Calcium Antagonists Inhibit Elevated Potassium Efflux From Aorta of Aldosterone-Salt Hypertensive Rats

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The purpose of this study was to evaluate the effect of calcium antagonists on basal tension and the elevated $^{42}$K efflux in aorta from aldosterone-salt hypertensive rats. Diltiazem decreased the basal tension ($2.0 \pm 0.4$ g) as well as the phasic contractile activity and returned the elevated $^{42}$K efflux ($0.018 \pm 0.002$ min) toward control values ($0.019 \pm 0.001$ min, $p<0.001$). The diltiazem median inhibitory concentration (IC$_{50}$) for basal tension ($0.04 \pm 0.02$ nM), however, was sevenfold less than the IC$_{50}$ for basal $^{42}$K efflux ($0.22 \pm 0.08$ nM, $p<0.01$). The basal $^{45}$Ca influx in aorta from aldosterone-salt hypertensive rats ($120 \pm 4$ nM/1 cell H$_2$O/min) was also decreased by diltiazem in a concentration-dependent manner, whereas the $^{45}$Ca influx in aorta from control-salt rats ($135 \pm 3$ nM/1 cell H$_2$O/min) was not altered. Similarly, the dihydropyridine nisoldipine eliminated the basal tension ($2.7 \pm 0.5$ g) and returned the elevated basal $^{42}$K efflux from the hypertensive aorta toward control levels ($0.010 \pm 0.0003$ min, $p<0.001$). The nisoldipine IC$_{50}$ for basal tension ($0.016 \pm 0.01$ nM) was 160-fold less than the IC$_{50}$ for basal $^{42}$K efflux ($1.8 \pm 1.2$ nM, $p<0.001$). Neither diltiazem nor nisoldipine altered the basal $^{42}$K efflux or contractile activity of aorta from control-salt rats. These results suggest that the basal tension and elevated $^{42}$K efflux in aorta from aldosterone-salt hypertensive rats are supported by the entry of extracellular calcium into the tissue through potential-operated calcium channels.

Although multiple factors are involved in the pathogenesis of hypertension, it has become apparent that functional changes in vascular smooth muscle (VSM) are important to the etiology of this disease. One reflection of this alteration is the increase in the steady-state turnover of potassium, which develops during the prehypertensive stage of both genetic and mineralocorticoid-salt models of hypertension. It has been proposed that this elevated K$^+$ efflux reflects a general increase in membrane permeability caused by a decrease in membrane-bound calcium observed in vascular tissue.

The presence of calcium-dependent potassium channels has been identified in several tissues, but the demonstration of these channels in smooth muscle has been relatively recent. These channels are normally closed at the resting level of intracellular calcium. However, stimulation of VSM by an agonist elevates the intracellular calcium concentration, which initiates contraction and opens calcium-dependent potassium channels. Reports of depolarization, increased calcium influx, and elevated intracellular calcium in VSM from hypertensive rats suggested that calcium entry through potential-operated calcium channels may support the elevated potassium efflux through this pathway. The purpose of our study was to evaluate the effect of calcium antagonists on the basal $^{42}$K efflux from aorta of aldosterone-salt hypertensive rats (AHR). The effect of calcium antagonists on basal tension was also determined.

