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Heaslip, Richard Joseph

MODULATION OF INTRACELLULAR CALCIUM BY VASOCONSTRICTORS AND VASODILATORS IN THE RAT AORTA

The Ohio State University

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MODULATION OF INTRACELLULAR CALCIUM BY
VASOCONSTRICTORS AND VASODILATORS IN
THE RAT AORTA

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Richard Joseph Heaslip, B.A.

*****
The Ohio State University
1982

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To my parents, Marie E. and Richard J. Heaslip.

They have always been my best teachers.

and

To Nancy, whose love, support and encouragement lighten every burden.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>I. VASCULAR SMOOTH MUSCLE FUNCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. SMOOTH MUSCLE CONTRACTION</td>
<td>3</td>
</tr>
<tr>
<td>A. The Role of Calcium in Smooth Muscle Contraction</td>
<td>3</td>
</tr>
<tr>
<td>B. The Biochemical Basis for Calcium-Induced Smooth Muscle Contraction</td>
<td>5</td>
</tr>
<tr>
<td>C. Sources of Activator Calcium in Smooth Muscle</td>
<td>11</td>
</tr>
<tr>
<td>III. MECHANISMS OF VASODILATATION</td>
<td>16</td>
</tr>
<tr>
<td>IV. SELECTED VASODILATORY AGENTS</td>
<td>23</td>
</tr>
<tr>
<td>A. Calcium Entry Blockers: Verapamil, Nifedipine and SKF24260</td>
<td>24</td>
</tr>
<tr>
<td>B. Intracellular Calcium Antagonants: 2-Substituted Methyleneoxyindenes</td>
<td>26</td>
</tr>
<tr>
<td>C. Cyclic Nucleotides: cAMP and cGMP</td>
<td>27</td>
</tr>
<tr>
<td>D. Papaverine</td>
<td>29</td>
</tr>
<tr>
<td>E. Sodium Nitroprusside</td>
<td>30</td>
</tr>
<tr>
<td>V. STATEMENT OF THE PROBLEM</td>
<td>31</td>
</tr>
</tbody>
</table>

vi
I. EVIDENCE FOR THE EXISTENCE OF TWO DISTINCT POOLS OF INTRACELLULAR CALCIUM ACCESSIBLE TO MOBILIZATION BY NOREPINEPHRINE.......................................................... 33

A. Introduction................................................................. 33

B. Methods................................................................. 34

1. Response of aorta to NE in calcium-free medium................................. 35

2. Effect of phentolamine on aortic response to NE......................................... 36

3. Concentration-response experiments to NE in calcium-free medium................. 37

4. Effect of withdrawal of extracellular calcium on aortic tissue contracted by NE and KCl.......................................................... 38

5. Dissociation of the phasic and sustained components of the NE response in calcium-free medium........................................... 38

6. Cumulative concentration-response curves to NE in calcium-free medium after elimination of the phasic components..... 39

C. Results................................................................. 40

1. Response of aorta to NE in calcium-free medium........................................ 40

2. Effect of phentolamine on aortic response to NE........................................ 41

3. Concentration-response experiments to NE in calcium-free medium............... 46

4. Effect of withdrawal of extracellular calcium on aortic tissue contracted by NE and KCl.......................................................... 46
**CHAPTERS**  

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5. Dissociation of the phasic and sustained components of the NE response in calcium-free medium</td>
</tr>
<tr>
<td>6. Cumulative concentration-response curves to NE in calcium-free medium after elimination of the phasic components</td>
</tr>
<tr>
<td>D. Discussion</td>
</tr>
<tr>
<td>II. EFFECTS OF VASODILATORY AGENTS ON THE PHASIC AND SUSTAINED CONTRACTIONS INDUCED BY NOREPINEPHRINE IN THE ABSENCE OF EXTRACELLULAR CALCIUM</td>
</tr>
<tr>
<td>A. Introduction</td>
</tr>
<tr>
<td>B. Methods</td>
</tr>
<tr>
<td>1. Spasmolytic activity of vasodilator agents towards NE- and KCl-induced contractions in the presence of extracellular calcium</td>
</tr>
<tr>
<td>2. Antispasmodic activity of vasodilatory agents towards NE-induced phasic and sustained contractions in the absence of extracellular calcium</td>
</tr>
<tr>
<td>3. Effect of vasodilator agents on the NE concentration-response curve in calcium-free medium after elimination of the phasic component of NE contraction</td>
</tr>
<tr>
<td>4. Effect of phenoxybenzamine pretreatment on the NE concentration-response curve in calcium-free medium after the elimination of the phasic component of contraction</td>
</tr>
<tr>
<td>C. Results and Discussion</td>
</tr>
<tr>
<td>1. Dibutyryl-cAMP, 8-Br-cGMP, Papaverine and Sodium Nitroprusside</td>
</tr>
<tr>
<td>a) Results</td>
</tr>
<tr>
<td>b) Discussion</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NE-Induced Aortic Contractions in Calcium-Free-EGTA Medium in Presence or Absence of Intermittent Re-exposure to Calcium</td>
<td>54</td>
</tr>
<tr>
<td>2. Log IC50 Values With Their 95 Percent Confidence Intervals for the Inhibition of NE-Induced Contractions in the Rat Aorta in the Presence or Absence of Extracellular Calcium by a Variety of Vasodilatory Agents</td>
<td>81</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Proposed Regulation of Smooth Muscle Contractility by Calcium and cAMP</td>
<td>22</td>
</tr>
<tr>
<td>2.</td>
<td>Contractile Response of the Rat Aorta to Potassium and NE in Calcium-Free Medium Containing 0 to 10 mM EGTA</td>
<td>42</td>
</tr>
<tr>
<td>3.</td>
<td>Representative tracings of Contractions of the Rat Aorta Produced by NE in Calcium-Free EGTA (1.0 mM) Medium</td>
<td>44</td>
</tr>
<tr>
<td>4.</td>
<td>Noncumulative Concentration-Response Curves of the Phasic and Sustained Components of the NE-Induced Contractions of the Rat Aorta in Calcium-Free EGTA (1.0 mM) Medium</td>
<td>48</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of Withdrawal of Extracellular Calcium from the Medium on Rat Aortic Tissue Contracted by NE or KCl Under Conditions of Continued Exposure to the Agonists</td>
<td>50</td>
</tr>
<tr>
<td>6.</td>
<td>Representative Tracings of the Effects of Repeated Stimulation of the Rat Aortic Tissue with 3x10⁻⁶M NE in Calcium-Free Sustained Components of the NE response</td>
<td>52</td>
</tr>
<tr>
<td>7.</td>
<td>Representative Tracing of Concentration-Response Effect of NE on the Rat Aorta in Calcium-Free EGTA Medium After Elimination of the Phasic Component of the NE Response</td>
<td>56</td>
</tr>
<tr>
<td>8.</td>
<td>Diagram of Experimental Protocol Used to Assess the Antispasmodic Activity of Vasodilatory Agents Towards NE-Induced Phasic and Sustained Contractions in the Absence of Extracellular Calcium</td>
<td>70</td>
</tr>
<tr>
<td>9.</td>
<td>Diagram of the Experimental Protocol Used to Assess the Effects of Vasodilator Agents on the NE Concentration-Response Curve in Calcium-Free EGTA Buffer after Elimination of the Phasic Component of Contraction</td>
<td>75</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of db-cAMP on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the Presence of Extracellular Calcium</td>
<td>83</td>
</tr>
<tr>
<td>Figure</td>
<td>Effect of Papaverine on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the Presence of Extracellular Calcium</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>12</td>
<td>Effect of 8-Br-cGMP on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the Presence of Extracellular Calcium</td>
<td>87</td>
</tr>
<tr>
<td>13</td>
<td>Effect of Sodium Nitroprusside on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the Presence of Extracellular Calcium</td>
<td>89</td>
</tr>
<tr>
<td>14</td>
<td>Effect of db-cAMP on Cumulative NE Concentration-Response Curves in Calcium-Free Medium After Elimination of the Phasic Component of Contraction</td>
<td>92</td>
</tr>
<tr>
<td>15</td>
<td>Effect of 8-Br-cGMP on Cumulative NE Concentration-Response Curves in Calcium-Free Medium After Elimination of the Phasic Component of Contraction</td>
<td>94</td>
</tr>
<tr>
<td>16</td>
<td>Effect of Phentolamine on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the Presence of Extracellular Calcium</td>
<td>103</td>
</tr>
<tr>
<td>17</td>
<td>Effect of Phentolamine on Cumulative NE Concentration-Response Curves in Calcium-Free Medium After Elimination of the Phasic Component of Contraction</td>
<td>106</td>
</tr>
<tr>
<td>18</td>
<td>Schild Plot for Phentolamine-Induced Shifts of the Cumulative NE Concentration-Response Curve in Calcium-Free Medium After Elimination of the Phasic Component of Contraction</td>
<td>108</td>
</tr>
<tr>
<td>19</td>
<td>Effect of Phenoxybenzamine Pretreatment on the Cumulative NE Concentration-Response Curve in Calcium-Free Medium After Elimination of the Phasic Component of Contraction</td>
<td>110</td>
</tr>
<tr>
<td>20</td>
<td>Effect of Nifedipine on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the Presence of Extracellular Calcium</td>
<td>119</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>21.</td>
<td>Effect of SKF24260 on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the presence of Extracellular Calcium.</td>
<td>121</td>
</tr>
<tr>
<td>22.</td>
<td>Effect of Verapamil on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the presence of Extracellular Calcium.</td>
<td>123</td>
</tr>
<tr>
<td>23.</td>
<td>Effect of bu-MDI on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the presence of Extracellular Calcium.</td>
<td>126</td>
</tr>
<tr>
<td>24.</td>
<td>Effect of pr-MDI on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the presence of Extracellular Calcium.</td>
<td>128</td>
</tr>
<tr>
<td>25.</td>
<td>Effect of Q-pr-MDI on Contractions of the Rat Aorta Induced by NE in the Presence or Absence of Extracellular (Bath) Calcium or by Potassium in the presence of Extracellular Calcium.</td>
<td>130</td>
</tr>
<tr>
<td>26.</td>
<td>Effect of pr-MDI on Cumulative NE Concentration-Response Curves in Calcium-Free Medium After Elimination of the Phasic Component of Contraction.</td>
<td>134</td>
</tr>
<tr>
<td>27.</td>
<td>Effect of Verapamil on Cumulative NE Concentration-Response Curves in Calcium-Free Medium After Elimination of the Phasic Component of Contraction.</td>
<td>136</td>
</tr>
<tr>
<td>28.</td>
<td>Contractile Response of the Rat Aorta to U44069 and KCl in the Absence of Extracellular Calcium.</td>
<td>155</td>
</tr>
<tr>
<td>29.</td>
<td>Effects of Nifedipine, Q-bu-MDI and bu-MDI on the Contractile Response of the Rat Aorta to U44069 in the Absence of Extracellular Calcium.</td>
<td>157</td>
</tr>
</tbody>
</table>
**INTRODUCTION**

**Vascular Smooth Muscle Function**

The continued viability of the cells of an organism is dependent upon the delivery of nutrients to those cells and upon the removal of toxic metabolic by-products from them. In higher organisms this transport function is performed by the blood as it circulates through the cardiovascular system. Since the proper function, and at times the life of a cell requires that the transport of oxygen, nutrients and toxic by-products be adjusted according to the metabolic needs of the cells of the various organ systems, it is important that an organism maintain control over regional blood perfusion.

The most direct method by which an organism can control a regional blood flow is by regulating the diameter of the blood vessels which perfuse that area (Guyton, 1976). Small changes in blood vessel diameter produce marked changes in the amount of blood flowing through that vessel; a reduction of diameter by 30 percent results in an 80 percent reduction in blood flow (Guyton, 1976).

Dynamic control over blood vessel caliber is possible by regulating the contractile activity of vascular smooth muscle in the blood vessels. This muscle tissue is the predominant structural feature of both the arteries and the veins of the circulatory system, and since it primarily encircles the blood vessel lumen, its
contraction results in a reduction of vessel caliber (Copenhaver et al., 1978).

Besides regulating blood flow through various individual vascular beds, vasoconstriction also serves as one of a number of mechanisms whereby an organism is able to regulate its systemic blood pressure. By decreasing the caliber of blood vessels, vasoconstriction greatly increases the resistance of those vessels to blood flow; a 30 percent reduction in vessel diameter results in a 416 percent increase in its resistance to blood flow (Guyton, 1976). When vasoconstriction occurs in a large number of vascular beds the increased resistance to blood flow results in an enhanced "back-pressure" in the arterial portions of the circulatory system, and an elevation of the systemic blood pressure (Guyton, 1976).

The following study explores the regulatory processes which govern both the induction and the inhibition of vascular smooth muscle contraction. Since abnormalities of vascular smooth muscle contraction have been associated with a number of common pathological conditions, including hypertension (Haeusler and Finch, 1972) and vasospastic disorders (Likoff, 1976), studies such as these give insight both into possible sites of pathogenic lesions in the tissue and into potential therapeutic approaches to the treatment of these diseases.
II. Smooth Muscle Contraction

A. The Role of Calcium in Smooth Muscle Contraction

Studies investigating the mechanisms of smooth muscle activation have largely centered upon the role of calcium as the intracellular stimulus for smooth muscle contraction. In smooth muscle, as in skeletal (Sandow, 1965; Bianchi, 1975) and cardiac muscles (Katz, 1970), agonists are proposed to initiate contraction by increasing the concentration of free ionized calcium in the myoplasm (Bohr, 1964; Bohr and Webb, 1978; Bolton, 1979).

The role of calcium in smooth muscle contraction was suggested in early work, when it was demonstrated that the removal of calcium from the salt solution bathing the muscle tissue resulted in the attenuation or loss of contractility (Bozler, 1960; Berger and Marshall, 1961; Edman and Schild, 1961; Chujyo and Holland, 1962; Waugh, 1962; Bohr, 1963). In addition, it was found that, within limits, the magnitude of contraction induced by a variety of agonists was a function of the concentration of calcium to which the muscle was exposed (Woolley, 1958; Durbin and Jenkinson, 1961; Edman and Schild, 1962; Waugh, 1962; Sperelakis, 1962a; Clegg et al., 1963; Godfraind and Kaba, 1972). Since tissue responses to a number of stimulatory agents depend upon the presence of extracellular calcium, it was concluded that a universal role for calcium in smooth muscle contraction is likely (Bohr, 1964).

Consistent with this conclusion has been the repeated demonstration that the flux of calcium into the cell through the sarcolemma increases during smooth muscle contraction (Robertson,
1960; Briggs and Melvin, 1961; Briggs, 1962; Sperelakis, 1962b; Chuyo and Holland, 1962; Urakawa and Holland, 1964). Furthermore, this calcium flux is proportional to the degree of tissue stimulation. Upon norepinephrine (NE) stimulation of smooth muscle tissue, the rate of influx of calcium into the sarcoplasm is dependent upon the concentration of agonist (Hurwitz et al., 1972; Godfraind, 1976). Similarly, when muscle strips are completely depolarized with high potassium buffers, the resulting degree of contraction is proportional to the entry of calcium into the cell (van Breemen, 1977).

Perhaps the best in vitro evidence for the regulation of smooth muscle contraction by calcium comes from tissue preparations in which the ability of the sarcolemma to inhibit the entry of extracellular calcium into myoplasm is chemically compromised. In these tissue preparations, called "skinned muscle preparations", the concentrations of free intracellular calcium approximates that of the calcium concentrations in the extracellular fluid. Thus, by regulating the amount of calcium in the buffer bathing the tissue a quantitative assessment of the concentration of calcium necessary for intracellular activation of smooth muscle can be determined. Using prolonged glycerine treatment to prepared skinned fibers of hog carotid artery, Filo et al. (1965) demonstrated that free calcium concentrations greater than $10^{-7}$M induced contraction, with maximum activation occurring at $10^{-6}$M calcium. Endo et al. (1977) later used a saponin skinning procedure to demonstrate that rabbit pulmonary artery and guinea pig taenia caecum are each activated in a concentration-dependent manner by intracellular calcium concentrations of $10^{-7}$M to
$10^{-5}$M. Similar results have also been reported using Triton X-100-skinned rabbit taenia coli (Gordon, 1978) and A23187-skinned guinea pig taenia caecum (Saida, 1981). Since calcium binding to smooth muscle myosin and myosin hydrolysis of ATP also increase in the range of $10^{-7}$M to $10^{-4}$M calcium (Murphy et al., 1969; Sparrow et al., 1970; Matsumoto et al., 1976; Sobieszik and Small, 1976; Litten et al., 1977), it has been suggested that smooth muscle contraction is stimulated in proportion to free intracellular calcium concentrations within these ranges (Bolton, 1979).

B. The Biochemical Basis For Calcium-Induced Smooth Muscle Contraction

While the calcium dependence of smooth muscle contraction has been recognized for some years, it is only recently that a biochemical basis for calcium-induced tension generation has been proposed (Adelstein and Hathaway, 1979). Like skeletal and cardiac muscle, contraction of smooth muscle requires the interaction of actin and myosin contractile proteins (Somlyo and Somlyo, 1968; Somlyo, 1975). Smooth muscle actin is a globular 42,000 dalton protein which is polymerized into a double-stranded helical filament in the smooth muscle cell (Hartshorne and Gorecka, 1980). Similar to the actin of striated muscles, this actin is associated with a second filamentous protein, tropomyosin, which lies aligned in the groves of the actin double helix (Hartshorne and Gorecka, 1980). These actin-tropomyosin protein filaments are, however, distinctly different from those of skeletal and cardiac muscles in ways which will be discussed later.
Myosin of smooth muscle is a 460,000 dalton protein which is composed of three pairs of smaller protein subunits. The backbone of the myosin complex is formed by two 200,000 dalton subunits which pair to form a filamentous tail that branches into two globular heads (Somlyo, 1980). These heads extend towards the polymerized actin filaments of the muscle in such a way that under "activated" conditions they may sequentially form crossbridges with consecutive subunits of the actin polymer. In this way, by a ratchet-like mechanism, the two filamentous structures can slide relative to each other and cause muscle contraction (Somlyo and Somlyo, 1968; Bowman and Rand, 1980a). The energy for the work thus produced is provided by an ATPase activity which is characteristic of the activated myosin-actin interaction (Bohr et al., 1962; Murphy et al., 1969; Bowman and Rand, 1980a). It is proposed that the chemical energy which is liberated by the hydrolysis of ATP activates the globular heads of the myosin protein, energizing them for the "power stroke" which pulls the actin filament along the myosin filament (Mulvany and Halpern, 1976). A high rate of ATP hydrolysis (for example, 100 nmole per mg of myosin per minute) by myosin in the presence of actin is thus considered to be the biochemical or "test tube" equivalent of contraction, while a low rate (2 to 3 nmole per mg myosin per minute) in the presence of these proteins is considered equivalent to relaxation (Adelstein and Hathaway, 1979).

