Altered Biochemical and Functional Responses in Aorta from Hypertensive Rats

ALLAN W. JONES, BRINDA B. GEISBUHLER, SHIVENDRA D. SHUKLA, AND JACQUELYN M. SMITH

SUMMARY Factors that lead to supersensitivity of vascular smooth muscle to norepinephrine during aldosterone-salt--induced hypertension in rats appear to reside beyond ligand-α-adrenergic receptor binding, which we have shown previously to be normal. The objective of this study was to determine whether significant shifts occur in the coupling between receptors and the production of putative second messengers. Measures of [3H]myo-inositol phosphates in aorta (endothelium removed) exhibited a concentration-dependent increase to norepinephrine, with the 50% response shifted significantly to the left in the hypertensive group (7.0 ± 0.9 x 10^-7 M in 8 control rats vs 1.1 ± 0.2 x 10^-7 M in 8 hypertensive rats; p<0.001). The production of [32P]phosphatidic acid was also shifted (6.5 ± 2.5 x 10^-7 M in 16 control vs 1.9 ± 0.8 x 10^-7 M in 12 hypertensive rats; p<0.05). The functional responses of 42K efflux and contraction to norepinephrine were also significantly shifted threefold to 15-fold in the hypertensive group (p<0.001), but the 50% response typically occurred at a 10 to 100 times lower concentration than that for the production of myo-inositol phosphates and phosphatidic acid. The amplification between receptor occupancy and functional responses apparently occurs beyond the production of phosphoinositide metabolites. The fivefold shift in the 50% response of biochemical end points for the hypertensive group accounted for most of the shift (sixfold) in the functional end points. It is concluded that the increased efficacy in the hypertensive group resulted more from shifts in the relation between receptor occupancy and production of phosphoinositide metabolites than from shifts in the action of these metabolites on functions that control 42K efflux and contraction. (Hypertension 11: 627-634, 1988)

KEY WORDS • norepinephrine • supersensitivity • myo-inositol phosphates • phosphatidic acid • potassium-42 efflux • contraction • vascular smooth muscle • aldosterone

SUPERSENSITIVITY of vascular smooth muscle to catecholamines has been associated with several models of hypertension.1-3 Moreover, it precedes the elevation of blood pressure in the mineralocorticoid-salt hypertensive rat, thus implicating supersensitivity as a pathogenic factor.1-3 A systematic characterization of α-adrenergic receptors revealed no significant alteration in receptor type (α1), equilibrium dissociation constants, or maximum binding (receptor concentration) of aortic smooth muscle from the aldosterone-salt hypertensive rat (AHR).6,7 Analyses of the ligand binding and dose-response curves revealed that the agonist dissociation constant (Ks) for norepinephrine (NE) was not changed in AHR, while the efficacy was 4.4 times higher. These findings support the conclusion that postreceptor events underlie the supersensitivity in AHR.

There has been an explosion of information relating phosphoinositide metabolites to cellular regulation in many tissues, including smooth muscle.10-12 The regulation of phospholipase C (PLC) by α-adrenergic receptor occupancy is a potential site for the development of supersensitivity. PLC acts on phosphatidylinositol 4,5-bisphosphate to form myo-inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which are thought to be important regulators of calcium release and protein kinase C, respectively.10-14 IP3 is rapidly metabolized to myo-inositol 1,4-bisphosphate (IP2), myo-inositol monophosphate (IP), and myo-inositol. This last reaction is inhibited by lithium,15 and in its presence NE increased IP turnover (and by infer-
ence, IP₃) in rat aorta.¹⁶-¹⁸ DAG, the other product of PLC, is converted to phosphatidic acid (PA), which has also been suggested to regulate cell calcium.¹⁹-²⁰ Increased [³²P]PA (and by inference, DAG) was associated with NE-induced contractures and calcium fluxes in aortic smooth muscle from rabbit and with contraction in rat aorta.¹⁷ Although NE-stimulated increases in IP₃ and DAG have not been reported in rat aorta, such changes (along with IP and PA) were observed during angiotensin II stimulation of rat aortic cells that were maintained in tissue culture.²¹ The objective of this study was to determine whether supersensitivity to NE results from an altered coupling (efficacy) between α₁-adrenergic receptor occupancy and the production of the metabolic products, IP, IP₃, and PA. Preliminary findings have appeared in abstracts.²²-²⁴

