to contractile agents observed in hypertension, the majority of the studies has demonstrated alterations in Ca\textsuperscript{2+} influx in smooth muscle of genetically hypertensive rats. In the present work, we evaluated whether the mechanisms of Ca\textsuperscript{2+} influx reported in genetic models of hypertension are also present in the 2K1C model. In view of such considerations, the aim of this study was to test the hypothesis that an increase in Ca\textsuperscript{2+} influx by direct activation of voltage-gated Ca\textsuperscript{2+} channels with Bay K 8644, a dihydropyridine selective L-type Ca\textsuperscript{2+} channel opener, provides contribution for a greater contraction in aortas from 2K1C. The membrane potential of smooth muscle cells appears to be an important regulator of the conformational state of L-type Ca\textsuperscript{2+} channels and consequently the dihydropyridine affinity.\textsuperscript{15,16} As a second aim, the effect of 10 mmol/L KCl on the resting membrane potential, leading to a greater contractile response in 2K1C aortas, which may be related to a less negative contribution for a greater contraction in aortas from 2K1C.

Methods

Animals

Renovascular hypertension was induced in rats following the 2K1C Goldblatt model.\textsuperscript{17} Briefly, male Wistar rats (180 to 200 g) were anesthetized, and after a midline laparotomy, a silver clip with 0.20 mm ID was placed around the left renal artery. The 2K rats were submitted to laparotomy only. Six weeks after surgery, the systolic arterial pressure was measured by the tail-cuff method. Rats were considered to be hypertensive when systolic pressure was >160 mm Hg. It is established that in this phase of hypertension neither the renin nor the ACE activities are increased in plasma and/or aorta of 2K1C rats.\textsuperscript{18} The experiments were performed in this phase of hypertension to rule out a major interference of the renin-angiotensin system on our results.

Isolated Aorta Rings

Following the arterial pressure recordings, the rats were killed by decapitation, and thoracic aortas were isolated. Aorta rings, 4 mm in length, were cut and mounted for isometric tension recording. The rings were placed in bath chambers (10 mL) for isolated organs containing physiological salt solution (PSS) at 37°C, continuously gassed with 5% CO\textsubscript{2}. The rings were equilibrated during the 60-minute equilibration period. Following the arterial pressure recordings, the rats were killed by anesthetized, and after a midline laparotomy, a silver clip with 0.20 mm ID was placed around the left renal artery. The 2K rats were submitted to laparotomy only. Six weeks after surgery, the systolic arterial pressure was measured by the tail-cuff method. Rats were considered to be hypertensive when systolic pressure was >160 mm Hg. It is established that in this phase of hypertension neither the renin nor the ACE activities are increased in plasma and/or aorta of 2K1C rats.\textsuperscript{18} The experiments were performed in this phase of hypertension to rule out a major interference of the renin-angiotensin system on our results.

Reactivity Studies

At the beginning of the experiments, the aortas were stimulated with 0.1 mmol/L norepinephrine to test their functional integrity. In all experiments, the vascular endothelium was gently removed to avoid its possible modulatory effect on the Bay K 8644 contractile response. Aorta rings were stimulated with increasing concentrations of Bay K 8644 (0.1 mmol/L to 0.5 mmol/L), cumulatively added to the bath in PSS containing 4.7 or 10 mmol/L KCl. This particular concentration of KCl was chosen because it is not far from the physiological range and should induce depolarization in aortas.

Cell Isolation, Culture, and Ca\textsuperscript{2+} Measurement

VSMCs were isolated by enzymatic digestion from aorta. Briefly, the aortas were dissected and longitudinally opened. The endothelium and the adventitia were removed. The tissue was then minced into small pieces and incubated in Ca\textsuperscript{2+} free Hanks’ solution containing 0.6 mg/mL collagenase, 0.6 mg/mL papain, 0.3 mg/mL soybean trypsin inhibitor, 5 mmol/L taurine, 10 mg/mL bovine serum albumin (type I), and 0.2 mmol/L dithiothreitol. The tissue was gently shaken in this solution for 40 minutes at 37°C. After incubation, the vessel fragments were washed, and cells were released by mechanical dispersion with a Pasteur pipette. The result cell suspension was centrifuged at 200g and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 2 mmol/L glutamine, 20 mmol/L HEPES (pH 7.3), 10 000 U/mL penicillin, and 10 000 μg/mL streptomycin. The cells were plated on glass coverslips and kept in a humidified 37°C incubator gassed with 5% CO\textsubscript{2}. The cells were used between 6 and 8 days after plating and were maintained in a serum-free medium for 24 hours before the experiment. On the day of the experiment, VSMCs were incubated with 5 μmol/L Fura-2 AM in 1 mg/mL BSA for 40 minutes at 37°C and then washed for 20 minutes. Culture dishes containing Fura 2–loaded cells were placed in a temperature-regulated (37°C) chamber mounted on the stage of a Nikon inverted microscope. Each fluorescence measurement was performed on groups of 4 to 6 cells isolated in the window of a dual wavelength spectralfluorometer (Deltaram, Photon Technology Intl), at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The cells were continuously superfused at 2 mL/min with Hanks’ solution. Fura 2 fluorescent signals originating from the cells were collected and stored using a software package from Photon Technology International (Felix). [Ca\textsuperscript{2+}], was calculated according to Grynkiewicz et al.\textsuperscript{19} The calculated K\textsubscript{d} for fura 2 in our system was 300 mmol/L.