Although the actin and myosin molecules of smooth muscle are structurally similar to actin and myosin of skeletal and cardiac muscle, there are major differences in the mechanisms of activation of
these proteins by calcium. Tropomyosin in skeletal and cardiac muscle is associated with an additional protein called troponin, which in the absence of calcium inhibits the interaction (and the ATPase activity) of actin and myosin (Ebashi and Endo, 1968; Fuchs and Briggs, 1968). Troponin consists of three subunits; troponin T (a tropomyosin-binding subunit), troponin C (a calcium binding subunit), and troponin I (an inhibitory subunit). The activation of myosin ATPase activity occurs when calcium (at levels greater than or equal to $10^{-7}$M) binds to troponin C, thus inducing a re-arrangement of the troponin complex and removing the inhibitory influence on the ATPase (Bowman and Rand, 1980a).

In contrast to skeletal and cardiac muscles, however, smooth muscle is not primarily regulated by a calcium-reversible inhibition of the actin-myosin interaction by troponin (see Barany and Barany, 1981). Indeed, no firm evidence has been proposed even for the existence of troponin in smooth muscle cells (Adelstein and Hathaway, 1979). Rather, smooth muscle contractility appears to be regulated predominantly by a reversible phosphorylation of the 20,000 dalton protein subunits of the myosin haloprotein (Small and Sobieszek, 1977; Barron et al., 1980; Adelstein, 1979). Phosphorylation of this protein subunit, called LC20, increases the ATPase activity of the actomyosin complex as much as 100-fold, thus providing the energy necessary for cycling of the crossbridges formed between actin and myosin during smooth muscle contraction (Sobieszek and Small, 1976; Gorecka et al., 1976; Chacko et al., 1977; Small and Sobieszek, 1977; DiSalvo et al., 1978; Mrwa et al., 1980; Chacko, 1981).
The degree of phosphorylation of myosin is determined by an endogenous enzyme system which is proposed to be comprised of two major components; a myosin light chain kinase (MLCK) enzyme which phosphorylates LC20, and a myosin light chain phosphatase (MLCP) which reverses this phosphorylation. MLCK is activated within the cell when the concentration of intracellular calcium rises (Small and Sobieszek, 1977). This activation requires mediation by a second protein, a 16,500 dalton calcium-binding protein called calmodulin (Dabrowska et al., 1978; Hathaway and Adelstein, 1979; Adelstein et al., 1978). These two proteins, MLCK and calmodulin, are thus proposed to constitute the primary effector components for calcium-induction of smooth muscle contraction. According to this model, when intracellular calcium concentrations are elevated to $10^{-7}$M or $10^{-6}$M, contraction is initiated by the following sequence: (1) calcium binds to calmodulin; (2) the calcium-calmodulin complex binds to the inactive MLCK, thus activating this enzyme; (3) activated MLCK phosphorylates the myosin LC20, activating actomyosin ATPase; and (4) the sequential formation and detachment (cycling) of actin and myosin crossbridges which is associated with ATPase activity results in contraction of the muscle cell (Adelstein and Hathaway, 1979). Relaxation occurs when calcium levels are reduced to $10^{-8}$M or lower. Calcium then dissociates from calmodulin, deactivating MLCK, and the myosin LC20 is dephosphorylated by the calcium-independent enzyme, MLCP (Small and Sobieszek, 1977; Chacko et al., 1977; Sherry and Hartshorne, 1980; Pato and Adelstein, 1980; see Introduction, section III for further discussion).
Using NE or KCl to induce muscle shortening, an increase in LC20 phosphorylation has also been demonstrated to correlate in vitro with the induction of contraction in a number of tissues, including various arteries (Barron et al., 1979, 1980; Mrwa et al., 1980; Driska et al., 1981; Dillon et al., 1981; Murray and England, 1980), trachea (DeLanerolle and Stull, 1980) and vas deferens (Chacko et al., 1977). Phosphorylation was found to precede tension development (Aksoy et al., 1982; Dillon et al., 1981), and the magnitude of agonist-induced phosphorylation, like that of the agonist-induced contraction, was shown to be a function of the concentrations of extracellular calcium (Barron et al., 1979, 1980). In addition, as might be expected, antagonists of calmodulin activity inhibit LC20 phosphorylation in a manner which parallels their antispasmodic activity (Cassidy et al., 1980; Barron et al., 1980).

While the discovery of LC20 phosphorylation has provided a great deal of information about the mechanisms of smooth muscle contraction, evidence has also been presented for the existence of additional calcium-dependent regulatory mechanisms. Investigators have demonstrated that actin-activated ATPase activity of phosphorylated myosin is itself stimulated by the calcium ion (Sobieszek and Small, 1976; Chacko et al., 1977; Chacko and Rosenfeld, 1982). This stimulation occurs maximally when two calcium ions are bound per myosin and it does not require a regulatory protein such as calmodulin.

An additional proposal for the regulation of smooth muscle contraction has been made by Mikawa et al. (1978) and Nonomura and Ebashi (1980). These investigators have found that a second protein
system, the leiotonin system, also calcium-dependently activates smooth muscle actomyosin ATPase activity. Moreover, the leiotonin system (which consists of leiotonin A, an 80,000 dalton protein, and leiotonin C, an 18,000 dalton calcium-binding protein) appears to activate actomyosin by binding to the actin protein, and so it is possible that smooth muscle activity is regulated by calcium through both the actin and myosin proteins. In support of this possibility, Marston et al. (1980) have also proposed the existence of troponin I- and troponin C-like proteins in the porcine aorta. Interestingly, phosphorylation of the actin filaments of this preparation results in an increase in the number of high affinity calcium binding sites while decreasing the concentration of calcium necessary for half-maximal activation of actomyosin ATPase activity (Walter and Marston, 1981). Further details of the components of this phospho-regulatory system have not yet been elucidated.

While the LC20 phosphorylation mechanism for smooth muscle contraction remains the most thoroughly studied and the most universally applied of the proposed mechanisms, recent results have shown that at least in some tissues additional calcium-dependent regulatory processes are functioning. In the swine carotid artery, Aksoy et al. (1982) have demonstrated that LC20 phosphorylation correlates best with muscle shortening velocity, and not necessarily with the level of tension maintained by the tissue. In fact, while KCl- and histamine-induced tonic tension was maintained for prolonged periods of time as long as calcium was present in the bathing medium, LC20 phosphorylation continually decreased from its initially induced
peak values until it approached near-baseline levels (Aksoy et al., 1982; Mras et al., 1982). These investigators proposed that, at least in this tissue, LC20 phosphorylation determines the number of actively cycling actomyosin crossbridges, while the maintenance of attached crossbridges in the contracted state is determined by another calcium-dependent process (Aksoy et al., 1982; Dillon and Murphy, 1982).

Although at this time it seems safe to assume that LC20 phosphorylation does play a major role in the regulation of smooth muscle contraction, it should be emphasized that additional regulatory mechanisms are likely to be involved. As yet, no single theory for the regulation of smooth muscle contraction suffices to explain the biochemical and physiological data thus far collected (Hartshorne and Mrwa, 1982).

C. Sources of Activator Calcium in Smooth Muscle

While the initiation and maintenance of tension by smooth muscle has been found to be intrinsically dependent upon the presence of elevated levels of intracellular calcium, the sources of such calcium remain a subject of continuing investigation. The role of extracellular calcium in the induction of smooth muscle contraction has been studied extensively by altering the concentration of calcium in the buffer solution bathing the tissue. Using this approach it has been shown that the magnitude of the contraction induced upon stimulation of the muscle with high-potassium buffers is dependent upon the concentration of calcium in the bathing medium (Durbin and Jenkinson, 1961; Edman and Schild, 1961; Waugh, 1962; Sperelakis,
Since smooth muscle becomes completely refractory to stimulation with potassium after extracellular calcium has been removed using calcium-free buffers or buffers which contain the calcium chelating agents EGTA or EDTA, it has been concluded that potassium-induced contractions are dependent upon the presence of extracellular calcium (Edman and Schild, 1962; Hinke, 1965; Hudgins and Weiss, 1968; Hudgins, 1969; van Breemen, 1969; Godfraind and Kaba, 1972). In support of this proposal, potassium-stimulated contractions of smooth muscle can be inhibited completely by agents such as lanthanum, verapamil and D600, which block potassium-stimulated influx of extracellular calcium (van Breemen, 1960; Goodman and Weiss, 1971; Mayer et al., 1977; van Breemen, 1977).

The inclusion of high concentrations of potassium in the medium bathing the tissue reduces the potassium gradient across the cell sarcolemma, thus causing a depolarization of the membrane which correlates with an increase in its calcium permeability (Kuriyama, 1963; Hermsemeyer, 1976; Casteels et al., 1977a; Bolton, 1979). A similar increase in membrane permeability which results in muscle contraction can be produced by electrical depolarization of the cell (Ito et al., 1977), and so it has been concluded that potassium-stimulated contraction is likely to be the result of the opening of a voltage-dependent calcium channel in the membrane, followed by the flow of calcium down its concentration gradient through this channel and into the cell (Bolton, 1979).
The magnitude of NE-stimulated smooth muscle contraction is also dependent upon the concentration of extracellular calcium. Maximum NE-induced muscle contraction is realized when extracellular calcium concentrations are in the millimolar range, while smaller contractions occur in lesser concentrations of calcium (Hinke, 1964; Hiraoka et al., 1968; Hurwitz et al., 1972; Keatinge, 1972b; Godfraind and Kaba, 1972; Sutter, 1976; Godfraind, 1976). In contrast to KCl stimulation, however, a variety of vascular smooth muscle tissues retain their ability to respond submaximally to NE stimulation in the absence of extracellular calcium and even in the presence of calcium chelating agents (Hinke, 1964; Hudgins and Weiss, 1968; Hudgins, 1969; van Breemen, 1969; Keatinge, 1972; Deth and van Breemen, 1974; Bose and Innes, 1975; Casteels et al., 1977a). Since the calcium entry blocker, lanthanum, also does not completely abolish responsiveness of these tissues to NE (either in the presence or absence of extracellular calcium) it can be concluded that the portion of the NE-induced contraction which is lanthanum-resistant is due to the mobilization of intracellular calcium stores (van Breemen et al., 1972; Deth and van Breemen, 1974, 1977; Godfraind, 1976). Indeed, stimulation with NE has been correlated with a release of calcium from intracellularly sequestered calcium pools (Deth and van Breemen, 1974, 1977; Karaki et al., 1979; Casteels and Droogmans, 1981).

In contrast to the tonically maintained contraction induced by NE in the presence of extracellular calcium (see Watkins and Davidson, 1980), NE-induced contractions of vascular smooth muscle in the absence of extracellular calcium have been reported to be phasic and
short lived (Hinke, 1964; Hudgins and Weiss, 1968; van Breemen, 1969; Deth and van Breemen, 1974, 1977; Droogmans et al., 1977). Furthermore, these phasic contractions can be elicited only once after the removal of extracellular calcium. Repetitive phasic contractions based solely upon intracellular calcium have been found to be dependent upon intermittent reloading of intracellular calcium pools by brief re-exposure of the tissue to extracellular calcium (Deth and van Breemen, 1974, 1977; Droogmans et al., 1977). Since under calcium-free conditions without subsequent calcium reloading the efflux of intracellular calcium increases only during the initial stimulation with NE, it is proposed that mobilizable intracellular calcium is extruded from the cell and perhaps sequestered into other non-mobilizable calcium pools (Deth and van Breemen, 1974, 1977; Droogmans et al., 1977; van Breemen et al., 1980). Based on these findings it is proposed that in these tissues the mobilization of intracellular calcium mediates the rapidly induced phasic component of the NE-stimulated contraction, while the influx of extracellular calcium provides the constant stimulus necessary for the tonic maintenance of tension (Bolton, 1979; van Breemen et al., 1980).

Unlike the potassium-induced influx of extracellular calcium, NE-induced influx is not necessarily dependent upon changes in the membrane potential. While concentrations of NE as high as $2 \times 10^{-7}$M induce contractions of arterial smooth muscle which are dependent upon extracellular calcium, these same concentrations have little effect on membrane polarization (Su et al., 1964; Mekata and Nin, 1972; Casteels et al., 1977a,b; Droogmans et al., 1977; Kuriyama et al., 1977). This
observation suggests that NE can selectively increase the calcium permeability of the arterial smooth muscle sarcolemma via calcium channels which are operated by the NE receptor, the alpha receptor (Bolton, 1979; Fleisch, 1974). Consistent with this proposal is the demonstration that the transport of activator calcium into the cell is proportional to NE-receptor binding (Hurwitz et al., 1972). In addition, a pharmacological distinction between the potassium-stimulated voltage-dependent calcium channel and the NE-stimulated receptor-operated calcium channel has recently been made by Meisher et al. (1981), who demonstrated that while the calcium influx inhibiting verapamil analogue, D600, is a more selective inhibitor of potassium-induced $^{45}\text{Ca}$ uptake by the rabbit aorta, amrinone (an agent previously known as a positive cardiac inotrope) more selectively inhibits NE-stimulated $^{45}\text{Ca}$ influx. Further studies will be necessary in order to better define the role of the NE receptor-operated calcium channel in other non-arterial smooth muscle systems.

It should be noted that high concentrations of NE (e.g. $10^{-6}\text{M}$) do induce depolarization of arterial smooth muscle. In this instance a voltage-dependent channel might also be expected to contribute to the NE-induced increase in calcium permeability of the sarcolemma (see Bolton, 1979).

Finally, it should be noted that a number of other smooth muscle agonists have also been proposed to mobilize intracellular calcium, including histamine (Droogmans et al., 1977), caffeine (Endo et al., 1977; Deth and Lynch, 1977) and the prostaglandin endoperoxide analogue U44069 (van Breemen et al., 1980; Loutzenhiser and van
Interestingly, U44069 has recently been found to induce a sustained contraction of the rabbit aorta even in the absence of extracellular calcium (van Breemen et al., 1980; Loutzenhiser and van Breemen, 1981). This finding contradicts the proposal (see above) that extracellular calcium is necessary for maintenance of tonic contractile tension, and suggests a more significant role for intracellular calcium in the maintenance of tissue tension. However, at this time further details of the mechanism of action for U44069 or the other agonists have not been elucidated.

III. Mechanisms of Vasodilatation

The mechanisms by which vasodilatory agents inhibit vascular contraction might be classified into two general categories; (1) mechanisms which reduce the concentrations of calcium made available to the contractile elements, and (2) mechanisms which inhibit the ability of the contractile elements to respond to elevated levels of intracellular calcium. A number of possible ways in which vasodilatory agents could produce each of these general effects have been suggested.

Perhaps the most direct means by which intracellular calcium concentrations can be limited is by directly antagonizing the ability of muscle agonists to initiate extracellular calcium influx or intracellular calcium mobilization. A large number of muscle stimulants, including NE, acetylcholine, histamine, prostaglandins and angiotensin, have been proposed to initiate muscle contraction by first binding to specific receptor molecules located on or in the cell
(see Bowman and Rand, 1980b). Once bound with a muscle stimulant, these "receptors" are proposed to initiate cellular contraction by mediating the opening of membrane calcium channels and/or the mobilization of intracellular calcium (Bolton, 1979). While it is beyond the scope of this review to elaborate the numbers and characteristics of the receptors which have been identified, it is important to note that for most of these receptors additional chemical compounds have been found which also specifically interact with the receptor, but which do not induce its activation. In appropriate concentrations these latter compounds, the receptor antagonists, displace the stimulatory agonistic compounds from the receptors. Thus, receptor activation is inhibited, and the stimulation of calcium influx and/or mobilization is blocked (Bowman and Rand, 1980b).

As has been previously described, potassium-induced calcium influx is not mediated by a specific receptor, but rather is dependent upon depolarization of the plasma membrane. Accordingly, agents which hyperpolarize cell membranes might be expected to antagonize potassium-induced calcium influx and the resultant contraction. While to date no vasodilatory agents have been identified to be selective hyperpolarizing agents, amyl nitrite and inorganic sodium nitrite are both capable of repolarizing depolarized arterial tissue (Keatinge, 1966). Under conditions of depolarization, the vasorelaxant activity of these agents may therefore be mediated, at least in part, via a hyperpolarization mechanism.

As previously described (see section IIA), calcium influx has been associated with agonist-induced smooth muscle contraction. Since
the maintenance of maximum contractile tension in vascular smooth muscle is dependent upon the availability of extracellular calcium, blocking the entry of this calcium can be expected to reduce the degree of tissue contraction. In fact, lanthanum, a trivalent rare earth element which inhibits calcium fluxes through membrane channels, has been demonstrated to possess vasodilatory activity which resembles the removal of calcium from the extracellular fluids (Deth and van Breemen, 1974, 1977).

Intracellular calcium concentrations might also be lowered by mechanisms which stimulate the extrusion or sequestration of intracellular calcium. Calcium extrusion in cardiac muscle cells is dependent upon external sodium and the presence of ATP (McNauton, 1978). The free energy necessary for pumping calcium out of the cell apparently is derived both from the hydrolysis of ATP and from the entry of sodium down its electrochemical gradient. Evidence exists that similar mechanisms may be operational in vascular smooth muscle (Brading, 1978; van Breemen et al., 1979; Keatinge and Harman, 1980). A reduction in extracellular sodium concentrations (and thus the sodium gradient) reduces the efflux of calcium from arterial muscle (Briggs and Melvin, 1961; Reuter et al., 1973) and causes an increase in muscle tone (Bohr et al., 1958). Inhibition of the sodium-potassium exchange pump which maintains the sodium gradient across the sarcolemma also induces a slow contraction of arterial muscle which is presumably caused by a reduced efflux of calcium upon the accumulation of intracellular sodium (Axelsson et al., 1967; Hendrickx and Casteels, 1974). It is possible, then, that vasodilators alter
calcium extrusion either indirectly, by stimulating the sodium-potassium exchange pump to increase the transmembrane sodium gradient, or directly, by stimulating the calcium pumping mechanism itself (Keatinge and Harmon, 1980). While evidence for the stimulation of calcium efflux as a mechanism of action of vasodilators has been scant, it is interesting to note that Overbeck et al. (1976) have reported that the activity of the sodium-potassium exchange pump in vascular tissues from hypertensive animals is often less than that in normotensive tissues.

The demonstration that a number of organelles are capable of reversibly binding calcium in smooth muscle has lead to the interesting suggestion that these organelles may participate in the contraction or relaxation process by regulating the levels of free intracellular calcium (see Bolton, 1979). Smooth muscle sarcoplasmic reticulum has been found to have a rate of calcium uptake and a calcium binding capacity which are compatible with such a role (Batra and Daniel, 1971; Devine et al., 1972; Fitz Patrick et al.; Somlyo et al., 1976; Aoki et al., 1976; Janis and Daniel, 1977; Somlyo, 1978). In fact a number of smooth muscle stimulants, including NE (Baudouin-Legros and Meyer, 1973; Saad and Huddart, 1981) and prostaglandins (Carsten, 1973a, 1973b, 1974) have been found to decrease calcium binding in isolated sarcoplasmic reticulum membrane fragments (microsomes), while papaverine (Anderson, 1972) and cAMP (Anderson, 1972; D'Auriac et al.; Baudouin-Legros and Meyer, 1973; Webb and Bhalla, 1976; Nishikori et al., 1977) have been found to increase such binding. However, a universal role for the sarcoplasmic reticulum in
the mediation of drug effects has been doubted by some, since stimulants and relaxants have not consistently been shown to decrease and increase microsomal calcium binding, respectively (e.g., for cAMP: Clyman et al., 1976; Allen, 1977). Further research is needed to resolve this point. Fitzpatrick and Szentivani (1977) have reported cAMP-stimulated microsomal uptake requires the presence of a protein kinase. It is possible that contradictory results may be due to the presence or absence of protein kinase or some other essential component in the isolated membrane preparation being studied (Keatinge and Harmon, 1980).