Methods

Animal and Tissue Preparations

The AHR model has been described previously. The left kidney was removed from anesthetized male Sprague-Dawley rats (150–170 g, Sasco, St. Louis, Missouri), and an osmotic minipump (Alza Corp.,...
Palo Alto, California) was implanted subcutaneously. Each rat was given a 1% NaCl solution (supplemented with 0.3% KCl to maintain potassium balance) in rats that received aldosterone to drink. The d-aldosterone (Sigma Chemical Co., St. Louis, Missouri) was dissolved in polyethylene glycol and was infused at a rate of 0.25 μg/hr for a minimum of 2 weeks (most rats were studied at 4 weeks). This protocol produced a significant elevation in systolic blood pressure, which was determined by a tail-cuff method. Each AHR had a minimum systolic pressure of 180 mm Hg. Control-salt (CS) rats were unilaterally nephrectomized and given a 1% NaCl solution to drink. Previous experiments indicated that vehicle infusions did not produce detectable differences in blood pressure, so infusions of the vehicle were omitted. The average systolic pressure of CS rats was 120 mm Hg. Rats were decapitated and the thoracic aorta quickly removed and placed in low calcium (0.25 mM Ca2+) dissection solution. For experiments involving 42K, the dissection solution was also K+ free to reduce endogenous potassium in preparation for 42K equilibration. Loose connective tissue and fat were dissected from the vessel, which was then slit lengthwise. After endothelial cells were removed from the strips by lightly stroking the intimal surface with moistened filter paper, the vessel was cut in half and mounted on a stainless steel holder.

Solutions
Normal physiological solution had the following composition (mM): Na+ 146.2, K+ 5.0, Mg2+ 1.2, Ca2+ 2.5, Cl- 143.9, HCO3- 13.5, H2PO4- 1.2, and glucose 11.4. Solutions were gassed with a mixture of 97% O2 and 3% CO2 to yield a pH of 7.4. For a calcium-free solution, CaCl2 was replaced with 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Aqueous stock solutions were made for diltiazem (gift from Marion Laboratories, Kansas City, Missouri), and nisoldipine (gift from Miles Pharmaceuticals, West Haven, Connecticut) stock solutions (1 mM) were in 100% ethanol. The highest concentration of ethanol in the experimental solutions was 0.1% for nisoldipine, which equaled 1 μM. The ethanol had no effect on control tissues.

Isotope Fluxes
Potassium efflux. These procedures were published previously and evaluated. The tissues were incubated for 2.5–3 hours at 37° C in physiological solution containing 42K (20 μCi/ml) (University of Missouri Reactor, Columbia, Missouri). After a 2-second rinse, each tissue was passed through a series of vigorously gassed tubes containing nonradioactive physiological solution. After 40 minutes, the calcium antagonist diltiazem or nisoldipine was included in the physiological solution. Preliminary experiments indicated that 15 minutes was sufficient to reach a maximal response to the calcium antagonist. Also, the dose-response curve derived from cumulative addition of a calcium antagonist to one strip was similar to that derived from exposure of individual strips to one concentration each. The more efficient protocol was adopted in which the tissues were exposed to each concentration of the antagonist for three consecutive 5-minute collection periods and then passed into tubes that contained the next higher concentration. Preliminary studies determined that the basal 42K efflux from aorta of AHR was stable for the duration of the experiment so a time correction factor was unnecessary. Because nisoldipine is light sensitive, all nisoldipine experiments were done in subdued light.

A γ spectrophotometer (Packard Instruments Co., Inc., Downers Grove, Illinois) was used to count 42K activity. Washout curves and rate constants (k/min) were calculated as reported previously on an IBM personal computer (Boca Raton, Florida). The rate constants at the 20–40-minute periods were used to determine steady-state turnover for statistical comparisons. To calculate the response to the antagonist (ΔK/min), the basal rate constant was subtracted from the rate constant obtained during the third collection period (10–15-minute exposure) at each antagonist concentration. The response to each concentration of antagonist (ΔK), was normalized in terms of the maximal response to the antagonist (ΔK max) and represented as a percentage. The median inhibitory concentration (IC50) was determined for each tissue by linear interpolation between the log concentration just below and just above the 50% response. 42Ca influx. Each half of an aorta was mounted on a holder, and the tissue was incubated for 2–3 hours at 37° C in a modified physiological solution with Ca2+=1.25 mM and H2PO4-=0. During the last 20 minutes of incubation, one half aorta was placed in a similar solution that contained diltiazem. Each half aorta was then placed in modified physiological solution (± diltiazem) that contained 45Ca (5 μCi/ml, New England Nuclear, Boston, Massachusetts). The influx was quickly terminated after 90 seconds by placement of the tissues in cold (1° C) wash solution (in which the Ca2+=5 mM and PO4=0) for 30 minutes. At the end of the wash period, the tissues were dried for 2 hours at 110° C and then weighed. The 45Ca was released by addition of 0.5 ml H2O2 (30% wt/vol) followed by a 10-minute microwave treatment. The sample was extracted into a 0.1 M HNO3 solution for 10 minutes, neutralized with 1N NaOH, and diluted into 10 ml scintillation cocktail. Standards were prepared similarly. The 45Ca tissue content (μM calcium/kg dry wt/min) at time 0 was determined by linear regression (corrected tissue counts vs. time). The 45Ca influx was normalized in terms of liter cell H2O because the aorta from AHR exhibited a 34% expansion of the cellular content. Normalization of the 45Ca influx as micromoles per liter cell H2O/kg dry wt for CS rats and 1.06 for AHR as reported by Garwitz and Jones.
FIGURE 1. Line graph showing effect of diltiazem (DZ) on basal $^{42}$K efflux in aorta from aldosterone-hypertensive rats (●) (n=6) and control-salt rats (○) (n=7). Fifteen-minute time periods for cumulative addition of DZ are noted in figure. Values represent means with representative SEM shown by vertical bar. Means are joined by straight lines.