Materials and Methods

Animal and Tissue Preparations

Male Sprague-Dawley rats (weight, 150–175 g; Sasco, St. Louis, MO, USA) were made hypertensive by removal of one kidney, infusion of aldosterone (0.25–1.0 μg/hr) by a minipump (Alza, Palo Alto, CA, USA), and drinking 1% NaCl. The NaCl solution was supplemented with 0.3% KCl to maintain body weight. After 3 to 4 weeks, systolic blood pressures measured by a tail-cuff device (Narco Bio-Systems, Houston, TX, USA) were typically 190 mm Hg in hypertensive rats (AHR) and 120 mm Hg in controls, which had a nephrectomy and were given 1% NaCl to drink. On the morning of the experiment, the rats were decapitated and the thoracic aorta was removed and trimmed free of fat and connective tissue while incubating in a low calcium (0.25 mM), potassium-free buffered solution. The tissues were cut into either strips or rings (for contraction), and the endothelium was removed by blotting the inner surface with filter paper moistened in the dissection solution. Strips were mounted on stainless steel holders for ⁴²K efflux or wire loops for IP and PA assays. Rings (approximately 3.5 mm long and 0.14 mm thick for controls or 0.18 mm thick for AHR) were mounted on the feet of an isometric force transducer (Grass, Quincy, MA, USA) and stretched by means of a micrometer to 1.3 times resting length.

Solutions

The normal physiological solution had the following composition (in 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 aerated with 95% O₂, 5% CO₂ mixture to obtain a pH of 7.4. Propranolol (3 μM), EDTA (0.1 mM), and ascorbic acid (1 mM) were added to all solutions to block β-adrenergic receptors and to inhibit oxidation of NE. LiCl (10 mM) was added to this solution for some protocols, while H₂PO₄⁻ was omitted during incubation with [³²P]PO₄ (ICN, Irvine, CA, USA). Myo-inositol (10 mM) was added to quench the effects of residual unincorporated [¹⁴C]myo-inositol (specific activity, 15 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO, USA).

Potassium Efflux

The potassium efflux protocol has been published¹-³ and evaluated.²² The strips were incubated for 3 hours at 37°C in the physiological solution containing ⁴²K (20 μCi/ml; University of Missouri Research Reactor, Columbia, MO, USA). The tissues were then passed through a series of vigorously aerated tubes, some containing experimental solutions with myo-inositol or lithium (or both) to mimic the protocols for measures of tissue myo-inositol phosphates. Exposures to NE alone were followed by washes in normal physiological solutions before the next concentration of NE was added. When lithium was present, each tissue was exposed to only one concentration of NE (4 strips/aorta). Tubes and residual tissue activity were counted on a gamma spectrophotometer (Packard Instruments, Downers Grove, IL, USA). Washout curves and rate constants, k, were calculated on an IBM personal computer (Boca Raton, FL, USA). Dose-response relations were derived by dividing the maximum change in rate for a given NE by the maximum change in rate for the highest NE. The median effective concentration, EC₅₀, was determined for each tissue by linear interpolation between log response values just below and just above the 50% response.

Contraction

The mounted tissues were placed in a vigorously aerated physiological solution for 1 to 2 hours. Successively higher concentrations of NE were added cumulatively to the bath until a steady response occurred (10–15 minutes), followed by the next concentration until a maximal response occurred. For the series involving preincubation with myo-inositol (10 mM) and lithium (10 mM), 20 and 10 minutes, respectively, an initial concentration of NE was tested on each strip (4 strips/aorta) followed by testing with supermaximal NE (3 μM). The percent response was calculated by dividing the initial force by the force developed in 3 μM NE.