Vasodilatory agents may also reverse or inhibit vascular muscle contraction by reducing the responsiveness of the contractile elements to elevations in intracellular calcium concentrations. Elevated intracellular calcium concentrations result in a variety of biochemical changes within the cell which are proposed to mediate cell contraction (see section II B). Theoretically, significant inhibition of any of a number of these events could diminish the contractile response to the stimulus and result in vasodilation.

Since calcium activation of MLCK requires mediation by the calcium binding protein calmodulin, inhibitors of calmodulin activity should render smooth muscle insensitive to calcium stimulation. This expectation has been verified by Cassidy et al. (1980), who found that phenothiazine calmodulin antagonists inhibit calcium–induced LC20 phosphorylation and tension generation in skinned smooth muscle strips, and that this inhibition could be reversed with exogenous calmodulin.
Direct inhibition of MLCK activity would also result in a reduction of LC20 phosphorylation and smooth muscle tension generation in the presence of activator calcium. No pharmacologic compound which directly inhibits the MLCK enzyme has yet been identified. However, an additional kinase enzyme called cPK, which regulates MLCK activity has been found to be endogenous to a variety of smooth muscles (see Adelstein and Hathaway, 1979). Phosphorylation of MLCK by cPK results in a two-to three-fold decrease in MLCK activity (Adelstein et al., 1978; Silver and DiSalvo, 1979), which is apparently the result of a diminished ability of the enzyme to bind the calcium-calmodulin activating complex (Vallet et al., 1981; Adelstein and Hathaway, 1979). Interestingly, cPK-mediated phosphorylation of MLCK is cAMP-dependent (Adelstein et al., 1978; Silver and DiSalvo, 1979; Vallet et al., 1981) and so it may represent the biochemical mechanism by which agents which elevate intracellular cAMP concentrations (e.g. beta adrenergic agonists, phosphodiesterase inhibitors and cAMP analogues) are able to induce vasodilatation (Silver et al., 1982; see fig. 1).

Finally, agents which would activate MLCP, or those which would inhibit actomyosin ATPase activity by interacting with either actin or myosin, would also be anticipated to induce vasodilatation of smooth muscle. To date, no pharmacological or physiological vasodilatory influences have been found to operate through these proteins. However, Walsh et al. (1981) have recently reported that chicken gizzard actin is also a substrate for a cAMP-dependent protein kinase. The regulatory significance of this finding remains to be established.
Fig. 1. Proposed regulation of smooth muscle contraction by calcium and cAMP.
It is important to note that since agonists may differ considerably in the mechanisms by which they increase the concentration of intracellular calcium and in the sources of the calcium which they utilize, significant differences in potency may exist when a given vasodilatory agent is used to antagonize contractions induced by different agonists. This is especially true in the cases of vasodilatory agents which specifically inhibit the ability of a given agonist to stimulate the tissue (e.g., selective receptor antagonists) or agents which specifically block individual sources of activator calcium (e.g., selective calcium channel blockers). Thus, vasodilatory agents which block voltage-dependent calcium channels can be expected to exhibit greater potency against potassium-induced contractions than against NE-induced contractions, while alpha receptor antagonists or specific blockers of NE receptor-operated channels should exhibit greater potency against contractions induced by NE. Differences in potency of vasodilators against contractions induced by specific agonists can therefore be used to gain insight about the mechanism of action of the vasodilatory agents (Rahwan, 1982).

IV. Selected Vasodilatory Agents

Although examination of the proposed mechanisms of action of a large number of vasodilatory agents is beyond the scope of this review, it will be beneficial to briefly discuss those vasodilators which have been used in the studies which follow. For a more complete treatise the reader is referred to the recent review by Bolton (1979).
A. Calcium Entry Blockers: Verapamil, Nifedipine and SKF24260

The calcium entry blockers are a chemically diverse group of agents which seem to share the ability to inhibit calcium influx through potential-dependent channels of the plasma membrane. While much of the evidence for a calcium channel site of action for these compounds is derived from electrophysiological studies on cardiac tissue, a growing body of evidence suggest a similar activity in vascular smooth muscle (see Fleckenstein, 1977). The calcium antagonistic compounds verapamil, nifedipine and SKF24260 (a nifedipine analogue) each inhibit potassium-induced contractions with greater potency than NE-induced contractions (Peiper et al., 1971; Haeusler, 1972; Golenhofen et al., 1973; Bilek et al., 1974; Golenhofen and Hermstein, 1975; Fleckenstein et al., 1976; Bowman and Rand, 1980c). Since potassium-induced contractions are mediated by membrane voltage-dependent calcium channels, while NE-induced contractions are mediated through both receptor-operated channels and intracellular calcium release, this selectivity is interpreted to be the result of relatively specific inhibition of calcium influx through voltage-dependent channels (Bolton, 1979; but see Flaim and Craven, 1981; Godfraind and Dieu, 1981). Verapamil and/or nifedipine induce a parallel shift of the calcium concentration-response curve in potassium-depolarized vascular (Sanner and Prusa, 1980), cardiac (Bristow and Green, 1977), intestinal (Ishizawa and Miyazaki, 1977) and vas deferens (Spedding, 1982) smooth muscle, indicating a calcium antagonistic type of action. Further evidence for a plasma membrane site of action is derived from the finding that verapamil, the
verapamil analogue D600, and nifedipine each inhibit potassium-stimulated $^{45}$Ca influx (Thorens and Haeusler, 1979; Rosenberger et al., 1979; Meisneri et al., 1981). Neither verapamil nor nifedipine is able to inhibit calcium-induced activation of skinned vascular smooth muscle (Weder and Grun, 1973). Verapamil (Mas-Olivera and Nayler, 1980) and the nifedipine analogue, nitrendipine (Bolger et al., 1982; Murphy and Snyder, 1982), have been demonstrated to bind specifically and saturably to high affinity sites in a number of isolated membrane preparations.

It should be noted that while the majority of reports on the activity of verapamil and nifedipine are consistent with, and indeed indicative of, a plasma membrane calcium channel blocking effect, a few studies have suggested the possibility of an intracellular site of action. Verapamil and nifedipine were found to inhibit the contractile activity of human mesenteric arteries and veins even in the absence of extracellular calcium (Mikkelsen et al., 1979). In the rat mesenteric bed verapamil inhibited NE-and potassium-induced contractions equipotently (Kondo et al., 1980), and Church and Zsoterm (1980) have interpreted data from rat and rabbit vascular tissues and from rat atrium as indicating intracellular sites of action for both verapamil and nifedipine.

Finally, it may be significant to note that verapamil may also have alpha receptor antagonist activity, since in a number of isolated membrane preparations it has been found to displace alpha receptor ligand binding (Blackmore et al., 1979; Fairhurst et al., 1980; Glossman and Hornung, 1980; Barnathan et al., 1982). In addition,
verapamil has been proposed to also block the fast sodium channels of cardiac muscle cells (Kaufmann et al., 1974; Kaumann and Serur, 1975). While verapamil has not been described as having sodium channel blocking properties in smooth muscle, it is clear that care must be taken before ascribing all of verapamil's effects to inhibition of calcium entry channels.

B. Intracellular Calcium Antagonists: 2-Substituted Methylene-dioxyindenes

The 2-substituted methylenedioxyindenes (MDIs) are calcium antagonistic compounds which are interestingly different from the classical calcium entry blockers previously described. The MDIs have been classified as calcium antagonists on the basis of their ability to inhibit a variety of calcium-dependent biological processes, including excitation-contraction coupling in vascular (Piascik et al., 1979) and nonvascular smooth muscle (Rahwan et al., 1979), cardiac muscle (Piascik et al., 1979; Lynch et al., 1982) and skeletal muscle (Rahwan and Gerald, 1981; Burchfield et al., 1982), and calcium-dependent (but not calcium-independent) stimulus-secretion coupling in the bovine adrenal medulla (Rahwan et al., 1977; Piascik et al., 1978). Such inhibition has been found to be reversible by increasing the concentration (and hence the availability) of extracellular calcium (Rahwan et al., 1977). In marked contrast to the calcium entry blockers previously described, however, the MDIs appear to act primarily at an intracellular site (see: Rahwan and Witiak, 1979; Rahwan et al., 1981; Rahwan, 1982). For example, the 2-n-butyl
substituted MDI (bu-MDI) depresses activation heat in stimulated skeletal muscle, indicating an inhibition of intracellular calcium release from the sarcoplasmic reticulum (Burchfield et al., 1982). The 2-n-propyl MDI (pr-MDI) inhibits caffeine-induced contractions of skeletal muscle in the presence and absence of extracellular calcium (Rahwan and Gerald, 1981) and barium-induced contractions of smooth muscle (Rahwan et al., 1977), which are both proposed to be mediated through the release of intracellular calcium. The MDIs also possess significant in-vivo activity against calcium-induced cardiac arrhythmias in dogs and rats (Lynch et al., 1981). These MDIs did not inhibit $^{45}$Ca uptake by adrenal medullary chromaffin cells (Rahwan et al., 1977).

While the mechanism of action of the MDIs has not yet been fully elucidated, it is interesting to note that bu-MDI has been found to bind to two calcium regulatory proteins, calmodulin and troponin C, and to inhibit calmodulin-dependent enzyme activity (Piascik et al., 1981; see section II B).

C. Cyclic Nucleotides: cAMP and cGMP

Investigators who have studied the role of cAMP as a vasodilatory agent have proposed that it may both lower the concentration of intracellular calcium and reduce the responsiveness of the smooth muscle to elevations in intracellular calcium concentration. The accumulation of cAMP in smooth muscle has been found to increase sodium/potassium ATPase activity in the plasma membrane (Scheid et al., 1979). This stimulation, by increasing the sodium gradient
across the sarcolemma, may increase the transmembrane sodium-calcium exchange (Brading, 1978; van Breemen et al., 1979), thereby lowering the concentration of intracellular calcium (see section III; Andersson and Nilsson, 1977).

Cyclic AMP has also been proposed to regulate the intracellular distribution of calcium. Baudouin-Legros and Meyer (1973) showed that the cAMP analogue, dibutyryl-cAMP (db-cAMP), stimulates calcium sequestration and inhibits calcium release by isolated aortic microsomes. This effect was subsequently shown to be associated with the reversible phosphorylation of microsomal protein by a cAMP-dependent protein kinase, and so it has been suggested that cAMP-dependent phosphorylation of microsomal proteins may play an important role in the regulation of intracellular calcium movements (Fitzpatrick and Szentivani, 1977; Bhalla et al., 1978).

Finally, as has been previously mentioned (see section III), cAMP-dependent phosphorylation of MLCK by cPK inhibits the calcium/calmodulin-induced activation of MLCK. Thus, at given concentrations of activator calcium, the presence of cAMP results in lesser MLCK stimulation, reduced LC20 phosphorylation and actomyosin ATPase activity, and presumably smaller contractions of vascular smooth muscle (Adelstein and Hathaway, 1979; Barany and Barany, 1981).

Like cAMP, cGMP activates protein kinase enzymes in smooth muscle (Andersson et al., 1980). It is similarly presumed that its vasodilatory activity is dependent upon the phosphorylation of specific muscle proteins (Kukovetz et al., 1981). Cyclic-GMP-dependent protein kinases have been found to phosphorylate endogenous
membrane proteins (Casnellie and Greengard, 1974). However, a specific consequence of this phosphorylation has not yet been identified. To date no evidence for cGMP-induced regulation of actomyosin ATPase activity has been found.

D. Papaverine

While papaverine has been recognized to be a vasodilatory agent for many years, its mechanism of action remains a subject of continuing investigation. Bolton (1979) has pointed out that many of papaverine's actions might be explained by the assumption that it blocks calcium ion channels of the sarcolemma. Increasing the extracellular calcium concentration partially reverses papaverine-induced relaxation of a number of smooth muscle preparations (Diamond and Marshall, 1969; Tashiro and Tomita, 1970; Ferrari et al., 1972), and papaverine has been shown to inhibit potassium-stimulated $^{45}\text{Ca}$ influx (Demesy and Godfraind, 1972; Thorens and Haeusler, 1979). However, since papaverine antagonizes contractions induced by agonists which act through a variety of receptor types, the calcium entry blockade produced by papaverine would need to be effective at a number of different membrane calcium channels. More likely, papaverine antagonizes the effects of calcium at an intracellular site which is common to the stimulation induced by all of the muscle agonists (Bolton, 1979). This proposal is supported by the demonstration that papaverine is an equipotent inhibitor of contractions induced by a variety of agonists (Massingham, 1973).
The smooth muscle relaxant activity of papaverine has been correlated with its ability to inhibit cAMP phosphodiesterase activity in smooth muscle (Uruno et al., 1974; Miyamoto et al., 1976; Polson et al., 1978; Kukovetz et al., 1981) and to promote cAMP accumulation (Kukovetz and Poch, 1970; Triner et al., 1972). Accordingly, the vasodilatory mechanisms ascribed to cAMP in the preceding section might also be expected to be functioning in papaverine-induced muscle relaxation.

E. Sodium Nitroprusside

Studies which have investigated the mechanism of sodium nitroprusside-induced vasodilatation have focused primarily on two aspects of nitroprusside activity; hyperpolarization of the smooth muscle membrane and stimulation of cyclic nucleotide accumulation. Nitroprusside has been demonstrated to hyperpolarize arterial smooth muscle by as much as 12 mV and has been proposed to be capable of relaxing smooth muscle by inhibiting the stimulus to contraction produced by depolarization (Haeusler and Thorens, 1976; Ito et al., 1978; see section II). The mechanism whereby this hyperpolarization is produced has not yet been identified, but may involve alterations in the chloride permeability of the membrane (Kreye, 1980). In addition, nitroprusside, like a variety of nitrogen-containing smooth muscle relaxants (e.g., nitroglycerin, isosorbide dinitrate, amyl nitrite and NaN02) promotes the formation of cGMP in the smooth muscle cell (Katsuki et al., 1977; Bohme et al., 1978). This accumulation is closely correlated to the degree of relaxation induced in a number of
tissues (Axelsson et al., 1979; Janis and Diamond, 1979; Kukovetz et al., 1979). In addition, 8-bromo-cyclic GMP (8-Br-cGMP), a lipophilic cGMP analogue, relaxes smooth muscle in a manner similar to these vasodilators (Schultz et al., 1979; Napoli et al., 1980). Interestingly, tissues made tolerant to the vasodilatory effects of nitroglycerin are no longer capable of accumulating cGMP when stimulated by this compound. These tissues do remain responsive to 8-Br-cGMP, however (Keith et al., 1982). As previously mentioned (section III), the vasodilatory activity of cGMP may be mediated through cGMP-dependent protein kinases in a manner analogous to that of cAMP. The sites and effects of cGMP-mediated phosphorylation have not been identified, however.

V. Statement of the Problem

Although NE has been demonstrated to induce the contraction of a number of arterial smooth muscles by mobilizing intracellular calcium, there is very little quantitative information available concerning the possible mechanisms by which NE may be producing this effect. Furthermore, very little is known about the ability of various vasodilatory agents to modulate muscle contractions which are mediated by intracellular calcium. Since the ability of a vasodilatory agent to regulate smooth muscle contraction can be expected to depend both upon the mechanism of activation of the muscle (i.e., the agonist being used) and upon the mechanisms of regulation by the vasodilator (i.e., the type of antagonist being used), studies concerning the activation and inhibition of those muscle contractions which are
mediated by intracellular calcium can yield valuable information about intracellular regulatory processes of smooth muscle contraction.

The current studies were undertaken in order to investigate the mechanism of NE-induced contractions of the rat aorta in the absence of extracellular calcium, and to explore the influence of vasodilator agents on these contractions. Specifically, the following questions were asked: (1) Does the rat aorta remain responsive to NE-induced contractions in the absence of extracellular calcium? (2) What is the mechanism of this response? (3) What role do the alpha receptors play in mediating this contraction? (4) What are the refilling characteristics of the intracellular calcium pools which initiate contraction? (5) What effects do vasodilatory agents with different sites of action (e.g., membrane calcium blockers vs intracellular calcium antagonists) have on these NE-induced contractions? (6) Can an intracellular site of action be predicted for a vasodilatory agent based upon its ability to inhibit contractions in the absence of extracellular calcium?
CHAPTER I

Evidence for the Existence of Two Distinct Pools of Intracellular Calcium in the Rat Aorta Accessible to Mobilization by Norepinephrine.

A. Introduction

Hudgins and Weiss (1968), Hudgins (1969) and van Breemen (1969) have demonstrated that while potassium-induced contractions of the rabbit aorta depend almost entirely on the presence of extracellular calcium, both extracellular and intracellular calcium pools are utilized during norepinephrine (NE)-induced contractions. In the absence of extracellular calcium, NE rapidly induces a short-lived phasic contraction of the rabbit aorta, while in the presence of extracellular calcium, this phasic contraction is followed by (and superimposed upon) a slower tonic contraction which is maintained for a prolonged period of time (Watkins and Davidson, 1980). It has been proposed that the phasic contraction is mediated by the release of intracellular calcium into the smooth muscle myoplasm (Deth and van Breemen, 1974; 1977).

While NE and KCl have also been shown to elicit contractions of the rat aorta by different mechanisms (Peiper et al., 1971), the ability of NE to contract the rat aorta in the absence of extracellular calcium has not been clearly established. Sutter (1976)
reported that NE could contract the rat aorta incubated in calcium-free physiological solution, while DeFelice and Joiner (1976) reported that under these conditions the tissue lost its contractile response. Godfraind and Kaba (1972) reported that the rat aorta retained its ability to contract in response to NE in calcium-free buffer, but became nonresponsive in the presence of 1 mM EDTA. Krishnamurty and Grollman (1976) recorded a slight contraction in the rat aorta in response to KCl, but not to NE, after the tissue was incubated for 30 minutes in calcium-free buffer containing 30 μM EDTA.

The studies described in this chapter were undertaken in order to clarify the mechanism of the contractile response of the rat aorta to NE and KCl in the absence of extracellular calcium.

B. Methods

Male Sprague-Dawley rats (Harlan Industries, Inc., Cumberland, Ind.), weighing 230-320 g were sacrificed by cervical dislocation. A 2.5 cm segment of the thoracic aorta was removed and rinsed in physiological buffer. Each aortic segment was cleaned of connective tissue and cut spirally to yield either two 3 mm x 15 mm strips or three 3 mm x 10 mm strips and each strip was mounted isometrically under 1 g tension in a 10 ml tissue bath (37°C) containing a normal physiological solution aerated with 5% CO₂ in O₂. A 1 to 1.5 hr. equilibration period was allowed prior to experimentation. Force generation was monitored using a Grass FT.03 isometric transducer coupled to a Grass Model 7D or a Grass Model 79D Polygraph recorder (Grass Instruments Co., Quincy, Mass.).
The normal (calcium-containing) physiological solution was composed of (mM): NaCl, 118; KCl, 4.7; MgCl₂·6H₂O, 0.54; NaH₂PO₄, 1.0; NaHCO₃, 25; EDTA (as antioxidant for NE), 0.027; CaCl₂·2H₂O, 2.5; dextrose, 11. Calcium-free solutions were made by excluding calcium and including the appropriate amounts of EGTA as indicated below. High potassium buffers were made by substituting KCl for NaCl on an equimolar basis.