Contraction

The thoracic aorta employed for contraction was treated similarly to that used for $^{42}$K efflux studies and was placed in low Ca$^2+$, potassium-free dissection solution and cleaned of fat and loose connective tissue. The aorta was then cut into rings (3.5 mm wide, 0.14 mm thick), and the endothelium was removed by lightly stroking the intimal layer with moistened filter paper. The rings were stretched one third of their resting diameter by means of a micrometer mounted on a force transducer. The tissue was placed in a vigorously gassed physiological solution maintained at 37° C and equilibrated for 90 minutes. Successively higher concentrations of the antagonist were added cumulatively to the bath. The tissue was exposed to each concentration of antagonist for a minimum of 10 minutes or until the contractile response reached a plateau. The tension recorded during the plateau was used in the calculation of the IC$_{50}$.

Statistics

The IC$_{50}$ values calculated for $^{42}$K efflux and tension studies were converted to log values for statistical comparison using the Student's $t$ test. Results are presented as the geometric mean ± SEM. A paired $t$ test was used to evaluate the effect of diltiazem on $^{45}$Ca influx. Ap<0.05 was required for significance.

Results

Diltiazem and $^{42}$K Efflux

The effect of diltiazem on basal $^{42}$K efflux in aorta from AHR and CS rats is presented in Figure 1. Basal $^{42}$K efflux was elevated in AHR (0.0176±0.002/min) compared with CS rats (0.0095±0.0004/min, p<0.005). This elevation was similar to previous findings. Diltiazem decreased the $^{42}$K efflux from AHR in a concentration-dependent manner (Figure 1), with an IC$_{50}$ of 0.22±0.08 μM. At high concentrations of diltiazem, the basal $^{42}$K efflux from AHR was reduced to within 15% of the CS rat aorta. The 1 mM supramaximal diltiazem concentration elevated $^{42}$K efflux in aorta from both AHR and CS rats, which may have resulted from membrane depolarization caused by the high concentration.

Diltiazem and $^{45}$Ca Influx

The basal $^{45}$Ca influx in aorta from CS rats (135±3 μM/l cell H$_2$O/min, n=17) was slightly higher than the basal $^{45}$Ca influx in aorta from AHR (120±3 μM/l cell H$_2$O/min, n=30, p<0.025). Expression of the influx in terms of micromoles per kilogram dry weight per minute reversed these differences: AHR was 127±4 versus 108±3 for CS rats (p<0.001). Diltiazem (1.0 μM and 10 μM) significantly decreased the basal $^{45}$Ca influx (Figure 2) in aorta from AHR (p<0.05) but not from CS rats. Diltiazem (0.1 μM) did not alter the basal $^{45}$Ca influx in aorta from either AHR or CS rats (data not shown).