Inositol Phosphate Assay

After the length was measured, each strip was placed in a physiological solution containing [³²P]myo-inositol (10 μCi/ml) for 2 hours followed by 10 minutes in a nonlabeled solution containing myo-inositol (10 mM). The strips were transferred into a physiological solution containing lithium (10 mM) for 10 minutes before a 30-minute incubation in a similar solution with one of the NE concentrations (NE = 0 for basal levels). The tissues were frozen by clamps cooled in liquid N₂ and were placed into 600 μl of chloroform/methanol (1:2) solution at 1°C for 30 minutes. The samples were then vortexed, and 0.9 ml of chloroform and 0.9 ml of water were added, with vortexing after each addition. The tissues were removed, and the samples were centrifuged to separate the layers. The aque-
Tracts were washed with 3 ml of chloroform, vortexed, orated under a stream of nitrogen gas. The residue was resuspended in 3 ml of chloroform and washed three times with 1 ml of 0.1 N HCl. Following each acid treatment of chloroform/methanol/HCl (12 N; 400:400:5 by volume). In this system PA separated with a Rf value of 0.5, which was identified by standards. Phospholipid spots were visualized by a 2-p-toluidinylnaphthylene-6-sulfonate spray (Sigma, St. Louis, MO, USA), scraped directly into vials containing 10 ml of scintillation solution and counted. Data were calculated as 32P contents in counts per minute per millimeter of aorta.

**Statistics**

Because the EC50 for NE was normally distributed on a log rather than an arithmetic scale, the log values of EC50 were used to make statistical comparisons. The arithmetic means were used for other comparisons. Unless noted otherwise, a p value below 0.05 was deemed significant based on the Student’s t test. The Bonferroni correction was applied where multiple comparisons were made, such as between a basal value and responses to a series of NE concentrations.

**Results**

**42K Efflux and Contraction**

NE increased 42K efflux and contraction in a concentration-dependent manner, as shown in Figure 1. The relations were shifted to the left (indicating supersensitivity) in AHR. Since these processes are calcium-dependent in rat aorta, the shift in both responses indicates that a common defect may exist between α-adrenergic receptor occupancy and calcium signaling.

**Phosphatidic Acid Assays**

Aortic strips were incubated 2 hours in a modified physiological solution containing [32P]P04 (50 μCi/ml, essentially PO4 free), with calcium reduced by 50%. Tissues could produce nearly normal force over this period (Jones, unpublished observations). The strips were then washed for 30 minutes in nonlabeled solution with 1.2 mM H2PO4-, followed by 10 minutes in a similar solution with one of the NE concentrations (NE = 0 for basal levels). Pilot studies indicated that [32P]PA reached a plateau between 5 and 15 minutes in NE. At the designated time the reaction was stopped by freeze clamping the aortic tissue. The extraction procedure was based on one used for rabbit aorta. The strips were mechanically homogenized in a solution of chloroform/methanol/HCl (12 N; 400:400:5 by volume) and centrifuged at 1500 rpm for 15 minutes. The supernatant was removed, and the residue reextracted in another chloroform/methanol/HCl solution (800:400:2.5). The extracts were combined and evaporated under a stream of nitrogen gas. The residue was resuspended in 3 ml of chloroform and washed three times with 1 ml of 0.1 N HCl. Following each acid wash, the sample was mixed and centrifuged and the upper (acid layer) removed. The combined acid extracts were washed with 3 ml of chloroform, vortexed, and centrifuged. The lower chloroform layer was added to the original chloroform extract and evaporated to dryness under nitrogen. The phospholipid residue was spotted on silica gel G plates (Analtech Corp, Newark, DE, USA) for thin layer chromatography using a solvent system of chloroform/pyridine/formic acid (50:30:5 by volume). In this system PA separated with a Rf value of 0.5, which was identified by standards. Phospholipid spots were visualized by a 2-p-toluidinylnaphthylene-6-sulfonate spray (Sigma, St. Louis, MO, USA), scraped directly into vials containing 10 ml of scintillation solution and counted. Data were calculated as 32P contents in counts per minute per millimeter of aorta.