The methods described below are identified by letters which correspond to the respective results in this chapter, and cross-referencing is made throughout the chapter.

1. Response of aorta to NE in calcium-free medium

Aortic strips which had been equilibrated in normal (calcium containing) buffer solution were rinsed 4 times at 4-minute intervals with 10 ml volumes of calcium-free buffer containing 0.0, 0.1, 1.0 or 10.0 mM EGTA, and equilibrated in the respective buffer for a total of 20 minutes. After equilibration, the tissues were exposed to either NE (3x10⁻⁶M) or potassium (100mM) in the calcium-free medium. The resultant contractions were recorded for 30 minutes. The tissues were then washed in calcium-free buffer containing the appropriate EGTA concentration and the baseline resting tension was again obtained. When appropriate, tension generation was corrected for slight shifts in tissue resting tension. Finally, the tissues were reincubated for 15 minutes in normal (calcium-containing) physiological solution and exposed again to NE (3x10⁻⁶M) or KCl (100mM) in order to ascertain maximal contractions of the tissues. Contractions in the absence of
extracellular calcium were expressed as a percentage of the maximum contraction induced by the respective agonists (3x10^{-6}M NE or 100 mM KCl) in the presence of 2.5 mM bath calcium.

In a separate set of experiments, aortic tissues which were washed and incubated as described above in calcium-free buffer containing 1.0 mM EGTA were contracted with 3 x 10^{-6}M NE for periods in excess of 90 minutes in order to determine the duration of the effects produced.

2. Effect of phentolamine on aortic response to NE

The ability of phentolamine (Regitine mesylate, Ciba Pharmaceutical Co., Summit, N.J.), an alpha-adrenergic receptor blocker, to inhibit the aortic contraction induced by 3 x 10^{-6}M NE following equilibration of the tissue in calcium-free (1.0 mM EGTA) medium was determined in two ways:

(1) Phentolamine was added in stepwise increasing concentrations to tissues which had been contracted with NE (3 x 10^{-6}M) for 30 minutes in calcium-free-EGTA buffer (n = 4). The phentolamine-induced relaxation was expressed as percent of the peak sustained contraction to NE prior to the addition of phentolamine. The viability of the tissue was then ascertained by washing the NE from the tissue with calcium-free-EGTA buffer and re-establishing the response of the tissue to NE (3 x 10^{-6}M) in normal (calcium-containing) medium.

(2) Phentolamine (10^{-5}M) was introduced into the calcium-free-EGTA medium and left in contact with the tissues for 10 minutes prior to introduction of 3 x 10^{-6}M NE for 30 minutes (n = 4). The tissue
was subsequently washed for 30 minutes in calcium-free-EGTA buffer, then re-incubated in normal (calcium-containing) buffer, and contracted with $3 \times 10^{-6}$ M NE. The biphasic contractions induced by NE in the presence of phentolamine in the calcium-free medium were expressed as a percentage of the maximum contraction produced by $3 \times 10^{-6}$ M NE in the absence of phentolamine and the presence of 2.5 mM bath calcium, and also calculated as a percentage of the biphasic NE responses in calcium-free medium in the absence of phentolamine (as determined in section A of the Methods and Results).

3. Concentration-response experiments to NE in calcium-free medium

Aortic strips were equilibrated in calcium-free buffer containing 1.0 mM EGTA as described above. NE was introduced to the bath at a given concentration (ranging from $3 \times 10^{-10}$ to $3 \times 10^{-4}$ M) to separate tissue preparations, and contractions were monitored for 30 minutes. The tissues were finally washed in calcium-free (1.0 mM EGTA) buffer, re-incubated in normal (calcium containing) physiological buffer, and contracted with $3 \times 10^{-6}$ M NE as above. Contractions in the absence of extracellular calcium were expressed as a percentage of the maximum inducible contraction to $3 \times 10^{-6}$ M NE in the presence of 2.5 mM extracellular calcium.

In separate tissues which had been similarly washed in calcium-free-EGTA medium and contracted by $3 \times 10^{-8}$ M NE for 20 minutes, NE was added in stepwise increasing concentrations to a final concentration of $3 \times 10^{-6}$ M and the resultant contractions were monitored.
4. **Effect of withdrawal of extracellular calcium on aortic tissue contracted by NE and KCl**

After a 1 hour equilibration period in normal (calcium containing) physiological buffer, one aortic strip of each pair was contracted with $3 \times 10^{-6}$M NE, while the other was contracted with 100 mM KCl. After 30 minutes, when each tissue had attained maximum contraction, the bathing medium of each tissue was replaced with a calcium-free medium containing 1.0 mM EGTA and the same concentrations of the respective agonists (NE or KCl). The resulting tissue relaxation (due to removal of extracellular calcium) was monitored with respect to time for 40 minutes, after which the tissues were washed with calcium-free (1.0 mM EGTA) buffer until they maintained a constant resting tension. Tissue tension during the 40-minute relaxation period (in the presence of agonist and the absence of calcium in the medium) was expressed as percent of the maximum contraction to the agonist attained in the presence of extracellular calcium.

5. **Dissociation of the phasic and sustained components of the NE response in calcium-free medium**

Pairs of aortic strips were removed from each rat and washed in calcium-free buffer containing 1.0 mM EGTA as previously described. NE ($3 \times 10^{-6}$M) was added to each tissue bath and the resulting control contraction was monitored for 30 minutes, followed by a 30 minute washout of NE with 1.0 mM EGTA-calcium-free buffer. Thereafter, each of the 2 strips from the paired aortic tissue of each rat was assigned
to one of two different treatment groups. Tissues in one group were exposed for 10 minutes to normal (calcium containing) physiological solution, while those in the second group were maintained in calcium-free buffer containing 1.0 mM EGTA for an equivalent amount of time. The tissues in each treatment group were then rinsed and re-equilibrated as described above using calcium-free 1.0 mM EGTA buffer. After 20 minutes the contractile response of each group to $3 \times 10^{-6}$ M NE was again determined. The entire cycle was repeated for a total of 4 contractions. The ability of the reintroduced NE to induce contraction each time was expressed as a percentage of the initial control contraction in calcium-free buffer containing 1.0 mM EGTA.

6. Cumulative concentration-response curves to NE in calcium-free medium after elimination of the phasic component

Cumulative concentration-response curves to NE in calcium-free medium were established as follows: Aortic strips were washed for 20 minutes in calcium-free buffer containing 1.0 mM EGTA. In order to separate the phasic and sustained components of the NE-induced contractions, $3 \times 10^{-6}$ M NE was added for 30 to 40 minutes in order to produce the usual initial phasic contraction and thereby deplete the calcium pool associated with this component of the biphasic response (see section 5 of Results and Discussion). The NE was then washed out for 30 minutes with calcium-free buffer containing 1.0 mM EGTA. NE was then added to the tissue baths in stepwise increasing concentrations and the resultant sustained contractions were monitored. Contractile activity to each NE concentration was
expressed as percent of the maximum contraction attained with the highest NE concentration in these concentration-response studies.

**Statistics**

All values are represented as mean ± SEM. Statistical tests are indicated in the Results and Discussion sections. ED$_{50}$ values and their 95% confidence intervals (whenever presented) were estimated using linear regression analysis of the probit-transformed percent response versus log NE concentration.

C. **Results**

1. **Response of aorta to NE in calcium-free medium**

   The contractility of the rat aortic strips to either high potassium or NE in each calcium-free buffer is presented in figure 2. Upon exposure to 100 mM KCl, tissues washed in calcium-free buffer contracted tonically, with the magnitude of the contraction attained being 14% the maximum attainable contraction in calcium-containing normal buffer. On the other hand, tissues washed in calcium-free buffers containing 0.1, 1.0 or 10.0 mM EGTA did not contract perceptibly to KCl. In contrast, in all four calcium-free buffer systems (0.0, 0.1, 1.0 or 10.0 mM EGTA), NE consistently induced contractions which were biphasic in nature; the initial phase being a rapidly produced phasic contraction which peaked within 30 seconds of NE exposure, and the second phase being a sustained contraction which required 25 to 30 minutes to reach maximum tension (see figure 3A).
While the maximum tension of the sustained contraction to NE in calcium-free medium (expressed as percent of the maximum response in calcium-containing medium) appears to differ somewhat in each buffer system, it is not reduced in a concentration-dependent manner by EGTA (see figure 2). Under calcium-free (1.0 mM EGTA) conditions, the maximum phasic and sustained contractile responses to $3 \times 10^{-6}$M NE were 26% and 24%, respectively, of the maximum responses obtained in calcium-containing medium.

The sustained contraction of the aorta to NE is stable for a prolonged time, fading by an average of only $19.0 \pm 2.1\%$ ($n = 8$) 60 minutes after attaining maximum tension generation in calcium-free medium in the presence of 1.0 mM EGTA. The sustained NE-contraction was consistently reversed by washing the tissue with calcium-free buffer (figure 3B).

2. **Effect of phentolamine on aortic response to NE**

Phentolamine reversed the sustained NE-induced contraction in a concentration-dependent manner when added cumulatively in calcium-free-EGTA medium to tissues previously contracted with $3 \times 10^{-6}$M NE (i.e., during the sustained contraction phase). Concentrations of $10^{-7}$, $10^{-6}$ and $10^{-5}$M ($n = 4$ each) of the alpha-blocker relaxed the sustained contractions by $11.0 \pm 3.4$, $59.2 \pm 2.1$ and $92.9 \pm 1.3$ percents, respectively (figure 3C). Furthermore, when $10^{-5}$M phentolamine was added to tissues in calcium-free (1.0 mM EGTA) buffer prior to NE ($3 \times 10^{-6}$M) exposure, the phasic and sustained contractions were reduced to $2.0 \pm 0.5\%$ and $1.8 \pm 0.3\%$, respectively,
Figure 2. Contractile response of the rat aorta to potassium and NE in calcium-free medium containing 0-10 mM EGTA. Contractions in the absence of extracellular calcium are expressed as a percentage of the maximal attainable control contractions to 100 mM KCl or $3 \times 10^{-6}$M NE, respectively, in the presence of 2.5 mM extracellular calcium. Values represent mean ± SEM, and number of experiments are in parentheses. See section 1 of Methods for details.
Figure 2.
Figure 3. Representative tracings of contractions of the rat aorta produced by NE (3 x 10^{-6}M, except where otherwise indicated) (filled circles) in calcium-free-EGTA (1.0 mM) medium. (A) Example of the biphasic components (phasic and sustained) of the NE response. See section 1 of Methods for details. (B) Reversal of the sustained phase of NE-induced contraction by washout of NE (arrows) with calcium-free-EGTA medium. See section 1 of Methods for details. (C) Reversal of the sustained phase of NE-induced contraction by graded concentrations of phentolamine (arrowheads). See section 2 of Methods for details. (D) Concentration-response effect of NE. See section 3 of Methods for details.
Figure 3.
of the maximum inducible contraction by NE under normal (calcium containing) conditions \( n = 4 \). These latter two values represent, respectively, 92.3% and 92.5% inhibition of those responses usually induced by NE under calcium-free (1.0 mM EGTA) conditions depicted in figure 2.

3. **Concentration-response experiments to NE in calcium-free medium**

Aortic strips which had been incubated in calcium-free buffer containing 1.0 mM EGTA contracted in a similar biphasic manner throughout the range of NE concentrations tested. Both the phasic and the sustained contractions were induced by NE in a concentration-dependent manner (figure 4) with \( ED_{50} \) values (and 95% confidence intervals) of \( 3.01 \times 10^{-8} \text{M} (2.29 \times 10^{-8} \text{M} - 3.95 \times 10^{-8} \text{M}) \) and \( 3.58 \times 10^{-8} \text{M} (2.48 \times 10^{-8} \text{M} - 5.15 \times 10^{-8} \text{M}) \), respectively. These values did not differ significantly at the 0.05 level. In separate experiments \( n = 4 \) where NE concentrations were increased during the sustained contraction phase induced by \( 3 \times 10^{-8} \text{M} \) NE, a concentration-dependent enhancement of contraction resulted (figure 3D).

4. **Effect of withdrawal of extracellular calcium on aortic tissue contracted by NE or KCl**

The effects of calcium withdrawal from the medium on a contracted tissue under conditions of continued agonist exposure are shown in figure 5. The KCl-induced contraction was completely reversed by calcium withdrawal within 20 minutes, while 21% of the NE-induced contraction persisted even after 40 minutes of calcium withdrawal.
The degree of reversion of NE-induced contraction was significantly less than that of the KCl-induced contraction throughout the 40-minute monitoring period (P < 0.01 at 2 minutes, and P < 0.0005 at subsequent time intervals, by paired Student t test). The maximum contraction produced by each agonist prior to calcium withdrawal (i.e., in the calcium-containing medium) was similar, that to KCl being 90.4 ± 3.5% that to NE as determined in a separate set of experiments (n = 12).

5. Dissociation of the phasic and sustained components of the NE response in calcium-free medium

Typical tracings from a pair of aortic strips from the calcium depletion experiments are shown in figure 6. Tissues previously maintained in calcium-free medium and then intermittently exposed to calcium for 10-minute periods with subsequent washing of extracellular calcium each time prior to re-exposure to NE, responded in the typical biphasic manner upon each NE re-introduction (tissue 1 in figure 6). In contrast, tissues which were not re-exposed to calcium (but were maintained throughout in calcium-free medium) contracted phasically only slightly (~10%) but retained 63.7 to 70.8% of their ability to produce a sustained contraction (table 1 and tissue 2 in figure 6). Despite incubations for as long as 5 hours in calcium-free (1.0 mM EGTA) buffer, the repeated exposure of these tissues to NE consistently resulted in the sustained contractile response which appeared, in fact, to become more prominent with time (table 1).

The ability of the tissues to contract biphasically after calcium reintroduction exhibited some decay under the present experimental
Figure 4. Noncumulative concentration-response curves of the phasic and sustained components of NE-induced contractions of the rat aorta in calcium-free-EGTA (1.0 mM) medium. Contractions in the absence of extracellular calcium are expressed as a percentage of the maximum attainable control contraction to $3 \times 10^{-6}$ M NE in the presence of 2.5 mM extracellular calcium. Each point represents the mean ± SEM of 8-10 experiments, using different tissues for each NE concentration. See section 3 of Methods for details. Inset: Cumulative concentration-response curve of the sustained component of NE-induced contraction of the rat aorta in calcium-free-EGTA medium after elimination of the phasic contractile component. See figure 7B and section 6 of Methods for details. Contractile activity at each NE concentration is represented as a percentage of the response to the highest ($3 \times 10^{-6}$ M) NE concentration used. Each point represents the mean ± SEM of 4 experiments.
Figure 4.

- PHASIC CONTRACTION
- SUSTAINED CONTRACTION

PERCENT OF CONTROL CONTRACTION

NOREPINEPHRINE CONCENTRATION (M)
Figure 5. Effect of withdrawal of extracellular calcium from the medium on rat aortic tissue contracted by NE or KCl under conditions of continued exposure to the agonists. Control contractions in the presence of 2.5 mM extracellular calcium (−8 to 0 minutes on x axis) were considered as 100%. Values at subsequent time intervals after withdrawal of extracellular calcium (2−40 minutes on the x axis) are represented on the y axis as a percentage of the maximum control contraction in response to the agonist obtained in presence of extracellular calcium. Each point represents the mean ± SEM (except where the SEM is too small) of 5 experiments. See section 4 of Methods for details. In the case of NE, all values represent the sustained component of the contractile response.
Figure 5.

**PERCENT OF CONTROL CONTRACTION**

- **NOREPINEPHRINE (3×10^{-6} M)**
- **POTASSIUM CHLORIDE (100 mM)**

**TIME (min)**
Figure 6. Representative tracings of the effects of repeated stimulation of the rat aortic tissue with $3 \times 10^{-6}$M NE (circle) in calcium-free-EGTA medium, and the dissociation of the phasic and sustained components of the NE response. Tissue 1: Prior to each NE stimulation in calcium-free medium, the tissue was briefly bathed in calcium-containing medium and the extracellular calcium washed away with calcium-free-EGTA buffer. Tissue 2: The tissue was exposed to calcium in the medium (which was then washed away) only prior to the first NE stimulation. Subsequent stimulations of tissue 2 with NE in calcium-free medium were not preceded by exposure of the tissue to extracellular calcium. Note the elimination of the phasic component of the action of NE in tissue 2. The time of NE introduction to the tissue bath is expressed as the total time elapsed after the initial washout of extracellular calcium by calcium-free-EGTA medium. See section 5 of Methods for details.
Figure 6.
NE-induced aortic contractions in calcium-free-EGTA medium in presence or absence of intermittent re-exposure to calcium

Repeated contractions were elicited by $3 \times 10^{-5}$ M NE in calcium-free EGTA medium in tissues which were either intermittently re-exposed for 10-min periods to calcium-containing medium and subsequently washed in calcium-free (1.0 mM EGTA) buffer or in tissues which were maintained in calcium-free (1.0 mM EGTA) buffer without intermittent re-exposure to calcium. See section E under "Methods" for further explanation. Values represent the mean ± S.E.M. of five experiments.

<table>
<thead>
<tr>
<th>Time of NE Introduction*</th>
<th>Tissues intermittently re-exposed to calcium</th>
<th>Tissues not re-exposed to calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phasic</td>
<td>Sustained</td>
</tr>
<tr>
<td>(min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>110</td>
<td>84.9 ± 2.5</td>
<td>86.8 ± 4.7</td>
</tr>
<tr>
<td>200</td>
<td>65.2 ± 4.6</td>
<td>78.9 ± 5.9</td>
</tr>
<tr>
<td>290</td>
<td>44.1 ± 6.3</td>
<td>67.1 ± 6.0</td>
</tr>
</tbody>
</table>

*The time of NE introduction to the tissue bath is expressed as the total time elapsed after initially washing the tissue in calcium-free EGTA buffer.

*NE addition was preceded by washing away the extracellular calcium with calcium-free EGTA buffer.
conditions (table 1), the decay being much more evident with the phasic component. Extending the calcium reintroduction period to 20 minutes (data not shown) did not affect this decay, and thus it is unlikely that this decay is caused by insufficient periods of calcium re-exposure.

While the lag for induction of the first phasic contraction prior to calcium reintroduction and the lags after each 10 minute calcium reintroduction were 4.0 ± 0.3 seconds and 4.7 ± 0.5 seconds, there was a 10.2 ± 0.4 second lag before the onset of the sustained contractions in the calcium depleted tissues (n = 12 for each).