Diltiazem and Contraction

Seventy percent of the aortic rings from AHR spontaneously developed basal tension, which was commonly (70–80%) associated with phasic contractile activity (Figure 3). The remaining 30% of AHR aorta did not develop any spontaneous tonic contractions. When present, the basal tension was relaxed a total of 2.0±0.4 g by diltiazem (Figure 3). Subsequent incubation of these tissues in 0 calcium/2 mM EGTA physiological solution produced no additional relaxation. Neither diltiazem nor 0 calcium solution altered the tension in CS rat aortic rings. The concentration-response curve for diltiazem inhibition of contraction was to the left of that for $^{42}$K efflux, as shown in Figure 4. The diltiazem IC$_{50}$ for contraction (0.04±0.02 μM) was significantly lower than the IC$_{50}$ for $^{42}$K efflux (0.22±0.08 μM, p<0.01).

Nisoldipine and $^{42}$K efflux

It was reported that some potassium channels were directly blocked by a calcium antagonist. Therefore, a class of calcium antagonist was studied that differed...
from diltiazem. Nisoldipine, a dihydropyridine, also reduced the elevated $^{42}$K efflux from AHR aorta in a concentration-dependent manner (Figure 5) with an IC$_{50}$ of 1.8±1.2 nM. Nisoldipine did not alter $^{42}$K efflux from CS rat aorta. A 15-minute exposure to the highest concentration of nisoldipine (1 $\mu$M) reduced the basal $^{42}$K efflux from AHR to within 25% of the CS rat aorta. This difference was significant ($p<0.025$), however.

**Nisoldipine and Contraction**

Nisoldipine eliminated the phasic contractile activity and relaxed the basal tension a total of 2.7±0.5 g in aortic rings from AHR. The IC$_{50}$ for nisoldipine was 0.016±0.01 nM. No additional change in contractile response occurred when the aortic rings were subsequently placed in 0 calcium/2 mM EGTA physiological solution. Neither nisoldipine nor 0 calcium solution altered the tension in CS rat rings. The concentration-response curve for nisoldipine inhibition of contraction was to the left (160-fold) of that for $^{42}$K efflux, as shown in Figure 6.

**Nisoldipine and KCl Stimulation**

We wished to determine whether depolarization-stimulated contraction and $^{42}$K efflux from CS rat aorta exhibited a similar difference in sensitivity to nisoldipine, or whether the difference was specific to AHR. Aorta from CS rats were stimulated with 30 mM KCl, which elevated tension (3.0±0.5 g, $n=6$) and $^{42}$K efflux (0.0213±0.0009/min, $n=5$) to levels observed in AHR aorta. The IC$_{50}$ for nisoldipine inhibition of tension and $^{42}$K efflux was 3.0±0.5 nM and 15±14 nM, respectively. The curve for the percent maximal inhibition of the KCl contraction by nisoldipine was fivefold to the left of the $^{42}$K efflux (Figure 7) in sharp contrast to the 160-fold shift in AHR (Figure 6).