**Statistics**

Because the EC50 for NE was normally distributed on a log rather than an arithmetic scale, the log values of EC50 were used to make statistical comparisons. The arithmetic means were used for other comparisons. Unless noted otherwise, a p value below 0.05 was deemed significant based on the Student’s t test. The Bonferroni correction was applied where multiple comparisons were made, such as between a basal value and responses to a series of NE concentrations.
The effects of NE were also evaluated in the presence of lithium, because the protocol for measuring IP and IP$_2$ employed lithium, an ion that is known to alter Na$^+-$K$^+$ transport. Lithium decreased basal $^{42}$K efflux in AHR ($p<0.01$), but not in controls (Figure 2). NE increased $^{42}$K efflux in a concentration-dependent manner and, as shown in Figure 3, the relation was shifted to the left in AHR ($p<0.005$). Lithium had no significant effect on the EC$_{50}$ in controls ($p<0.05$) for NE stimulation of $^{42}$K efflux and contraction (Table I), while lithium had no significant effect on the EC$_{50}$ in AHR. As a result of the effect of lithium on controls, the contractile response of AHR, which was shifted threefold in physiological solution, was not significantly different in lithium solutions (see Table 1).

Inositol Phosphates

Thirty-minute exposure to NE significantly increased the content of $[^3$H$]IP$ and $[^3$H$]IP_2$ in controls and AHR, as shown in Table 2. No significant change in $[^3$H$]IP_3$ was observed at 30 minutes (data not shown). The aorta exhibited sustained functional responses throughout the 30-minute exposure to NE, as shown in Figure 2. The basal and NE stimulated contents of $[^3$H$]IP$ and $[^3$H$]IP_2$ were significantly greater in AHR than in controls on the basis of aortic length (see Table 2). These differences were much reduced after normalizing the contents on the basis of cellular H$_2$O (values in parentheses in Table 2), which indicated that the differences in AHR resulted mainly from cellular hypertrophy.

The NE-stimulated responses for $[^3$H$]IP$ and $[^3$H$]IP_2$ in AHR (Figure 4) were significantly ($p<0.001$) shifted to the left of control responses, indicating supersensitivity. The EC$_{50}$ for controls was not consistently different from the $K_A$. In contrast, the EC$_{50}$ for controls was not consistently different from the $K_A$.

### Table 1. EC$_{50}$ Values for Norepinephrine Stimulation of Phosphatidic Acid and Myo-Inositol Monophosphate and 1,4-Bisphosphate Production, $^{42}$K Efflux, and Contraction in Rat Aorta