6. Cumulative concentration-response curves to NE in calcium-free medium after elimination of the phasic component

After virtual elimination of the phasic component of the NE response, the sustained response to NE in calcium-free-EGTA medium was concentration-dependent (figure 7B), with a maximum contraction representing 67.3 ± 3.1% of the initial sustained contractions determined prior to elimination of the phasic component (figure 7A). The ED$_{50}$ value (and 95% confidence interval) of NE calculated from these concentration-response curves (figures 7B and 4 inset) was 2.35 x 10$^{-8}$M (2.1 x 10$^{-8}$M - 2.63 x 10$^{-8}$M), and did not differ significantly from the ED$_{50}$ values of the phasic and sustained contractions elicited by exposure of the tissues to NE after initial washout of extracellular calcium (P > 0.05; see sections 3 of Methods and Results).
Figure 7. Representative tracing of concentration-response effect of NE on the rat aorta in calcium-free-EGTA medium after elimination of the phasic component of the NE response. Filled circles represent point of introduction of NE into the medium. Arrows represent washout of NE. (A) The normal biphasic initial response of the rat aorta to NE in calcium-free medium after washout of extracellular calcium. (B) Concentration-response effect of NE after elimination of the initial phasic component (see section 6 of Methods for details).
Figure 7.

A.

B.

NOREPINEPHRINE CONCENTRATION (M)

10 min.

3 \times 10^{-6}

10^{-8} \quad 3 \times 10^{-8} \quad 10^{-7} \quad 3 \times 10^{-7} \quad 3 \times 10^{-6}
D. Discussion

The results of the present investigation demonstrate that although the rat aorta is completely refractory to high levels of KCl after it is washed in calcium-free buffer containing 1.0 mM EGTA, it remains responsive to even low concentrations of norepinephrine. These findings are an extension of similar observations reported for the rat aorta (Sutter, 1976), for the rabbit aorta (Deth and van Breemen, 1974, 1977), and for other vascular and nonvascular smooth muscles (see Bolton, 1979), from which it was concluded that NE mobilizes smooth muscle intracellular calcium. DeFelice and Joiner (1976) reported that, in calcium-free buffer (even in the absence of EGTA), the loss of extracellular calcium from the rat aorta is nearly complete after 20 minutes. In the present study, the persistence of NE-induced contractions despite repeated washing in the presence of EGTA would therefore suggest that NE mobilizes intracellular calcium in the rat aorta. This conclusion is further supported by the finding that calcium withdrawal from the medium in the presence of EGTA (sections 4 of Methods and Results) abolishes within 20 minutes the KCl-induced contractions [which are dependent upon extracellular calcium (Bolton, 1979; also figure 2)] while NE-induced contractions persist for more than 40 minutes (figure 5). It is not clear at the present time why DeFelice and Joiner (1976) could not elicit contractions of the rat aorta with NE under calcium-free conditions, nor is it clear why Godfraind and Kaba (1972) could elicit contractions of the rat aorta with NE in calcium-free medium in the absence but not in the presence of EDTA.
The rat aorta apparently differs from that of the rabbit, however, in that after incubation in calcium-free EGTA buffers, the rat aorta responds to NE exposure with a biphasic contraction (figures 2 and 3). A similar biphasic contraction in calcium free buffer has recently been reported for rabbit ear artery (Casteels et al., 1981). The initial contraction is a phasic one which appears to be analogous to the contraction induced by NE in the rabbit aorta (van Breemen, 1969; Deth and van Breemen, 1974, 1977). It is rapidly produced upon NE introduction, is short lived (figure 3), and, once it is produced, it can be elicited again only after brief re-exposure to external calcium (figure 6). In contrast to the rabbit aorta, however, the rat aorta responds to NE in calcium-free medium with a second contraction which develops more slowly and is sustained (figure 3A). While the magnitude of this sustained contraction appears to vary somewhat with EGTA concentration, it persists despite EGTA concentrations as high as 10.0 mM (figure 2). This sustained contraction is not reversed in a concentration-dependent manner by EGTA, and since it is produced under conditions in which the KCl-induced contraction is completely inhibited, we conclude that, like the phasic contraction, the sustained contraction is produced as a result of the ability of NE to mobilize intracellular calcium. This concentration-dependent sustained contraction to NE (figure 4) is reversed by washing the NE from the tissue (figure 3B). Phentolamine abolishes both the phasic and sustained (figure 3C) components of the effect of NE on the aorta in calcium-free medium, indicating a probable involvement of the alpha-adrenergic receptor in the biphasic response of the rat aorta to
Based on the data of figure 2, the sustained contraction of aortic strips in response to $3 \times 10^{-6}$M NE in calcium-free medium containing 1 mM EGTA was 24% of the contractile response achieved in the presence of extracellular calcium. The data presented in figure 5 support this finding: tissues exposed continuously to KCl relaxed completely within 20 minutes of calcium withdrawal, while those exposed to NE retained 21% of their initial response (recorded in calcium-containing medium) even after 40 minutes in calcium-free EGTA buffer. Based on these findings it is tempting to speculate that as much as 24% of the contraction which is produced by NE in calcium-containing buffer is, in fact, due to NE-induced mobilization of intracellular calcium. However, it should be noted that intracellular calcium pools may contribute differently to contractions produced in the presence or in the absence of extracellular calcium, since extracellular calcium has been proposed to modulate the calcium binding capacity of some intracellular calcium pools [e.g. the inner aspect of the sarcolemma (Bianchi, 1969)].

The phasic contraction of the rabbit aorta to NE has been proposed to be transient in nature because: (1) the calcium content of the mobilized intracellular pool is limited; (2) a portion of the calcium pool mobilized by NE is extruded from the cell; and (3) the mobilized intracellular calcium is re-sequestered into non-mobilizable calcium pools within the cell (Deth and van Breemen, 1977; van Breemen et al., 1980). Similarly, in the rat aorta, NE induces $^{45}$Ca efflux from intracellular (lanthanum-resistant) stores (Godfraind, 1976).
which are normally limited in capacity (Godfraind and Kaba, 1972; DeFelice and Joiner, 1976). This mobilized intracellular calcium, however, is not re-sequestered into the same rapidly mobilizable intracellular pool, since a second or subsequent phasic contraction can be elicited only after reloading this pool by brief re-exposures of the tissue to extracellular calcium (figure 6).

In contrast to the NE-induced phasic contraction, however, the sustained contraction in the rat aorta can be repeatedly and reproducibly elicited for as long as 5 hours after the removal of calcium from the bathing medium (figure 6; table 1). This finding suggests a number of possible mechanisms for the sustained contraction. (1) NE could be inducing the mobilization of calcium from an intracellular pool which is able to re-sequester that same calcium upon NE withdrawal, with very little loss of calcium from the cell or to other non-mobilizable pools. (2) NE could be inducing the repeated mobilization of calcium from an intracellular pool which, despite the loss of calcium from the cell and to other non-mobilizable pools, is large enough to maintain tissue contractility for repeated and prolonged periods of time without calcium replenishment from extracellular sources. (3) Assuming the possibility that calcium-free conditions may affect intracellular calcium pools by allowing gradual and continuous leaching of subeffective amounts of calcium into the cytoplasm and eventual removal of this leached calcium by efflux from the cell or re-sequestration, it is possible to hypothesize that NE could elicit prolonged and repeated contractions by inhibiting efflux or re-sequestration of the leached calcium, thereby increasing
cytoplasmic calcium concentrations to levels that would activate the contractile elements. Alternatively, a combination of the above mechanisms might be occurring. The currently available data are not sufficient to establish one of the above possibilities as the definitive mechanism for the sustained contraction induced by NE. However, the long duration and the reproducible nature of the sustained response in calcium chelating buffer observed in the present study, and the necessity for a concentration of $10^{-7}$ to $10^{-6} \text{M}$ calcium proximal to the contractile elements to initiate contraction (Filo et al., 1965; Endo et al., 1977; Gordon, 1978), suggest that the probable mechanism of the sustained contraction induced by NE involves a conservation of intracellular calcium within the cell and a close association between the source of calcium for this response and the contractile apparatus.

Based upon the rapid mobilization and refilling characteristics of the NE-mobilizable calcium pool involved in the phasic contraction in the rabbit aorta, van Breemen et al. (1980) proposed the source of this intracellular calcium to be the inner aspect of the sarcolemma, or some structure closely associated with it. Casteels and Droogmans (1981) reached similar conclusions after studying the rabbit ear artery. In light of these proposals it is perhaps significant to note that with repeated exposure to NE under conditions which allow development of the phasic contraction (tissue 1 in figure 6; table 1; sections 5 in Methods and Results), this component of the NE response gradually diminishes in magnitude. On the other hand, under conditions in which only the sustained response to NE is elicited
(tissue 2 in figure 6; table 1; sections 5 in Methods and Results), this component of the NE response is not attenuated. This would suggest that the calcium pool associated with the phasic contraction is more peripherally located within the cell than the pool utilized for the sustained contraction, since it is more susceptible to the adverse effects of exposure to nonphysiological (calcium-free) buffers. The data presented seem consistent with the possibility that calcium associated with the phasic contraction is stored intracellularly at the inner aspect of the sarcolemma, while calcium associated with the sustained contraction is stored in a deeper intracellular structure such as the sarcoplasmic reticulum. Regardless of the morphological location of the calcium pool associated with each type of contraction, it appears that each calcium pool represents a functionally distinct entity within the cell.

It should be noted that although there appears to be two distinct intracellular pools of NE-mobilizable calcium in the rat aorta, a portion of the calcium mobilized from the pool involved with the phasic contraction is apparently retained within the cell for a sufficiently long period of time to contribute to the maximum tension eventually induced by the sustained contraction, as evidenced by the finding (table 1; figure 6; and sections 5 in Methods and Results) that the repeated sustained contractions decrease slightly in magnitude under conditions where the phasic contractions are decreasing in magnitude (tissue 1 in figure 6; table 1), but are maintained at a constant magnitude under conditions in which the phasic component is virtually eliminated (tissue 2 in figure 6; table 1).
Since tissues in which the calcium pool involved in the phasic contraction to NE has been depleted (see sections 6 of Methods and Results) remain responsive to NE in a concentration-dependent manner (figures 7B and 4 inset) by exhibiting sustained contractions, and the \( ED_{50} \) of NE under this condition is indistinguishable from that of the initial phasic and the initial sustained contractions elicited after removal of extracellular calcium, it is likely that each of these contractions is initiated by the same primary event, presumably the interaction of NE with the alpha-adrenergic receptor on the muscle membrane. This proposal is further supported by the finding that exposure of the aortic tissue which has been washed in calcium-free (1.0 mM EGTA) buffer to phentolamine prior to the introduction of NE to the bath inhibited both the phasic and sustained components of the NE-induced contractions to an equal degree.

In summary, the following conclusions may be derived from the present investigation: (1) In the absence of extracellular calcium, intracellular calcium contributes to the contractile effects of NE in the rat aorta, as evidenced by the ability of NE, but not potassium, to induce contractions under such conditions. (2) Two distinct intracellular calcium pools mediate the phasic and sustained components of the NE-induced contractile response of the rat aorta in calcium-free medium, as evidenced by the uncoupling of these two components under defined experimental conditions. (3) The phasic and sustained components of the contractile response of the rat aorta to NE in calcium-free medium are probably mediated by the same mechanism, as evidenced by the similarity of the \( ED_{50} \) of NE for evoking the
phasic contraction and the sustained contraction (the latter under conditions of either the presence or the absence of the phasic component). Furthermore, this initial common mechanism may involve, at least in part, the interaction of NE with the alpha-adrenergic receptor, as evidenced by the inhibition of both components of the biphasic response by phentolamine.
Effects of Vasodilatory Agents on the Phasic and Sustained Contraction Induced by Norepinephrine in the Absence of Extracellular Calcium.

A. Introduction

As described in the preceding chapter, NE induces a biphasic contraction of the rat aorta in calcium free buffers containing 1 mM EGTA. If these contractions are the result of calcium mobilization from two separate calcium pools, or alternatively, if they are the result of NE-induced mobilization of calcium via two distinct activation mechanisms, the possibility exists that a given vasodilator agent may inhibit one of the phases of the NE-induced contraction more specifically than the other phase. The demonstration of such a vasodilatory specificity would be interesting in that it could provide insight into both the mechanisms of action of the vasodilatory compounds and the mechanisms of smooth muscle activation by NE.

Vasodilatory agents which act solely by inhibiting agonist-induced calcium influx can be expected not to significantly affect NE-induced contractions in a calcium-free medium, since these contractions are obviously not dependent upon the entry of extracellular calcium. By contrast, vasodilators with intracellular sites of action, or those that induce vasodilation by mechanisms other
than by inhibiting calcium influx through the sarcolemma might be expected to inhibit contractions which are mediated by intracellular calcium. The ability or failure of a vasodilator agent to inhibit contractions which are mediated solely by intracellular calcium may thus represent the best in vitro evidence for distinction between intracellular and membrane (calcium channel) sites of action.

In order to address these questions, the effects of various vasodilatory agents on NE-induced contractions of the rat aorta in the absence of extracellular calcium was investigated in studies which are described in the present chapter.

B. Methods

Rat aortic tissue segments were isolated and mounted for the measurement of isometric tension generation as described in the Methods section of Chapter I. Normal (calcium-containing) and 40 mM potassium buffers were also prepared as previously described. Calcium-free-EGTA buffer was made by substituting 1 mM EGTA for calcium in the normal buffer.

Stock solutions of N\textsuperscript{6},O\textsuperscript{2'}-dibutyryladenosine 3'5'-cyclic monophosphate (db-cAMP) (Sigma Chemical Co., St. Louis, Mo.), papaverine (Eli Lilly and Co., Indianapolis, Ind.), 8-bromoguanosine 3'5'-cyclic monophosphate (8-Br-cGMP) (Sigma Chemical Co.), Sodium nitroprusside dihydrate (Nipride, Roche Laboratories, Nutley, N.J.), phentolamine (Regitine mesylate, Ciba Pharmaceutical Co., Summit, N.J.), verapamil (Isoptin, Knoll Pharmaceutical Co., Whippany, N.J.), the quaternary 2-n-butyl 3-trimethylamino-5,6-methylenedioxyindene
iodide (Q-bu-MDI; synthesized in our laboratory as per Witiak et al., 1982) and the tertiary 2-n-propyl and 2-n-butyl substituted 3-dimethylamino-5,6-methyleneoxyindene hydrochlorides (pr-MDI and bu-MDI, respectively; synthesized as per Witiak et al., 1974) used in the following experiments were prepared fresh daily in normal saline solution. Phenoxybenzamine (Dibenzyline, Smith Kline and French Laboratories, Philadelphia, Pa.), nifedipine (Pfizer Pharmaceuticals, New York, N.Y.), and SKF24260 (a nifedipine analogue) (Smith Kline and French Laboratories) solutions were prepared by dilution into saline of a $10^{-3}$M stock solution prepared in absolute ethanol immediately prior to use. Stock solutions of nitroprusside, nifedipine SKF24260 were protected from light, and experiments using these compounds were performed under subdued light to prevent degradation. L-Norepinephrine bitartrate (NE) (L-arterenol bitartrate) (Sigma Chemical Co.) solutions were made in normal saline containing 0.05 percent sodium metabisulfite as an antioxidant.

All concentrations cited in the text represent final tissue bath concentrations (molar) calculated for the respective salts of each compound.

1. Spasmolytic activity of vasodilator agents against NE- and KCl-induced contractions in the presence of extracellular calcium.

The ability of db-cAMP, papaverine, 8-Br-cGMP, nitroprusside, phen tolamine, nifedipine, SKF24260, verapamil, Q-bu-MDI, bu-MDI and pr-MDI to relax tissues which had been contracted by either high-potassium (40 mM) buffer or $10^{-7}$M NE in the presence of extracellular
calcium was assessed by exposing the contracted tissue to cumulatively increasing concentrations of the vasodilator agent being tested, and monitoring the resultant degree of tissue relaxation. Tissue tension after each concentration of vasodilator was expressed as a percentage of the tissue tension prior to the addition of the vasodilator, and corrected for anticipated time-dependent loss of tension ("fade") as determined in separate control experiments whenever that loss was expected to represent 6 percent or more of the initial tension generation.

For statistical analysis, percent-response data falling between 15 and 85 percent for each tissue were also transformed to their appropriate probit values, and IC50 values were determined for each tissue using a linear regression analysis of the probit response vs. log concentration data. IC50 values for each vasodilatory agent were expressed as the mean of the IC50 values determined for that agent (n = 4-8) with its 95 percent confidence limit.

2. Antispasmodic activity of vasodilatory agents against NE-induced phasic and sustained contractions in the absence of extracellular calcium.

The experimental protocol used to assess the ability of vasodilator agents to inhibit NE-induced phasic and sustained contractions is diagrammed schematically in figure 8. Tissues which had been equilibrated in normal physiological solution were washed 3 times over an initial 10 minute period and then incubated for 20 minutes in calcium-free buffer containing 1 mM EGTA in order to remove
Figure 8. Diagram of experimental protocol used to assess antispasmodic activity of vasodilatory agents towards NE-induced phasic and sustained contractions in the absence of extracellular calcium. See Section 2 for detailed description. Arrows indicate times when tissues were rinsed in calcium-free buffer containing 1 mM EGTA.
Figure 8.
extracellular calcium as previously described (see Chapter I). Tissues were then exposed to $3 \times 10^{-7}$ M NE and the resultant phasic and sustained contractions were monitored for 30 minutes. Tissues were next washed in Ca-free-EGTA buffer for 30 minutes in order to re-establish the baseline resting tension. These initial NE-induced contractions, corrected for slight shifts in resting tension by extrapolating a line between pre- and post-contraction resting tensions, were then considered as "reference" NE-induced contractions for each tissue. Tissues were next washed twice over a 15 minute period in normal (calcium-containing) physiological solution in order to re-load the calcium pool associated with the phasic component of contraction. Each tissue was then again washed three times over a ten minute period and incubated for a 20 minute period in calcium-free-EGTA buffer to remove extracellular calcium. A chosen concentration of the vasodilator to be tested was introduced to the bath during this latter 20 minute incubation period and left in contact with the tissue while $3 \times 10^{-7}$ M NE was re-introduced to the bath. The resulting phasic and sustained contractions were again monitored for 30 minutes. Finally, tissues were washed in calcium-free buffer containing EGTA in order to re-establish baseline resting tension. The magnitudes of the phasic and sustained contractions thus determined in the presence of vasodilator were expressed as a percentage of the tension developed in the phasic and sustained portions of the initial "reference" contractions of each tissue.

During the evaluation of each vasodilator, a number of tissues were not treated with any vasodilator ("untreated control") in order to
assess the magnitude of the second phasic and sustained contractions as a percentage of the first reference phasic and sustained contractions in corresponding untreated tissues.

IC50 values for inhibition of the phasic and sustained contractions by each vasodilator were determined by first expressing the contraction remaining in each tissue after vasodilator treatment as a percentage of the mean phasic and sustained contractions remaining in tissues which were run simultaneously but not treated with a vasodilator ("control"). These percentages were then transformed to their corresponding probit values and analyzed vs. the log of vasodilator concentration by linear regression analysis to determine the IC50 value and its corresponding 95% confidence interval for each vasodilator.