Electrophysiological Studies

The resting membrane potential of the cells was measured with conventional glass microelectrodes with resistances between 40 to 80 MΩ when filled with 2 mol/L KCl. Aorta rings without endothelium were carefully slit along the longitudinal axis and pinned down on the bottom of an experimental chamber (300 μL) with the luminal side upward. The chamber was continuously perfused with PSS gassed with 95% O\textsubscript{2}/5% CO\textsubscript{2} at a flow rate of 1 to 2 mL min\textsuperscript{-1}. The membrane potential was measured with an electrometer (DUO-773-WPI Inc), sampled at a frequency of 500 Hz with an A/D converter (Digidata 1200-Axon Instrument) under control by Axoscope 1.1 program (Axon Instrument Inc) and stored on a computer hard disk for offline analysis.

Solutions and Drugs

The composition of the PSS was the following (in mmol/L): 130.0 NaCl, 1.6 CaCl\textsubscript{2}, 4.7 KCl, 1.17 MgSO\textsubscript{4}, 1.18 KH\textsubscript{2}PO\textsubscript{4}, 14.9 NaHCO\textsubscript{3}, 0.026 EDTA, and 5.5 dextrose (pH 7.4). The Hanks’ solution’s was as follows (in mmol/L): 145.0 NaCl, 1.6 CaCl\textsubscript{2}, 5.0 KCl, 1.0 MgCl\textsubscript{2}, 0.5 NaH\textsubscript{2}PO\textsubscript{4}, 10.0 dextrose, and 10.0 HEPES (pH 7.4).

Data Analysis

Results are expressed as mean±SEM, and n indicates the number of animals. Statistical significance was evaluated by unpaired t test analysis by GraphPad Prism 2.00 (1995) software, and values of P<0.05 were considered to be significant.

Results

Systolic blood pressure was found to be significantly higher in 2K1C rats (203±4 mm Hg, n=28, P<0.001) than in 2K rats (116±3 mm Hg, n=24) 6 weeks after surgery. Figure 1A shows that in 4.7 mmol/L KCl PSS, Bay K 8644 induced concentration-dependent contractions, greater in 2K1C aortas (ME: 1.77±0.15 g, P<0.05; pD\textsubscript{2}: 8.29±0.07, P<0.01; n=11) than in 2K aortas (ME, 1.25±0.13 g; pD\textsubscript{2}, 7.93±0.08; n=10). In 10 mmol/L KCl PSS, the responses were also concentration dependent (Figure 1B) but were similar in aortas from 2K (ME, 1.14±0.21 g; pD\textsubscript{2}, 8.38±0.07; n=9) and 2K1C (ME, 1.28±0.13 g; pD\textsubscript{2}, 8.56±0.08; n=9). In-
creasing the KCl concentration to 10 mmol/L decreased the maximal contractile response to Bay K 8644 only in 2K1C aortas (P < 0.05), but the pD2 values were increased in both 2K (P < 0.001) and 2K1C (P < 0.01). The basal cytosolic free calcium concentration was higher in cells from 2K1C (293.4 ± 25.83 nmol/L, n = 32, P < 0.001) than in cells from 2K (205.4 ± 12.83 nmol/L, n = 35). Figure 2A shows that 100 nmol/L Bay K 8644 induced a larger increase in the cytosolic free calcium in cells from 2K1C (190.60 ± 45.65 nmol/L, n = 5, P < 0.05) than in cells from 2K (92.57 ± 14.67 nmol/L, n = 7). In the presence of 10 mmol/L KCl, 100 nmol/L Bay K 8644 also increased the cytosolic free calcium, but no differences were observed between the groups (2K: 125.20 ± 32.17 nmol/L, n = 8; 2K1C: 134.90 ± 45.12 nmol/L, n = 5) (Figure 2B). Ten nmol/L KCl induced an increase in cytosolic free Ca2+ that was similar in 2K (35.62 ± 13.98 nmol/L, n = 8) and in 2K1C (41.31 ± 13.96 nmol/L, n = 5). As shown in Figure 3, the resting membrane potential of cells from 2K1C aortas (−35.19 ± 4.19 mV, P < 0.003, n = 19) were significantly less negative than those from 2K aortas (−48.32 ± 8.88 mV, n = 44).