**Discussion**

Our results suggest that 75–85% of the elevation in basal potassium efflux was secondary to increased calcium entry into the AHR aorta. Two types of calcium antagonists, diltiazem and nisoldipine, reduced the elevated potassium efflux as well as spontaneous contraction toward control values. It is likely that diltiazem and nisoldipine decreased potassium efflux in aorta from AHR by inhibition of calcium entry through potential-operated calcium channels (POC) because calcium antagonists do not alter resting calcium entry through “leak” channels, and at the IC$_{50}$ for inhibition of basal K$^+$ efflux in AHR, they selectively block calcium entry through POC and not through other channels. Activation of POC to increase calcium entry into control aorta from CS rats were stimulated with 30 mM KCl, which elevated tension (3.0±0.5 g, $n=6$) and $^{42}$K efflux (0.0213±0.0009/min, $n=5$) to levels observed in AHR aorta. The IC$_{50}$ for nisoldipine inhibition of tension and $^{42}$K efflux was 3.0±0.5 nM and 15±14 nM, respectively. The curve for the percent maximal inhibition of the KCl contraction by nisoldipine was fivefold to the left of the $^{42}$K efflux (Figure 7) in sharp contrast to the 160-fold shift in AHR (Figure 6).
VSM was shown to increase potassium efflux as well. Both calcium entry and potassium efflux from control VSM were inhibited by concentrations of diltiazem (IC50=0.6 μM) and nisoldipine (IC50=2 nM) equivalent to those that inhibited the basal potassium efflux in AHR aorta (diltiazem IC50=0.2 μM, nisoldipine IC50=1.8 nM). Likewise, the nisoldipine IC50 was similar to the binding constant for single POC in VSM. The basal 45Ca influx in AHR (but not CS rats) was also inhibited by diltiazem, although at a higher IC50 than potassium efflux. This shift may reflect technical limitations for the detection of small changes in calcium influx with our method. Because activation of POC normally requires depolarization, these results imply that the membrane potential in AHR aorta was less polarized than that in CS rat aorta or that the POC exhibited a different dependence on membrane potential. No measurements are available of resting membrane potential in VSM from AHR, and measurements in spontaneously hypertensive rats were inconsistent, with reports of a decrease or no change. Verification that the AHR aorta is depolarized or that POC in AHR behave differently at a normal membrane potential will require detailed electrophysiological study.

Although our studies were not sufficiently detailed to identify the type of channels underlying this response, the slow type of POC and a calcium-dependent potassium channel are likely candidates. Although both fast and slow calcium channels have been identified in VSM, the greater sensitivity of the slow channel to calcium antagonists would suggest that activation of slow channels underlies the increased diltiazem-sensitive calcium influx in the AHR aorta. Many potassium channels have been identified in mammalian tissues. However, the identification of calcium-dependent potassium channels in rat aorta, and the high sensitivity of basal potassium efflux in AHR to calcium antagonists suggest that calcium-dependent potassium channels mediate most of the increased potassium efflux in AHR. It is unknown whether the elevation in residual potassium efflux from AHR aorta exposed to nisoldipine (1 μM) resulted from incomplete blockage of calcium entry or from abnormal function of a calcium-independent potassium channel. The identification of the particular types of potassium channel will again require detailed electrophysiological study.

Spontaneous tension that developed in most aorta from AHR was eliminated by diltiazem and nisoldipine and therefore appeared to result from calcium entry via POC. Similar findings were observed in arteries from spontaneously hypertensive rats. Our previous studies on control rats indicated that the calcium-dependent potassium efflux and contraction were closely correlated. Therefore, it was expected that basal tension and potassium efflux in AHR would exhibit similar sensitivity to calcium antagonists. However, a sevenfold (diltiazem, Figure 4) to 160-fold (nisoldipine, Figure 6) difference in sensitivity existed between these two measures that was attributable mainly to a supersensitivity of the basal tension to calcium antagonists. For example, the diltiazem and nisoldipine IC50 for inhibition of basal potassium efflux in AHR was similar to that for KCI-stimulated potassium efflux from CS rats, whereas the IC50 for inhibition of basal tension in AHR was 10–37-fold less than that for inhibition of KCI-stimulated contraction in control rat aortas (Figure 7). The reason for the increased sensitivity of basal tension in AHR to calcium antagonists is not readily apparent. Actions on sites other than POC require much higher concentrations. Although inhibitory effects of a dihydropyridine were observed on chemically skinned aorta from spontaneously hypertensive rats, contributions from residual POC may not have been eliminated. McMahon and Paul had to modify the skinning procedure significantly to overcome this problem for studies of rat aorta.

In conclusion, this investigation demonstrated that spontaneous tension and elevation of basal potassium efflux developed in aorta from AHR and that both processes could be returned toward control levels by the calcium antagonists diltiazem and nisoldipine. The altered functions appeared to be second-
ary to increased calcium entry via POC. Basal tension was more sensitive to inhibition by calcium antagonists than potassium efflux, but the mechanisms remain to be elucidated.

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