Since, as described in Chapter I, aortic tissue slowly loses its ability to contract phasically under these conditions of repeated NE-stimulation, each tissue was used for only one pair of test contractions.

3. Effect of vasodilator agents on the NE concentration-response curve in calcium-free medium after elimination of the phasic component of the NE contraction.

The effects of db-cAMP ($1.8 \times 10^{-5}$M - $5 \times 10^{-5}$M), 8-Br-cGMP ($10^{-5}$M - $10^{-4}$M), phentolamine ($10^{-7}$M - $10^{-6}$M), verapamil $3 \times 10^{-5}$M - $10^{-3}$M) and pr-MDI ($5 \times 10^{-5}$M - $3 \times 10^{-4}$M) on the NE concentration-response relationship were assessed using the experimental protocol diagrammed in figure 9. Tissues were washed free of extracellular
calcium and equilibrated in calcium-free-EGTA buffer as described earlier (section 2). In order to deplete the calcium pool associated with the phasic contraction (and thereby to render the tissue monophasically responsive to NE), $10^{-6}$ M NE was introduced to the baths for a 30 minute period. Each tissue was then washed for 30 minutes in calcium-free-EGTA buffer to re-establish baseline resting tension. Tissues were next re-exposed to $10^{-6}$ M NE in order to obtain a reference contraction which represented a near-maximum inducible sustained contraction for each tissue under these conditions (see Chapter I). Tissues were then again washed free of NE using calcium-free-EGTA buffer. After each tissue had again relaxed (30 minutes), a chosen concentration of one of the five vasodilators to be tested (or no vasodilator for untreated control experiments) was introduced to the bath for 20 minutes. In the continued presence of the vasodilator, NE was then cumulatively added to the bath and the induced sustained contraction at each NE concentration was monitored. Finally, tissues were again washed in calcium-free-EGTA to establish unstimulated resting tension. Following each washout period (prior to each period of NE exposure) resting tension was re-established at 1 g. Since resting tension of aortic strips exposed to calcium-free-EGTA buffer for such extended periods of time tended to slowly rise (approximately 75 mg. during the first 4 hours), $10^{-6}$ M phentolamine was occasionally introduced to the baths at the end of each experiment to confirm that the NE-induced tension had been completely reversed during the final washout period. NE-induced tension generation was corrected for any shifts in baseline as described earlier.
Figure 9. Diagram of experimental protocol used to assess the effects of vasodilator agents on the NE concentration-response curve in calcium-free-EGTA buffer, after elimination of the phasic component of contraction. See Section 3 for detailed description. Arrows indicate times when tissues were rinsed in calcium-free buffer containing 1 mM EGTA.
REFERENCE

Ca\(^{++}\)-free 10\(^{-6}\) M NE Ca\(^{++}\)-free
(Ca\(^{++}\)-free) wash (Ca\(^{++}\)-free)

UNTREATED ±VASODILATOR (Ca\(^{++}\)-free)

F

Ca\(^{++}\)-free wash

CUMULATIVE ADDITION OF NE

10\(^{-6}\) M NE (Ca\(^{++}\)-free)

(10\(^{-6}\) M NE (Ca\(^{++}\)-free)

15 min

15 min

TREATED

UNTREATED CONTROL

Figure 9.
For graphic purposes, tension generation at each concentration of NE was expressed as a percentage of the reference sustained contraction for each tissue. For each concentration of vasodilator, the average of these percentages (± S.E.M.) at each NE concentration was graphed (e.g., figure 10).

ED50 values for NE in the presence of the vasodilator were calculated by expressing the tension generated at each concentration of NE as a percentage of the maximum tension generated by that tissue in the presence of the vasodilator. As before, the probit transformation of these percentages were subjected to linear regression analysis vs. the log of the NE concentration to determine ED50 values for each tissue, and mean ED50 value for each treatment group was determined. The significance of the shifts of the NE ED50 produced by each vasodilator was determined using analysis of variance.

During the experiments with phentolamine, when individual strips of the paired tissues taken from each rat had been assigned one to a phentolamine treatment group and the other to the control (no phentolamine) group, an estimate of the shift in the ED50 of the NE concentration-response curve induced by a given concentration of phentolamine was determined by comparing the NE ED50 in the presence of phentolamine to that determined under the control (no phentolamine) conditions. Using this comparison, dose ratios were determined and a Schild plot was constructed in order to estimate the pA2 value for phentolamine (Schild, 1947; Arunlakshana and Schild, 1959). While dose ratios thusly determined for phentolamine concentrations of 10^{-7}M
and 3 x 10^{-7} M were not affected by the inclusion of 10^{-6} M propranolol in the tissue bath during the NE concentration-response determination, dose ratios determined for 10^{-6} M phenolamine in the absence of propranolol were larger than those determined in the presence of propranolol. This result indicates a contribution of beta-receptor stimulation at NE concentrations required to reverse the effects of 10^{-6} M phenolamine, and so dose ratios were determined for this phenolamine concentration (10^{-6} M) in the presence of 10^{-6} M propranolol.

4. **Effect of phenoxybenzamine pretreatment on the NE concentration-response curve in calcium-free medium after the elimination of the phasic component of NE contraction.**

Pairs of aortic strips were removed from each rat, mounted in tissue baths, and washed in calcium-free-EGTA buffer as previously described. 10^{-6} M NE was then added to each bath and the resulting contractions were monitored for 30 minutes before the tissues were again washed in calcium-free-EGTA buffer and allowed to relax to baseline resting tension. In these experiments, this initial NE exposure was used to both deplete the calcium pool associated with the phasic contraction, and to determine an estimate of the maximum sustained contractile tension generated by each tissue for use as a reference contraction. One strip from each paired aortic tissue was then exposed to 3 x 10^{-9} M phenoxybenzamine for 15 minutes while, as a control, the other strip was exposed to a dilution of ethanol in saline equivalent to the diluent of the phenoxybenzamine stock
solution. Each strip was next washed 4 times over a 30-minute period with calcium-free-EGTA buffer to remove the phenoxybenzamine which had not bound covalently to the alpha receptor. NE was then cumulatively added to the baths and the induced sustained contraction was monitored.

As described earlier, NE-induced tension generation was expressed as a percentage of the reference sustained contraction for each tissue. ED50 values for each curve were also estimated as previously described for cumulative NE-induced contractions (section 3). To determine whether phenoxybenzamine pretreatment caused a shift in the NE concentration-response curve, the ED50 values of NE in the phenoxybenzamine-treated tissues were compared to those of their respective paired control tissues and analyzed for a statistically significant difference using a paired t-test (n=6). In addition, data obtained from paired control and phenoxybenzamine-treated tissues was analyzed according to the methods of Parker and Waud (1971) in order to obtain estimates of the -log K_A of NE under calcium-free conditions.

C. Results and Discussion

1. Dibutyryl-cAMP, 8-Br-cGMP, Papaverine and Sodium Nitroprusside.

a.) Results

The ability of db-cAMP, 8-Br-cGMP, papaverine and nitroprusside to inhibit contractions of aortic strips which had been induced by potassium or by NE in the presence of 2.5 mM bath calcium, or by NE in the absence of bath (extracellular) calcium are shown in figures 10-
13. Concurrent with the findings of Chapter I, tissues which had been
stimulated with NE according to the scheme depicted in figure 8 did
not contract as forcefully upon the second exposure, even in the
absence of vasodilators. In addition, the tension of the second
contraction of each control tissue as a percentage of the first
contraction was observed to vary slightly when control tissues run
simultaneously with vasodilator-treated tissues were compared. The
reasons for this variation are not known. However, since the standard
error of the average tension generation by untreated control tissues
during the course of experiments with vasodilators was small,
statistical analysis for each vasodilator was performed using data
from the simultaneously-run untreated tissues as the reference.

Each of the vasodilatory agents tested inhibited the contractions
induced in the absence of extracellular calcium in a concentration-
dependent manner. The IC50 values for db-cAMP, 8-Br-cGMP, papaverine
and nitroprusside for the inhibition of contractions induced by
potassium or NE in the presence of extracellular calcium, or by NE in
the absence of extracellular calcium are shown in the upper portion of
Table 2. (The IC50 data for vasodilatory compounds tested in this and
subsequent sections are presented collectively in Table 2 to make it
easier to compare the IC50 data for vasodilatory agents with different
mechanisms of action).

The IC50 values of db-cAMP, 8-Br-cGMP, papaverine and
nitroprusside for inhibition of NE-induced contractions that are
dependent upon intracellular calcium were consistently equal to or
less than the respective IC50 value of each of these vasodilators
Log IC50 values (molar concentrations) with their 95 percent confidence intervals for the inhibition of NE-induced contractions of the rat aorta in the presence or absence of extracellular calcium by a variety of vasodilatory agents. Inhibition of (10⁻⁷ M) NE-and (40 mM) potassium-induced contractions in the presence of extracellular (bath) calcium were determined by cumulative addition of each vasodilatory agent to tissues contracted by one of these agents (n=4-8). Inhibition of the phasic and sustained contractions induced by NE in calcium-free buffer containing 1 mM EGTA was determined by preincubating tissues with given concentrations of a vasodilator prior to the introduction of NE to the bath, and comparing the resultant contraction to NE-induced control contractions previously obtained from the same tissue (see figure 8, n=6-8 tissues per concentration of each vasodilator). IC50s were defined as the concentration of vasodilator which inhibited NE-or potassium-induced contractions by 50 percent. See sections 1 and 2 under "Methods" for details. Significant differences in IC50 values may be identified by the failure of their 95 percent confidence intervals to overlap.

<table>
<thead>
<tr>
<th>Agonist and Buffer</th>
<th>Normal buffer (2.5 mM calcium)</th>
<th>Calcium-free buffer (1.0 mM EGTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>potassium (40 mM)</td>
<td>NE (10⁻⁷ M)</td>
</tr>
<tr>
<td>dibutyryl-cAMP</td>
<td>-4.21±0.08</td>
<td>-4.52±0.15</td>
</tr>
<tr>
<td>Papaverine</td>
<td>-5.08±0.19</td>
<td>-5.41±0.12</td>
</tr>
<tr>
<td>8-Br-cGMP</td>
<td>-3.86±0.14</td>
<td>-4.25±0.14</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>-8.02±0.25</td>
<td>-7.88±0.27</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>-4.35±0.16</td>
<td>-7.62±0.28</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>-8.70±0.29</td>
<td>-6.79±0.33</td>
</tr>
<tr>
<td>SKF24260</td>
<td>-8.12±0.30</td>
<td>-6.84±0.76a</td>
</tr>
<tr>
<td>Verapamil</td>
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<td>-5.97±0.42</td>
</tr>
<tr>
<td>bu-MDI</td>
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</tr>
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<td>Q-bu-MDI</td>
<td>-3.77±0.10</td>
<td>-3.89±0.28</td>
</tr>
</tbody>
</table>

a. Maximum inhibition obtained was 60 percent. The −log IC50 reported represents an estimate of concentration which produced relaxation of contraction by 50 percent. 50 percent of maximum vasodilatory effect of SKF24260 occurred at 1.9 x 10⁻⁸ M., (log 50% max. effect 95 percent confidence interval=7.73±0.36).

b. Inhibition curve was biphasic.
determined for the inhibition of potassium-induced contractions (Table 2). Similarly, the IC50 of each of these vasodilators for inhibition of NE-induced contractions in the presence of extracellular calcium was also less than or equal to the IC50 of that vasodilator against the potassium-induced contraction. However, no consistent relationship between a vasodilator's IC50 for NE-induced contraction in the presence of extracellular calcium and for NE-induced contractions in the absence of extracellular calcium was observed (Table 2). 8-Br-cGMP and nitroprusside were more potent inhibitors of contractions induced by NE in the absence of extracellular calcium than in the presence of this cation (figures 12 and 13), while papaverine and db-cAMP (vs. the phasic contraction) (figures 10 and 11) were more potent inhibitors of NE-induced contraction when extracellular calcium was present, than when it was absent (Table 2).

In calcium-free-EGTA buffer, papaverine, 8-Br-cGMP, and nitroprusside inhibited both components of the NE-induced biphasic contraction to an equal extent (Table 2, figures 11-13). By contrast, at concentration of $10^{-5}$M to $10^{-4}$M db-cAMP was found to be a more potent inhibitor of the sustained component of the contraction than of the phasic component ($P<0.05$, Student's $t$ test) (figure 10). The IC50 value of db-cAMP for the sustained contraction ($2.5 \times 10^{-5}$M) differed significantly from that of the phasic contraction ($6.3 \times 10^{-5}$M) as evidenced by the failure of their 95% confidence intervals to overlap (Table 2).

In calcium-free-EGTA buffer, after elimination of the phasic component of the NE-induced contraction (Section C of Methods and
Figure 10. Effect of db-cAMP on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contraction induced by potassium (40mM) (□□□□) or NE (10⁻⁷M) (○○○○) in the presence of extracellular calcium was determined by adding db-cAMP in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each db-cAMP addition is expressed as a percentage of tissue tension before db-cAMP addition, as described in Methods section 1. Inhibition of the NE-induced phasic (■■■■) and sustained (●●●●) contractions in calcium-free (1 mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of db-cAMP and then introducing NE (3 x 10⁻⁷M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the db-cAMP test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 10.
Figure 11. Effect of papaverine on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contraction induced by potassium (40mM) (□□□) or NE (10^-7M) (○○○) in the presence of extracellular calcium was determined by adding papaverine in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each papaverine addition is expressed as a percentage of tissue tension before papaverine addition, as described in Methods section 1. Inhibition of the NE-induced phasic (■■■) and sustained (●○○) contractions in calcium-free (1 mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of papaverine and then introducing NE (3 x 10^-7M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the papaverine test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 11.
Figure 12. Effect of 8-Br-cGMP on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contraction induced by potassium (40mM)(□-□-□) or NE (10⁻⁷M)(○-○-○) in the presence of extracellular calcium was determined by adding 8-Br-cGMP in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each 8-Br-cGMP addition is expressed as a percentage of tissue tension before 8-Br-cGMP addition, as described in Methods section 1. Inhibition of the NE-induced phasic (■-■-) and sustained (●-●-) contractions in calcium-free (1 mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of 8-Br-cGMP and then introducing NE (3 x 10⁻⁷M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the 8-Br-cGMP test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 12.
Figure 13. Effect of nitroprusside on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contraction induced by potassium (40mM) (■ ■ ■) or NE (10⁻⁷ M) (○○○) in the presence of extracellular calcium was determined by adding nitroprusside in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each nitroprusside addition is expressed as a percentage of tissue tension before nitroprusside addition, as described in Methods section 1. Inhibition of the NE-induced phasic (■ ■ ■) and sustained (○○○) contractions in calcium-free (1 mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of nitroprusside and then introducing NE (3 x 10⁻⁷ M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the nitroprusside test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 13.
figure 9) the NE concentration-response relationship was inhibited noncompetitively by both db-cAMP (figure 14) and 8-Br-cGMP (figure 15). Concentrations of $1.8 \times 10^{-5}$ M, $3 \times 10^{-5}$ M and $5 \times 10^{-5}$ M db-cAMP depressed the maximum NE-induced tension generation to, respectively, 84, 45 and 28 percent of that developed under control conditions. The ED50 values of the NE concentration-response curves determined in the presence of these concentrations of db-cAMP were $2.6 \times 10^{-8}$ M, $3.0 \times 10^{-8}$ M and $4.4 \times 10^{-8}$ M, respectively (based upon the maximum tension generated to NE in the presence of db-cAMP, as described in Methods section 3). Only the NE ED50 determined for the highest concentration of db-cAMP was significantly different from the value of $2.1 \times 10^{-8}$ M determined for control (untreated) tissues ($P<0.05$; analysis of variance). At concentrations of $10^{-5}$ M, $3 \times 10^{-5}$ M and $10^{-4}$ M, 8-Br-cGMP depressed maximum NE-inducible tension to 55, 35 and 29 percent of control, respectively. For these concentrations of 8-Br-cGMP, respective ED50 values of the NE concentration-response curves (based upon maximum tension generated in each tissue; Methods section 3) were $2.3 \times 10^{-8}$ M, $2.5 \times 10^{-8}$ M and $3.3 \times 10^{-8}$ M. These values did not differ significantly from the ED50 value of $2.1 \times 10^{-8}$ M determined simultaneously for NE in untreated control tissues ($P>0.05$; analysis of variance).

Maximum tension generation during the cumulative NE concentration-response curves of untreated control tissues was found to be greater than the maximum tension generated in these tissues during their respective reference contractions (see figures 14 and 15). For this reason, tension generation in control tissues plateaus
Figure 14. Effect of db-cAMP on cumulative NE concentration-response curves in calcium-free medium after elimination of the phasic component of contraction. Cumulative NE concentration-response curves were determined as diagrammed in figure 9 and described in Methods section 3. Briefly, tissues which had been maintained in calcium-free (1 mM EGTA) buffer and preincubated for 20 min. in the desired concentration of db-cAMP, were exposed to cumulatively increasing concentrations of NE and the resulting sustained contractions were monitored. Tension was expressed as a percentage of the tension generated during a previously-determined reference contraction for each tissue induced by 10^{-6}M NE after the elimination of the phasic component of contraction.
Figure 14.
Figure 15. Effect of 8-Br-cGMP on cumulative NE concentration-response curves in calcium-free medium after elimination of the phasic component of contraction. Cumulative NE concentration-response curves were determined as diagrammed in figure 9 and described in Methods section 3. Briefly, tissues which had been maintained in calcium-free (1 mM EGTA) buffer and preincubated for 20 min. in the desired concentration of 8-Br-cGMP, were exposed to cumulatively increasing concentrations of NE and the resulting sustained contractions were monitored. Tension was expressed as a percentage of the tension generated during a previously-determined reference contraction for each tissue induced by $10^{-6}$ M NE after the elimination of the phasic component of contraction.
Figure 15.
at values greater than 100 percent of the reference contraction. This finding is consistent with the findings of Chapter 1, which reported that with repeated NE stimulation under calcium-free conditions, maximum tension generation of the tissue during the sustained contraction increases.

b.) Discussion

The findings that db-cAMP, 8-Br-cGMP, papaverine and nitroprusside each inhibit contractions which are dependent solely upon intracellular calcium at concentrations similar to those that inhibit contractions that also depend upon extracellular calcium supports the proposals that these agents are able to induce vasodilatation via intracellular mechanisms of action. A variety of possible vasodilatory sites and mechanisms of action have been proposed for each of these agents (see Introduction, section IV). The significant potency of each of these four agents in calcium-free medium makes it possible to exclude some of these mechanisms as the primary means of vasodilation in this system.