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of control aortas</th>
<th>Control (M)</th>
<th>No. of AHR aortas</th>
<th>AHR (M)</th>
<th>Control/AHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>16</td>
<td>6.5 ± 2.5 × 10$^{-7}$</td>
<td>12</td>
<td>1.9 ± 0.8 × 10$^{-7}$*</td>
<td>3.4</td>
</tr>
<tr>
<td>IP$^+$</td>
<td>6</td>
<td>11 ± 2 × 10$^{-7}$</td>
<td>7</td>
<td>2.0 ± 0.1 × 10$^{-7}$†</td>
<td>5.5</td>
</tr>
<tr>
<td>IP$_2$†</td>
<td>7</td>
<td>7.0 ± 0.9 × 10$^{-7}$</td>
<td>8</td>
<td>1.1 ± 0.2 × 10$^{-7}$</td>
<td>6.4</td>
</tr>
<tr>
<td>$K_A$</td>
<td>7</td>
<td>4.8 ± 1.5 × 10$^{-7}$</td>
<td>9</td>
<td>4.8 ± 1.2 × 10$^{-7}$</td>
<td>1.0</td>
</tr>
<tr>
<td>$^{42}$K</td>
<td>7</td>
<td>35 ± 12 × 10$^{-9}$</td>
<td>7</td>
<td>2.4 ± 0.5 × 10$^{-9}$ ‡</td>
<td>14.6</td>
</tr>
<tr>
<td>$^{42}$K†</td>
<td>6</td>
<td>6.8 ± 0.9 × 10$^{-9}$</td>
<td>6</td>
<td>1.9 ± 0.4 × 10$^{-9}$§</td>
<td>3.6</td>
</tr>
<tr>
<td>T</td>
<td>8</td>
<td>15 ± 2 × 10$^{-9}$</td>
<td>7</td>
<td>5.2 ± 2.4 × 10$^{-9}$</td>
<td></td>
</tr>
<tr>
<td>T†</td>
<td>13</td>
<td>6.2 ± 1.3 × 10$^{-9}$</td>
<td>13</td>
<td>3.6 ± 0.7 × 10$^{-9}$</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Values are means ± SEM. PA = phosphatidic acid; IP = myo-inositol monophosphate; IP$_2$ = myo-inositol 1,4-bisphosphate; $K_A$ = agonist dissociation constant derived from the effects of a noncompetitive antagonist, Dibenamine. on norepinephrine stimulation of $^{42}$K efflux; T = contraction; AHR = aldosterone-salt hypertensive rats.
* $p<0.05$, † $p<0.001$, § $p<0.005$, || $p<0.02$, compared with control values.
†Measured in the presence of 10 mM Li.
Table 2. Effects of 3 μM Norepinephrine on [3H]Myo-Inositol Phosphate Content in Aortas from Control and Aldosterone-Salt Hypertensive Rats

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control No. of aortas</th>
<th>AHR No. of aortas</th>
<th>IP</th>
<th>IP2</th>
<th>IP</th>
<th>IP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal (cpm/mm length)</td>
<td>18</td>
<td>22</td>
<td>139±22</td>
<td>21±3</td>
<td>225±21*</td>
<td>63±7*</td>
</tr>
<tr>
<td>Basal (cpm/mg cell H2O)</td>
<td>(556±88)</td>
<td>(84±12)</td>
<td>(562±52)</td>
<td>(158±18)*</td>
<td>(158±18)*</td>
<td></td>
</tr>
<tr>
<td>NE (cpm/mm length)</td>
<td>18</td>
<td>21</td>
<td>211±23</td>
<td>146±13</td>
<td>408±30*</td>
<td>274±18*</td>
</tr>
<tr>
<td>NE (cpm/mg cell H2O)</td>
<td>(844±92)</td>
<td>(584±52)</td>
<td>(1020±75)</td>
<td>(685±45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANE (cpm/mm length)</td>
<td>18</td>
<td>146±13*</td>
<td>184±18*†</td>
<td>211±16*‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANE (cpm/mg cell H2O)</td>
<td>(500±52)‡</td>
<td>(528±40)‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM. Tissues were exposed to NE for 30 minutes in a physiological saline solution with 10 mM Li. *p<0.01, compared with control values. †AHR contain approximately 1.6 times more cellular H2O per length than do control rats (0.40 mg H2O/mm vs 0.25 mm H2O/mm in control rats). ‡Values corrected for cellular hypertrophy appear in parentheses. See Table 1 for key to abbreviations. 

Discussion

Phosphatidic Acid

NE also altered [32P]PA in a concentration-dependent manner, which was shifted to the left in AHR (Figure 5). The 3.4-fold shift in EC50 for AHR (see Table 1) was not as great as that for IP and IP2; however, the EC50 was significantly lower than the Kd (p<0.025) in contrast to control values (p = NS). Also of significance in Table 1, the EC50 values for functional end points (42K efflux, contraction) were 100-fold to 100-fold lower than those for the biochemical measures (IP, IP2, and PA). Despite this large difference, the average ratios of EC50 for controls to AHR exhibited reasonable agreement between functional (sixfold) and biochemical end points (fivefold).