Discussion
The above results show, to our knowledge for the first time, evidence to support the hypothesis that Ca2+ influx is increased in 2K1C aortas and provides a greater contribution to the increased contraction observed in them. We found a larger contractile response to Bay K 8644, a dihydropyridine sensitive L-type Ca2+ channel opener, in aortas from 2K1C. The present observations are in agreement with other studies that demonstrated an increase in the contractile response to Bay K 8644 in different models of hypertension such as SHR, SHR stroke-prone (SHRSP), and Lyon hypertensive rats. This finding is consistent with the differences observed in the
Bay K 8644–induced \([\text{Ca}^{2+}]\), increase in isolated cells from 2K1C compared with 2K. Our results clearly demonstrate that the \(\text{Ca}^{2+}\) influx induced by Bay K 8644, through voltage-gated channels, is increased in 2K1C aortas. These observations are in accordance with other investigators reporting that the elevation in \([\text{Ca}^{2+}]\), and the contractile response to Bay K 8644 were greater in the SHR femoral artery than in the arteries of normotensive rats. Because the increased intracellular concentration of free \(\text{Ca}^{2+}\) induced by the contractile agent, plays a key role in the development of vasoconstriction, we may conclude that the increased contraction in aortas from 2K1C is due to the observed increase in the \(\text{Ca}^{2+}\) influx induced by Bay K 8644.

Because the opening of voltage-gated channels in arterial smooth muscle is a function of the final membrane potential and normally requires membrane depolarization, a possible explanation for the increased \(\text{Ca}^{2+}\) influx, in response to Bay K 8644, appears to be that the 2K1C aortas are more depolarized in the resting state than are 2K. Indeed, a more depolarized membrane in aortas from 2K1C relative to 2K is clearly demonstrated in this work. The observation that the resting membrane potential of vascular smooth muscle from 2K1C aortas is depolarized could be consistent with a \(\text{Ca}^{2+}\) channel preferential state-dependence interaction with Bay K 8644. The most likely explanation for this suggestion is derived from studies in VSMCs that show that a decreased membrane potential is associated with an increased affinity for dihydropyridines. The influence of the membrane depolarization on the effect of Bay K 8644 is further supported by our finding that the elevation in KCl concentration increased the sensitivity of aortas to this agent, in both 2K and 2K1C.

Another interesting result from our study is that in the presence of 10 mmol/L KCl, there is no difference in the contractile responses to Bay K 8644 between aortas from 2K1C and 2K, which coincides with the similar increase in \(\text{Ca}^{2+}\) concentration observed under the same conditions. Our findings are in agreement with other reports that neither sensitivity nor maximal contraction to Bay K 8644 was found to differ between arteries from genetic hypertensive rats and from normotensive control when extracellular KCl was increased.

It is well documented that the sensitivity to KCl is increased in arteries from SHR. It is believed that a decreased cell membrane potential results in voltage-operated channels lying closer to their threshold. This may account for the enhancement of vascular responsiveness to stimuli known to activate voltage-operated \(\text{Ca}^{2+}\) channels. In contrast, we observed no differences in the increment in \([\text{Ca}^{2+}]\), when the cells from 2K and 2K1C are stimulated with either 10 mmol/L (present study) or 90 mmol/L (G.E. Callera, unpublished data, 2000). Besides this, the contractile effect of KCl in the concentration range of 10 to 120 mmol/L is also the same for the 2 groups of rats (G.E. Callera, unpublished data, 2000). In agreement with the present results, other authors have shown that the vascular resistance of isolated perfused kidney from Dahl rats the effects of Bay K 8644 were increased, whereas the response to KCl did not differ from that observed in normotensive controls. These findings suggest that the changes in responsiveness to Bay K 8644 cannot be entirely attributed to changes in membrane potential but that other factors may be involved as well. In fact, it has been shown an increased availability of \(\text{Ca}^{2+}\) channels in basilar arteries of 2K1C rats, which is also responsible for a larger calcium influx seen in those cells. In our case, we suggest that the augmented responsiveness to the \(\text{Ca}^{2+}\) channel agonist may be related to a particular state of voltage-sensitive \(\text{Ca}^{2+}\) channels in 2K1C aortas, attributable to changes in the membrane potential. This hypothesis finds support on the fact that the level of membrane potential can control both the conformational state of the channel as well as Bay K 8644 affinity.

Binding studies reported increased number of \(\text{Ca}^{2+}\) channels in arteries from SHR compared with normotensive rats. This observation raised an alternative possibility that there is an increase in the number of calcium channels available to interact with Bay K 8644 in aortas from 2K1C. Although we have not tested this hypothesis, we do not believe that an increase in the number of \(\text{Ca}^{2+}\) channels is related to the increased effects of Bay K 8644 in 2K1C because of the following: (1) at the same concentration, Bay K 8644 induces higher effects in 2K1C aortas; and (2) the responses to Bay K 8644 are similar in 2K and 2K1C when KCl concentration is increased.

In conclusion, the increased contractile response to Bay K 8644 in 2K1C aortas is due to an increased \(\text{Ca}^{2+}\) influx through voltage-gated \(\text{Ca}^{2+}\) channels observed in aorta rings and in isolated VSMCs, respectively. Our results also provide evidence that the more depolarized membrane in the aortas from 2K1C favors the activation of the voltage-dependent \(\text{Ca}^{2+}\) channels by Bay K 8644. The functional significance of this change in membrane potential described in aortas as well as in small arteries from hypertensive animals is unclear at present. Given the importance of membrane potential in regulating vascular tone, the evaluation of this question may provide hypothesis for designing tools to restore it to normal levels in hypertensive states.

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