The dibutyryl analogue of cAMP is a lipophilic cAMP derivative which is proposed to enter the smooth muscle cell, and which generally mimics the actions of cAMP in both whole tissue (Kreye and Schultz, 1972; Webb and Bohr, 1981) and isolated microsomal preparations (Baudouin-Legros and Meyer, 1973). In mimicking cAMP this compound might be proposed to regulate smooth muscle contractions via a number of potential mechanisms. Phosphorylation of MLCK by a cAMP-dependent protein kinase has been demonstrated to result in the inhibition of
MLCK in a number of smooth muscle preparations (Adelstein et al., 1978; Silver and DiSalvo, 1979; Adelstein and Hathaway, 1979; Barany and Barany, 1981; Vallet et al., 1981). Since MLCK activity has been correlated with the initiation and maintenance of smooth muscle tension (Adelstein and Hathaway, 1979; Barany and Barany, 1981; Hartshorne and Mrwa, 1982), cAMP-dependent inhibition of MLCK may be expected to result in the reversal or prevention of smooth muscle contraction. Cyclic AMP has also been proposed to stimulate intracellular calcium sequestration, since cAMP-dependent phosphorylation of microsomal proteins is correlated with the stimulation of calcium uptake by microsomal membrane preparations (Fitzpatrick and Szentivani, 1977; Bhalla et al., 1981). Such an increase in calcium sequestration could also induce vasodilatation by lowering the concentration of intracellular activator calcium. Either of these mechanisms might be expected to mediate vasodilatation regardless of whether the source of calcium is intracellular in location or is the extracellular fluid.

An additional mechanism by which cAMP has been proposed to mediate vasodilatation is derived from the finding that cAMP stimulates sodium/potassium ATPase activity of the smooth muscle plasma membrane (Somlyo et al., 1972; Webb and Bohr, 1981). This stimulation results in membrane hyperpolarization, and so it has been proposed that it may induce vasodilatation by reducing the stimulus for muscle contraction (Kreye, 1981). In the smooth muscle system currently being studied, however, it seems unlikely that this activity could represent the primary mechanism of vasorelaxation. The failure
of potassium to induce contraction of the aortic strip in calcium-free EGTA buffer (see Chapter I) argues against a role for membrane polarization as a primary regulator of tension generation in this preparation. Indeed, while under calcium-free conditions the tissue remains responsive to NE concentrations of $10^{-9}$ M to $10^{-7}$ M, these concentrations have been found not to significantly change membrane polarization in a number of smooth muscle preparations (Su et al., 1964; Mekata and Niu, 1972; Casteels et al., 1977 a,b).

Thus, our data supports the proposals of a primarily intracellular mechanism of action for db-cAMP. The noncompetitive inhibition of the NE concentration-response curve by db-cAMP (figure 14) is also consistent with the type of inhibition which would be expected from a vasodilatory agent which inhibits the response to NE at an intracellular site subsequent to alpha adrenoreceptor occupancy (Ariens and Simonis, 1964a).

Like cAMP, cGMP also activates smooth muscle protein kinase enzymes which phosphorylate endogenous membrane proteins (Casnellie and Greengard, 1974). It is presumed that the vasodilatory activity of cGMP is thus also the result of the simulation of specific phosphorylation reactions within the cell, and that 8-Br-cGMP, a cGMP analogue which is more lipophilic, is able to enter the cell and mimic the activity of the cGMP parent molecule (Kukovitz et al., 1981; Schultz et al., 1978). The demonstration that 8-Br-cGMP inhibits contractions which are dependent upon intracellular calcium (figure 12) is consistent with such a proposed intracellular site of action for this compound. Similar to db-cAMP, 8-Br-cGMP also inhibits
the NE concentration-response curve with noncompetitive kinetics (figure 15) consistent with an intracellular site of action (Ariens and Simonis, 1964a).

Papaverine has been proposed to have two potential sites of action whereby it might induce the relaxation of vascular smooth muscle. It has been pointed out by Bolton (1979) that many of the actions of papaverine may be explained by the assumption that it blocks calcium ion channels of the sarcolemma (see Introduction, section IV D). Indeed, papaverine has been found to inhibit potassium-stimulated $^{45}$Ca influx in vascular smooth muscle (Demesy and Godfraind, 1972; Thorens and Haeusler, 1979). However, inhibition of calcium influx obviously cannot be the primary vasodilatory mechanism of papaverine in the calcium-free system being studied, since extracellular calcium has been removed in this system (see Chapter 1).

The inhibition by papaverine of NE-induced contractions which are dependent upon intracellular calcium at concentrations similar to those which inhibit contractions dependent upon extracellular calcium again suggests that the primary site of action of papaverine is intracellular. As an inhibitor of cAMP phosphodiesterase in smooth muscle (Uruno et al., 1974; Miyamoto et al., 1976; Polson et al., 1978; Kukovetz et al., 1981), papaverine promotes the cellular accumulation of cAMP (Kukovetz and Poch, 1970; Triner et al., 1972). Papaverine-induced vasodilatation may therefore be mediated by intracellular mechanisms stimulated by cAMP. As previously described, a number of these mechanisms are consistent with the inhibition of contractions induced by intracellularly released calcium.
Nitroprusside has also been proposed to have both membrane and intracellular sites of action whereby it might mediate vasodilation (see Introduction, section IV E): it hyperpolarizes the arterial smooth muscle plasma membrane (Haeusler and Thorens, 1976; Ito et al., 1978) and it stimulates the accumulation of cGMP in vascular smooth muscle (Axelsson et al., 1979; Kukovetz et al., 1979; Keith et al., 1982). While again, it seems unlikely that hyperpolarization could have a major vasodilatory effect on the calcium-free system being studied, the stimulation of intracellular cGMP-dependent phosphorylation might be expected to underlie the vasodilatory mechanism of action of nitroprusside.

It is interesting to note that each of the vasodilatory agents under discussion inhibited NE-induced contractions in absence or in presence of extracellular calcium with a potency equal to or greater than that against potassium-induced contractions (Table 2). By contrast, compounds which act primarily by inhibiting calcium influx through the plasma membrane generally demonstrate significantly greater potency against potassium-induced contractions than against NE-induced contractions (Rahwan, 1982; see also Introduction, section IV A, and this chapter, section 3).

The significance of slight differences in the ability of each of these agents to inhibit NE-induced contractions in the presence as compared to the absence of extracellular calcium is unclear. However, it is also interesting that the cGMP analogue, 8-Br-cGMP, and an agent shown to stimulate intracellular cGMP formation, sodium nitroprusside, are each more potent inhibitors of contractions induced by NE in the
absence of extracellular calcium than in its presence. By contrast, the cAMP analogue, db-cAMP, and papaverine, an agent proposed to stimulate cAMP accumulation, are generally less potent (or at best equipotent) inhibitors of contractions induced by NE in the absence of calcium as in the presence of calcium.

The finding that both the phasic and the sustained components of the contraction induced by NE in the absence of extracellular calcium were inhibited by each of the agents under discussion is evidence that both types of contraction are susceptible to similar intracellular regulatory processes. Papaverine, 8-Br-cGMP and nitroprusside each inhibited both phases of the biphasic NE contraction to an equal degree (Table 2, figures 11-13) while db-cAMP was a significantly more potent inhibitor of the sustained contraction than the phasic contraction (Table 2, figure 10). The reason for this difference in potency for db-cAMP is not clear, however, it may represent a particular sensitivity of the sustained contraction to one of the vasodilatory mechanisms stimulated by db-cAMP. Such a finding would be interesting since it may be a clue to differences in the intracellular mechanisms for induction of the phasic and sustained contractions by NE. However, the failure of papaverine (which causes intracellular cAMP accumulation) to similarly inhibit the sustained contraction with greater potency than the phasic contraction makes it difficult to interpret the results with db-cAMP in terms of cAMP-induced vasodilatory processes. Further study is necessary if the reason for differences in potency of db-cAMP for inhibition of the sustained and phasic contractions is to be determined.
In summary, the following has been determined: 1) Both the phasic and the sustained components of the NE-induced biphasic contraction of the rat aorta in calcium-free buffer are inhibited in a concentration-dependent manner by four vasodilatory agents which have been proposed to have intracellular sites of action (db-cAMP, 8-Br-cGMP, papaverine and nitroprusside); 2) These vasodilators inhibit NE-induced phasic and sustained contractions in calcium-free buffer (which are dependent upon intracellular calcium) at concentrations equal to or less than those necessary to equally inhibit potassium-induced contractions (which are dependent upon extracellular calcium); 3) Dibutyryl-cAMP is a more potent inhibitor of the sustained component of the NE-induced contraction in calcium-free buffer than of the phasic component, while papaverine, 8-Br-cGMP and nitroprusside inhibit both components to an equal extent; and 4) Both db-cAMP and 8-Br-cGMP inhibit the NE concentration-response curve in calcium-free buffer in a noncompetitive manner, which is consistent with an intracellular site of action.

2. Alpha Receptor Antagonists: Phentolamine and Phenoxybenzamine

a.) Results

The effect of phentolamine on NE-induced contractions in the presence and absence of extracellular calcium and on potassium-induced contractions in the presence of extracellular calcium are shown in figure 16. While phentolamine inhibited each of these contractions in a concentration-dependent manner, it exhibited a much greater potency
Figure 16. Effect of phentolamine on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contractions induced by potassium (40mM) (-□-□-) or NE (10^-7M) (-○-○-) in the presence of extracellular calcium was determined by adding phentolamine in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each phentolamine addition is expressed as a percentage of tissue tension before phentolamine addition, as described in Methods, section 1. Inhibition of the NE-induced phasic (-■-■-) and sustained (-♦-♦-) contractions in calcium-free (1mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of phentolamine and then introducing NE (3 x 10^-7 M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the phentolamine test contraction, as diagrammed in figure 8 and described in Methods, section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 16.
against NE-induced contractions, whether in the presence or absence of extracellular calcium, than against contractions induced by potassium. This difference in potency is apparent in Table 2, where it can be seen that the IC50 values for phentolamine inhibition of NE-induced contractions are about 2.5 log units less than those for inhibition of potassium-induced contractions. Phentolamine was an equi-effective inhibitor of the phasic and sustained components of the NE-induced contraction in the absence of calcium, as evidenced by the overlap of the 95 percent confidence intervals of the IC50 values for phentolamine inhibition of these contractions (Table 2).

The effects of phentolamine on the cumulatively determined NE concentration-response curve after the elimination of the phasic component of contraction are shown in figure 17. In contrast to the results previously described for db-cAMP and 8-Br-cGMP (see preceding section), concentration of 10^-7 M, 3 x 10^-7 M and 10^-6 M phentolamine significantly (P<0.05) and concentration-dependently shifted the ED50 of the NE concentration-response curve from a control of 2.5 x 10^-8 M to 1.9 x 10^-7 M, 4.7 x 10^-7 M and 1.9 x 10^-6 M, respectively, without significantly depressing the maximum-inducible tension (P>0.05, analysis of variance). A Schild plot of the paired tissue data from these experiments (see Methods section 3, this chapter) yielded a straight line with a correlation co-efficient of 0.93 and a slope of 1.01 (figure 18). The pA2 value was determined from this plot to be 7.73. The effects of phenoxybenzamine pretreatment on the NE concentration response curve in the absence of extracellular calcium (see Methods, section 4) are shown in figure 19. While pretreatment
Figure 17. Effect of phentolamine on cumulative NE concentration-response curves in calcium-free medium after elimination of the phasic component of contraction. Cumulative NE concentration-response curves were determined as diagrammed in figure 9 and described in Methods, section 3. Briefly, tissues which had been maintained in calcium-free (1mM EGTA) buffer and preincubated for 20 min. in the desired concentration of phentolamine, were exposed to cumulatively increasing concentrations of NE and the resulting sustained contractions were monitored. Tension was expressed as a percentage of the tension generated during a previously-determined reference contraction for each tissue induced by $10^{-6}$M NE after the elimination of the phasic component of contraction.
Figure 17.
Figure 18. Schild plot for phentolamine-induced shifts of the cumulative NE concentration-response curve in calcium-free medium after elimination of the phasic component of contraction. Whenever the paired tissues obtained from a single rat were assigned one to a phentolamine-treated group and the other to a control (untreated) group during the determination of cumulative NE concentration-response curves (Methods, section 3, Figure 9) a dose ratio (DR) of the ED50s of NE in the presence and absence of phentolamine was determined. Log (DR-1) vs. phentolamine concentration was plotted, and, using linear regression analysis, an estimate of the phentolamine pA2 was determined according to the methods of Schild (1947). Numbers in parenthesis represent the n of dose ratios at each phentolamine concentration.
**Figure 18.**

A graph showing the relationship between **Log (DR-1)** and **Phentolamine Concentration (M)**. The graph includes data points and a line of best fit with the following parameters:

- $pA_2 = 7.73$
- $r = 0.93$
- $m = 1.01$

The graph indicates a linear relationship with error bars for certain data points.
Figure 19. Effect of phenoxybenzamine pretreatment on the cumulative NE concentration-response curve in calcium-free medium after elimination of the phasic component of contraction. Paired tissues taken from each rat were assigned one to a phenoxybenzamine-pretreated group and the other to an untreated control group (n=6 for each group). Each tissue was washed in calcium-free EGTA (1mM) buffer and exposed to $10^{-6}$M NE in order to eliminate the phasic component of contraction and to obtain a reference sustained contraction. After again washing the tissues in calcium-free EGTA buffer, tissues in the phenoxybenzamine-treated group were exposed to $3 \times 10^{-9}$M phenoxybenzamine (15 min.) and re-washed in calcium-free EGTA buffer. Both phenoxybenzamine-treated and untreated control tissues were next exposed to cumulatively increasing concentrations of NE and the resulting sustained contractions were monitored. Tension was expressed as a percentage of the tension generated during the initial NE-induced reference sustained contraction. See Methods, section IV for further details.
Figure 19.

PERCENT OF REFERENCE CONTRACTION

CONCENTRATION OF NOREPINEPHRINE (M)

UNTREATED CONTROL

PHENOXYBENZAMINE TREATED
with $3 \times 10^{-9} \text{M}$ phenoxybenzamine reduced the maximum NE-induced tension by 57 percent, this pretreatment did not significantly shift the ED50 of the NE concentration-response relationship ($P > 0.05$, paired $t$ test). The mean NE ED50 value for phenoxybenzamine-treated tissues (based upon 50 percent of maximum tension generated as per Methods, section 4) was $3.2 \times 10^{-8} \text{M}$, while that for untreated tissues was $2.6 \times 10^{-8} \text{M}$. The $-\log K_A$ of NE under these calcium-free conditions was determined to be $7.22 \pm 0.12$ (S.E.M.), which corresponds to a $K_A$ value of $6.0 \times 10^{-8} \text{M}$.

b.) Discussion

While it is evident from the preceding section concerning db-cAMP, papaverine, 8-Br-cGMP and nitroprusside that vasodilatory agents with intracellular mechanisms of action might reasonably be expected to inhibit NE-induced contractions which are dependent solely upon intracellular calcium, it should also be clear that compounds with alpha receptor antagonist activity could also inhibit these contractions. Preliminary evidence presented in Chapter 1 supports this proposal, since it was found that phentolamine, an alpha receptor blocker, concentration-dependently relaxed the NE-induced sustained contraction in calcium-free buffer (figure 3) and that $10^{-5} \text{M}$ phentolamine pretreatment similarly inhibited both the phasic and sustained components of the NE response in this buffer (see Results, section 1, Chapter I). The supposition of an alpha receptor involvement in these NE-induced responses is verified by the experiments with phentolamine and phenoxybenzamine described in this
section.

The demonstration of similar IC50 values for phenolamine inhibition of the phasic and the sustained portions of the NE-induced contraction is an anticipated extension of work described in Chapter I (see Methods and Results, section 2), which demonstrated that each of these phases of contraction were induced to an equal extent by a given concentration of NE, and therefore presumably by a given alpha receptor occupancy. Thus, inhibition of NE-induced activation of the alpha receptor by phenolamine is expected to similarly inhibit both the phasic and the sustained contractions. This inhibition was found throughout the range of phenolamine concentrations tested (figure 16).

A comparison of the IC50 values for phenolamine inhibition of NE-induced and potassium-induced contractions (Table 2) attests to the selectivity of the alpha antagonistic activity of phenolamine. The difference between these IC50 values, being approximately 2.5 log units, is much larger than the differences of up to 0.7 log units observed between IC50 values for inhibition of NE-induced and potassium-induced contractions by db-cAMP, papaverine, 8-Br-cGMP or nitroprusside (Table 2). Dibutyryl-cAMP, papaverine, 8-Br-cGMP and nitroprusside, having primarily intracellular sites of action, inhibit the effects of NE-induced and potassium-induced elevations in intracellular calcium concentrations with similar efficacy (figures 10-13, Table 2). In contrast, by specifically inhibiting the receptor for NE-induced activation of the smooth muscle, phenolamine inhibits NE-induced contractions at concentrations which do not effect
potassium-induced contractions. Thus, although alpha receptor antagonists and intracellularly-acting vasodilatory agents are both capable of inhibiting NE-induced contractions of the rat aorta in the presence and absence of extracellular calcium, selective alpha antagonists can be expected to demonstrate a much greater potency against NE-induced contractions than against those induced by potassium, while intracellular antagonists so not discriminate less between these two antagonists.

As a competitive alpha antagonist it is also expected that phentolamine inhibition of the NE-induced contraction in calcium-free buffer would be surmountable by increasing NE concentrations. This expectation is verified in figure 17, where it is shown that NE is capable of completely reversing, in a concentration-dependent manner, the inhibition of tissue contractility produced by increasing concentrations of phentolamine. Furthermore, a Schild plot of the shift in the ED50 of NE can be seen to yield a straight line with a slope of 1.0 (figure 18), which is consistent with the requirements of the competitive antagonist model proposed by Schild and his colleagues (Schild, 1947; Arunlakshana and Schild, 1959). The pA2 value of 7.7 thus determined is similar to the value of 7.8 and 8.0 previously determined for phentolamine inhibition of the alpha receptor in the rat aorta (Godfraind, 1976; Patil et al., 1972). It can therefore be concluded that phentolamine inhibits the NE-induced contractions in calcium-free buffer through a competitive inhibition of the alpha receptor. This conclusion is further supported by data obtained during experiments with phenoxybenzamine (figure 19). The -log K_A
value of $7.22 \pm 0.12$ (S.E.M.) determined for NE during phenoxybenzamine pretreatment experiments is similar to the $-\log K_A$ values of 7.0 to 6.5 determined for NE interaction with the alpha receptor of the rabbit aorta (Besse and Furchgott, 1967, 1976) and does not significantly differ from the $-\log K_A$ of $7.39 \pm 0.28$ (S.E.M.) determined for NE binding to the rat aorta alpha receptor (P. Rice, personal communication) in the presence of extracellular calcium.

The similarity of the $K_A$ values for NE in the presence and absence of extracellular calcium would indicate that the affinity of NE binding to the alpha receptor is similar under both conditions. However, it may be noted that the ED50 for NE-induced contractions under calcium-free conditions is significantly higher than the ED50 commonly reported for NE-induced contractions in the presence of extracellular calcium (see Sutter, 1976). The low ED50 value for NE-induced contractions in the presence of extracellular calcium, is due to the relatively good efficacy of NE on the alpha receptor under these conditions. In the presence of extracellular calcium, the aorta is stimulated to contract maximally after only a fraction of the total number of alpha receptors is activated by NE (Besse and Furchgott, 1976). Under these conditions pretreatment of the tissue with phenoxybenzamine to diminish the number of spare receptors results in a shift of the ED50 toward higher concentrations.