FIGURE 4. Effect of norepinephrine (NE; log scale) on percent change in [3H]myo-inositol monophosphate (IP) and 1,4-bisphosphate (IP2) contents of aorta from control rats (C; •, △) and aldosterone-salt hypertensive rats (AHR; ○, ◊). The EC50 is indicated for controls (●; n = 6–7) and AHR (○; n = 7–8). The agonist dissociation constant (Kd) for NE (X) is also shown (taken from Smith et al.7 with permission of the American Society of Pharmacology and Experimental Therapeutics). Vertical and horizontal bars indicate SEM. Means are joined by lines. The concentration-response relations were significantly shifted to the left in AHR (p<0.001).

FIGURE 5. Effect of a 10-minute exposure to norepinephrine (NE) on [32P]phosphatidic acid ([32PA] content and percentage of maximal response in aorta from control rats (C; ●) and aldosterone-salt hypertensive rats (AHR; ◊). Means (n = 6–16) are joined by straight lines; vertical bars indicate SEM. Asterisk indicates significant difference (p<0.05) for controls versus AHR. The concentration-response relation was significantly shifted to the left in AHR (p<0.001). The percent response (right panel) was computed by dividing the difference between the [32P]PA content at each NE and the basal content (NE = 0) by the maximum difference. Because some [32P]PA contents at low NE were less than the basal levels, this normalization led to apparently negative responses that were not statistically significant (p > 0.05). The EC50 was calculated for each rat by interpolation of the percent responses between 3×10^-2 M and 3×10^-3 M.
The concentration-dependent production of metabolites from two pathways (IP$_3$→IP$_2$→IP and DAG→PA; Figure 6) exhibited significant shifts to the left in AHR and implicate a common source, $\alpha_1$-adrenergic receptor–PLC coupling. Our earlier study argues against changes in $\alpha_1$-adrenergic receptor type, affinity, or number as major factors. Likewise, altered sensitivity at the level of the contractile proteins does not appear to be a major factor in NE supersensitivity. The EC$_{50}$ (0.36 $\mu$M) for intracellular calcium ([Ca$^{2+}$]) stimulation of contraction in chemically skinned aorta from controls was unchanged in AHR. This study is also consistent with our hypothesis that the major defects in calcium regulation of vascular smooth muscle from AHR result from altered membrane function.

A schematic model (admittedly oversimplified) for $\alpha_1$-adrenergic receptor regulation of vascular smooth muscle appears in Figure 6. $\alpha_1$-Adrenergic receptor occupancy is shown to control PLC activity, although current information indicates that guanosine 5'-triphosphate binding protein(s) may couple the $\alpha_1$-adrenergic receptor to PLC.$^{11, 12}$ One substrate, phosphatidylinositol 4,5-bisphosphate, is shown to form the products IP$_3$ and DAG, both putative second messengers. IP$_3$ for calcium release from sarcoplasmic reticulum,$^{13-15}$ and DAG for protein kinase C activity.$^8$ Protein kinase C has multiple substrates, including phospholipase A$_2$, and in rat aorta may influence contraction through action of arachidonate metabolites on calcium entry as well as by direct effects on contractile proteins.$^{12}$

The metabolites of IP$_3$ measured in this study are shown in Figure 6 to be serially produced, with lithium inhibiting the breakdown of IP to myo-inositol. As a cautionary note, IP$_2$ and IP can be produced directly from phosphatidylinositol monophosphate and phosphatidylinositol, respectively, without IP$_3$ being formed.$^8, 11$ It therefore is important for future studies to confirm that IP$_3$ levels increase or levels of phosphatidylinositol 4,5-bisphosphate decrease (or both occur) during $\alpha_1$-adrenergic occupancy. These changes have been reported for angiotensin II stimulation of rat aortic cells maintained in tissue culture.$^{12}$ Such cells, however, undergo major changes and usually exhibit only small responses to $\alpha_1$-adrenergic stimulation.

The metabolite of DAG that we measured, PA, has also been proposed to regulate calcium entry.$^{19, 20}$ and changes in PA were correlated with the maintained contractile responses and $^{45}$Ca influx in rabbit aorta.$^{21}$ The potential role of PA in such signaling has not received as much emphasis as the DAG–protein kinase C pathway, however. Significant DAG and resultant PA were produced by hydrolysis of phosphatidylinositol in rat aortic cells maintained in culture, and phosphatidylinositol 4,5-bisphosphate hydrolysis (see Figure 6) may produce only a limited amount of DAG and PA transiently.$^{22}$ As noted, future studies must confirm in intact arteries that DAG levels and protein kinase C activity increase in response to $\alpha_1$-adrenergic receptor occupancy. Our study and that of Campbell et al.$^{21}$ have confirmed that $[^{32}\text{P}]/PA$ rises significantly.

The two functional end points were interpreted to reflect the $\alpha_1$-adrenergic receptor effect on free [Ca$^{2+}$]. Although force development has been well established to be calcium-dependent, major questions have been raised concerning changes in sensitivity to [Ca$^{2+}$] during maintained contraction. Indeed, it has been proposed that force sustained during receptor occupancy occurs with little or no elevation in cytosolic calcium ([Ca$^{2+}$]), in Figure 6). Instead, calcium in the submembrane domain acting with DAG was proposed to regulate contraction through protein kinase C activity.$^{12}$ Although this proposal requires more evidence before acceptance, it underscores our emphasis on the calcium-dependent $^{42}$K efflux as a better measure of the effective calcium concentration in the submembrane domain ([Ca$^{2+}$]$_{m}$ in Figure 6).$^{1, 7, 27, 30}$

Calcium-dependent potassium channels have been.
identified in many cell types and were systematically characterized in arterial smooth muscle. These channels exhibited high conductance and stable response to alterations in [Ca2+], and in membrane potential, ENa, over the physiological range. NE caused a fourfold increase in calcium-dependent 42K efflux from rabbit aorta, with little or no change in ENa. For these reasons, the NE-stimulated 42K efflux may yield a reliable, although uncalibrated, index of [Ca2+] in aorta without major interference from changes in ENa. It is unknown, however, whether phosphorylation of calcium-dependent potassium channels increases potassium current, as reported in Helix neurons.

The overall effect of α,-adrenergic receptor occupancy on calcium would be to increase [Ca2+] and [Ca2+] in a combination of calcium release and influx. Because the binding of ligands to α,-adrenergic receptor follows the laws of mass action, 50% occupancy would be expected at NE with a Kd of 5 x 10^-7 M (see Table 1). NE just above and below this value (EC50 = 1-11 x 10^-7 M, see Table 1) caused a 50% increase in aortic IP3, IP2, and PA, which shows that the relation between the biochemical response and receptor occupancy approaches 1 to 1 over the midrange. In sharp contrast, the concentration-response relations (EC50, see Table 1) for functional measures of calcium were shifted 10-fold to 100-fold to the left of those for the production of phosphoinositide metabolites. Similar differences have been reported for intact aorta as well as in cultured cells from rat aorta. Our previous analyses of α,-adrenergic receptor binding indicated that only 6% occupancy in controls and 2% in AHR was required for 50% response of the calcium-dependent 42K efflux. The amplification between receptor occupancy and functional responses apparently occurs beyond the production of second messengers; that is, a relatively small change in tissue IP3, DAG, or PA seemingly has a large effect on functional responses. EC50 values in AHR for the biochemical end points were lower than the Kd for NE (see Table 1), in sharp contrast to those for controls. The shifts in EC50 for the biochemical end points in AHR (fivefold) accounted for most of the shifts in EC50 for the functional end points (sixfold). For these reasons, it is concluded that the increased efficacy reported by us for AHR resulted more from shifts in the relation between receptor occupancy and PLC products than from shifts in the action of these products on functions that control 42K efflux and contraction. The action of these products apparently was highly efficient under normal conditions, while the less efficient production of phosphoinositide metabolites was subjected to regulation by the aldosterone-salt treatment.

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