In contrast, under conditions in which there are no spare receptors, irreversible inhibition of alpha receptors with phenoxybenzamine depresses the maximum NE-inducible tension without producing a shift in the ED50 of the remaining concentration-response
relationship (Furchgott and Bursztyn, 1967; Ariens et al., 1964b).

Since under our calcium-free conditions the phenoxybenzamine-induced irreversible blockade of alpha receptors caused a depression of the NE concentration-response curve without shifting the NE ED50, it can be concluded that under these conditions there are no spare receptors for induction of this contraction. Since the efficacy of NE in stimulating smooth muscle contraction can be expected to depend on its ability to increase the concentration of free intracellular calcium, it is understandable that the removal of extracellular calcium may result in a condition in which NE has a lower efficacy. In normal (calcium-containing) buffers NE can stimulate calcium influx from the extracellular fluid as well as mobilize intracellular calcium to produce the rise in intracellular calcium concentrations which stimulates smooth muscle contraction (Deth and van Breemen, 1974, 1977; Godfraind, 1976; van Breemen, 1977; see also Introduction, section II C). In calcium-free buffer, however, a given concentration of NE must rely solely upon the mobilization of intracellular calcium to produce its stimulus for contraction (see Chapter I), and so the rise in intracellular calcium concentration produced by NE binding to the alpha receptor is likely to be smaller.

Interestingly, the demonstration of an absence of spare receptors for NE-induced contractions in calcium-free buffer indicates that under calcium-free conditions it is the number of alpha receptors, and not necessarily the size of the intracellular calcium pools which is the limiting factor for tension generation. We have previously demonstrated that the maximum sustained contraction induced by NE
under calcium-free conditions is equal to approximately 24 percent of the maximum NE-inducible tension in the presence of 2.5 mM extracellular calcium (see Chapter I, figure 2). By using other vasoconstrictors, it might then be found that intracellular calcium can support sustained contractions of the aorta which are greater than 24 percent of maximum.

It is important to also note that since there are no spare receptors for NE-induced contractions of the rat aorta in calcium-free buffer, noncompetitive inhibitors of alpha receptor function will depress the maximum NE-inducible tension without shifting the NE-concentration-response relationship (e.g., figure 19) in much the same way as db-cAMP (figure 14) and 8-Br-cGMP (figure 15). Thus, a noncompetitive depression of the NE concentration-response curve by a vasodilatory agent cannot alone be assumed to be indicative of an intracellular site of action for that agent unless the possibility that it inhibits alpha receptor function can be excluded.

In summary, from the results described for experiments with phentolamine and phenoxybenzamine it may be concluded that: 1) Both the phasic and the sustained contractions of the rat aorta induced by NE in the absence of extracellular calcium are the result of NE stimulation of the aortic alpha adrenoreceptor; 2) Phentolamine inhibits the NE-induced biphasic contractions of the rat aorta in calcium-free buffer with an activity (IC50 and pA2) similar to that for its inhibition of NE-induced contraction in the presence of extracellular calcium; 3) While the calcium-free conditions used in our experiments apparently do not substantially alter the affinity of
NE binding to its receptor (as measured by the $K_A$ value), these conditions do appear to reduce the efficacy (and intrinsic activity) of NE; and 4) Under our calcium-free conditions there are no spare receptors for the induction of the biphasic NE response, and so inhibition by a noncompetitive alpha receptor antagonist can be expected to depress the maximum of the NE concentration-response curve without shifting its ED50.

3. Calcium Entry Blockers and Intracellular Calcium Antagonists: Nifedipine, SKF24260, Verapamil, bu-MDI, pr-MDI and Q-bu-MDI

a. Results

The effects of the calcium entry blockers, nifedipine, SKF24260 and verapamil on NE-induced contractions in the presence or absence of extracellular calcium and on potassium-induced contractions in the presence of extracellular calcium are shown in figures 20-22. In the presence of extracellular calcium (2.5mM) each of these agents inhibited potassium-induced contractions with much greater potency than NE-induced contractions: log IC50 values for nifedipine, SKF24260 and verapamil inhibition of potassium-induced contractions were, respectively, 1.91, 1.28 and 1.08 units less than the respective log IC50 values for the inhibition of NE-induced contractions by these agents (Table 2). In contrast to verapamil, which completely inhibited the contraction induced by NE in the presence of bath calcium (figure 22), SKF24260 produced a maximum of only 60 percent inhibition of this contraction (figure 21). Nifedipine was not tested
Figure 20. Effect of nifedipine on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contractions induced by potassium (40mM)(-) or NE (10⁻⁷M)(-○-) in the presence of extracellular calcium was determined by adding nifedipine in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each nifedipine addition is expressed as a percentage of tissue tension before nifedipine addition, as described in Methods section 1. Inhibition of the NE-induced phasic (-■-) and sustained (-○-) contractions in calcium-free (1mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of nifedipine and then introducing NE (3 x 10⁻⁷M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the nifedipine test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 20.
Figure 21. Effect of SKF24260 on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contractions induced by potassium (40mM)(-□-□-) or NE (10^-7M)(-○-○-) in the presence of extracellular calcium was determined by adding SKF24260 in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each SKF24260 addition is expressed as a percentage of tissue tension before SKF24260 addition, as described in Methods section 1. Inhibition of the NE-induced phasic (-■-■-) and sustained (-○-○-) contractions in calcium-free (1mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of SKF24260 and then introducing NE (3 x 10^-7M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the SKF24260 test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 21
Figure 22. Effect of verapamil on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contractions induced by potassium (40mM) (-□-□-) or NE (10^-7M) (-○-○-) in the presence of extracellular calcium was determined by adding verapamil in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each verapamil addition is expressed as a percentage of tissue tension before verapamil addition, as described in Methods section 1. Inhibition of the NE-induced phasic (-■-■-) and sustained (-■-■-) contractions in calcium-free (1mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of verapamil and then introducing NE (3 x 10^-7M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the verapamil test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 22.
at high enough concentrations to allow the determination of its maximum inhibitory effect on NE-induced contractions in presence of extracellular calcium (figure 20).

Neither nifedipine nor SKF24260 produced a marked concentration-dependent inhibition of either the phasic or the sustained components of the NE-induced contractions in calcium-free buffer over the range of concentrations tested (figures 20 and 21), and so the IC50s for these agents under calcium-free conditions could not be determined. Verapamil inhibited the phasic and sustained contractions induced by NE in calcium-free buffer with log IC50 values of -4.17 and -4.00, respectively. These values are much greater than those for verapamil inhibition of either NE- or potassium-induced contractions in the presence of extracellular calcium (Table 2), indicating significantly lesser potency of this compound against contractions which are dependent upon intracellular calcium. The IC50s for verapamil inhibition of the phasic and sustained contractions in calcium-free buffer are not significantly different from each other, as is evidenced by the overlap of their 95 percent confidence intervals (Table 2).

The effects of intracellular calcium antagonists, bu-MDI and pr-MDI, and of the quaternary MDI derivative, Q-bu-MDI, on NE-induced contractions in the presence or absence of extracellular calcium and on potassium-induced contractions in the presence of extracellular calcium are shown in figures 23-25. Each of the three MDI derivatives tested was found to inhibit in a concentration-dependent manner the contractions induced by NE or potassium, whether in the presence or
**Figure 23.** Effect of bu-MDI on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contractions induced by potassium (40mM) (■■■) or NE (10^-7 M) (○○○) in the presence of extracellular calcium was determined by adding bu-MDI in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each bu-MDI addition is expressed as a percentage of tissue tension before bu-MDI addition, as described in Methods section 1. Inhibition of the NE-induced phasic (■■■) and sustained (○○○) contractions in calcium-free (1mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of bu-MDI and then introducing NE (3 x 10^-7 M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the bu-MDI test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 23.
Figure 24. Effect of Pr-MDI on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contractions induced by potassium (40mM) (-----) or NE (10^-7M) (-----) in the presence of extracellular calcium was determined by adding Pr-MDI in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each Pr-MDI addition is expressed as a percentage of tissue tension before Pr-MDI addition, as described in Methods section 1. Inhibition of the NE-induced phasic (-----) and sustained (-----) contractions in calcium-free (1mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of Pr-MDI and then introducing NE (3 x 10^-7M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the Pr-MDI test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 24.
Figure 25. Effect of Q-bu-MDI on contractions of the rat aorta induced by NE in the presence or absence of extracellular (bath) calcium or by potassium in the presence of extracellular calcium. Inhibition of contractions induced by potassium (40mM)(-□--□-) or NE (10⁻⁶M)(-○-○-) in the presence of extracellular calcium was determined by adding Q-bu-MDI in stepwise increasing concentrations to the buffer bathing tissues which had been pre-constricted (30 min.) with these agents. Tension after each Q-bu-MDI addition is expressed as a percentage of tissue tension before Q-bu-MDI addition, as described in Methods section 1. Inhibition of the NE-induced phasic (-■-■-) and sustained (-●-●-) contractions in calcium-free (1mM EGTA) buffer was determined by preincubating (20 min.) tissues with a chosen concentration of Q-bu-MDI and then introducing NE (3 x 10⁻⁷M) to the tissue bath. Tension is expressed as a percentage of tension developed during a reference contraction which was elicited from each tissue prior to the Q-bu-MDI test contraction, as diagrammed in figure 8 and described in Methods section 2. For calcium-free experiments each tissue was used for only one pair of test contractions.
Figure 25.

PERCENT OF REFERENCE CONTRACTION

CONCENTRATION OF Q-Bu-MDI (M)

- 0.1
- 1
- 10
- 100
absence of extracellular calcium. In the presence of extracellular calcium, both bu-MDI and pr-MDI were more potent inhibitors of potassium-induced contractions than of NE-induced contractions: the log IC50 values for inhibition of potassium-induced contractions by bu-MDI and pr-MDI were each 0.56 units less than for their inhibition of NE-induced contractions (Table 2). In contrast, Q-bu-MDI inhibited both NE- and potassium-induced contractions in the presence of extracellular calcium with similar potency: the log IC50 values for inhibition of contractions induced by NE and potassium were not significantly different, as evidenced by the overlap of their 95 percent confidence intervals (Table 2).

In the absence of extracellular calcium (in calcium-free EGTA buffer), bu-MDI, pr-MDI and Q-bu-MDI were each equipotent inhibitors of the phasic and sustained contractions induced by NE (Table 2). In addition, the IC50 values of each MDI for inhibiting NE-induced contractions (phasic and sustained) in the absence of extracellular calcium did not significantly differ from the IC50 values of that MDI for inhibiting NE-induced contractions in the presence of extracellular calcium (Table 2).

The quaternary butyl MDI (Q-bu-MDI) was a less potent inhibitor than its tertiary butyl analogue (bu-MDI) of contractions induced by potassium in the presence of extracellular calcium and of both the phasic and the sustained contractions induced by NE in the absence of extracellular calcium. However, bu-MDI and Q-bu-MDI were equipotent inhibitors of NE-induced contractions in the presence of extracellular calcium (as judged by the overlap of the 95 percent confidence
intervals of their IC50s; Table 2).

It might be noted that, in the absence of extracellular calcium, inhibition of the NE-induced sustained contraction by bu-MDI and of the phasic and sustained contraction by Q-bu-MDI was biphasic in nature (figures 23 and 25). The reason for this is not known.

The effects of pr-MDI and verapamil on the cumulative NE concentration response curve in calcium-free buffer after the elimination of the phasic component of contraction are shown, respectively, in figures 26 and 27. In concentrations of $5 \times 10^{-5}$M, $1.5 \times 10^{-4}$M, $2 \times 10^{-4}$M and $3 \times 10^{-4}$, pr-MDI inhibited maximum NE-inducible tension generation by 18, 20, 47 and 74 percent, respectively (figure 26). These concentrations also dose-dependently shifted the ED50 of the NE concentration-response curve from a control value of $1.6 \times 10^{-8}$M to $5.9 \times 10^{-8}$M, $1 \times 10^{-7}$M, $1.9 \times 10^{-7}$M and $3.7 \times 10^{-7}$M, respectively. At high concentrations, verapamil also depressed the maximum NE-inducible tension and shifted the ED50 of the NE concentration response curve (figure 27). At $3 \times 10^{-4}$M, $5 \times 10^{-4}$M and $10^{-3}$M, verapamil depressed maximum NE-inducible tension by and average of 3, 36 and 80 percent respectively. At $3 \times 10^{-5}$M, verapamil did not reduce maximum NE-inducible tension, but rather appeared to stimulate it slightly (approximately 13 percent). Verapamil concentrations of $3 \times 10^{-5}$, $3 \times 10^{-4}$M and $5 \times 10^{-4}$M caused a dose-dependent shift of the NE ED50 from a control value of $8.5 \times 10^{-9}$M to $5.2 \times 10^{-8}$M, $1.6 \times 10^{-7}$M and $3.7 \times 10^{-7}$M, respectively. At a concentration of $10^{-3}$M verapamil, only one tissue of five tested remained responsive to NE stimulation (four were completely refractory), and so an accurate estimate of the
Figure 26. Effect of pr-MDI on cumulative NE concentration-response curves in calcium-free medium after elimination of the phasic component of contraction. Cumulative NE concentration-response curves were determined as diagrammed in figure 9 and described in Methods section 3. Briefly, tissues which had been maintained in calcium-free (1mM EGTA) buffer and preincubated for 20 minutes in the desired concentration of pr-MDI, were exposed to cumulatively increasing concentrations of NE and the resulting sustained contractions were monitored. Tension was expressed as a percentage of the tension generated during a previously-determined reference contraction for each tissue induced by $10^{-6}$M NE after the elimination of the phasic component of contraction.
Figure 26.
Figure 27. Effect of verapamil on cumulative NE concentration-response curves in calcium-free medium after elimination of the phasic component of contraction. Cumulative NE concentration-response curves were determined as diagrammed in figure 9 and described in Methods section 3. Briefly, tissues which had been maintained in calcium-free (1mM EGTA) buffer and preincubated for 20 minutes in the desired concentration of verapamil, were exposed to cumulatively increasing concentrations of NE and the resulting sustained contractions were monitored. Tension was expressed as a percentage of the tension generated during a previously-determined reference contraction for each tissue induced by $10^{-6}$M NE after the elimination of the phasic component of contraction.
ED50 of NE could not be made for this verapamil concentration.

b.) Discussion

The demonstration that nifedipine, SKF24260 and verapamil are each more potent inhibitors of contractions induced by potassium than those induced by NE in the presence of extracellular calcium confirms results which have previously been reported for these agents on various vascular smooth muscles, including the rat aorta (Peiper et al., 1971; Haeusler, 1972; Golenhofen et al., 1973; Massingham, 1973; Bilek et al., 1974; Golenhofen and Hermstein, 1975; Schumann et al., 1975; Fleckenstein et al., 1976; Bowman and Rand, 1980c). Since potassium-induced contractions are dependent upon the entry of extracellular calcium through voltage-dependent calcium channels, while NE-induced contractions are thought to depend upon both the entry of extracellular calcium through receptor-operated calcium channels and the mobilization of intracellular calcium, the greater potency of these agents against potassium-induced contractions has been interpreted to be the result of a relatively selective inhibition of calcium influx through the plasma membrane voltage-dependent channels (see: Bolton, 1979; Introduction, sections II C and IV A). This interpretation is consistent with the electrophysiological effects of these agents on the slow calcium channels of cardiac muscle cells (see Fleckenstein, 1977). Evidence has also been presented, however, for the existence of other mechanisms whereby these agents may be producing their vasodilatory effects. Church and Zsoter (1980) have interpreted data obtained from rat and rabbit vascular tissues
and from rat atrium as being indicative of intracellular sites of action for verapamil and nifedipine. Mikkelsen et al. (1979) have reached similar conclusions, since verapamil and nifedipine inhibited the NE-induced contraction of human mesenteric arteries and veins in calcium-free buffer. It has also been proposed that verapamil may have alpha adrenoceptor antagonist activity, since in a number of isolated membrane preparations, verapamil has been demonstrated to displace alpha receptor ligand binding (Blackmore et al., 1979; Fairhurst et al., 1980; Glossman and Hornung, 1980; Barnathan et al., 1982).

The results of our experiments indicate that nifedipine, SKF24260 and verapamil do not have intracellular vasodilatory activity at concentrations which are effective in inhibiting contractions that are produced wholly or partially by the influx of extracellular calcium (i.e., by potassium or NE stimulation in calcium-containing buffer). Even at $10^{-4}$M, a concentration which is more than 10,000 times greater than that necessary for their inhibition of potassium-induced contractions, neither nifedipine (figure 20) nor SKF24260 (figure 21) markedly inhibited NE-induced contractions which were dependent solely on intracellular calcium. Although at $10^{-4}$M verapamil did inhibit NE-induced contractions in calcium-free buffer, this inhibition was not seen at verapamil concentrations which completely inhibited potassium-induced contractions (figure 22)(which were shown in Chapter 1 to be dependent upon extracellular calcium). These data are consistent with the proposed membrane calcium-channel blocking mechanism of action of each of these three agents, and do not support the opposite findings
of Mikkelsen et al. (1979) in human mesenteric arteries and veins.

In light of the inability of nifedipine or SKF24260 to inhibit NE-induced contractions which were dependent upon intracellular calcium, it may be significant to note that these agents partially inhibited contractions which were induced by NE in the presence of extracellular calcium (figures 20 and 21). Inhibition of these NE-induced contractions is presumably the result of antagonism of the influx of calcium through the plasma membrane. If, as proposed by Bolton (1979), this influx occurs largely through receptor-operated channels which are distinct from the voltage-dependent channels opened by potassium-induced depolarization, these data would indicate that nifedipine and SKF24260 are able to inhibit calcium influx through receptor-operated channels as well as through voltage-dependent channels.

Interestingly, against NE-induced contractions in the presence of extracellular calcium, SKF24260 produces 50 percent of its maximum vasodilatory effect at a concentration of $1.9 \times 10^{-8}$M (see footnote a, Table 2). This concentration is not significantly different from the IC50 value of $7.6 \times 10^{-9}$M (log IC50=−8.12) for SKF24260 inhibition of potassium-induced contractions (Table 2). Since SKF24260 appears to half-maximally inhibit each of these two calcium channels at similar concentrations, it would appear that this compound is relatively non-selective with respect to these two calcium channels. The failure of SKF24260 to completely inhibit the NE-induced contraction in the presence of extracellular calcium (figure 21) is presumably due to the contributions of intracellular calcium to these
contractions.

The maximum nifedipine-mediated inhibition of the contraction induced by NE in the presence of extracellular calcium cannot be determined from the data presented (figure 20), since doses of nifedipine greater than $10^{-6}$M were not tested. However, the concentration of nifedipine necessary to produce 50 percent of the maximum nifedipine-inducible inhibition can be conservatively estimated to be at least $3 \times 10^{-8}$M, since this concentration produces 50 percent of the inhibition observed at the highest tested dose of $10^{-6}$M nifedipine (figure 20). The concentration of nifedipine necessary to produce 50 percent of maximum nifedipine-inducible inhibition of the NE-stimulated contraction in the presence of extracellular calcium is thus more than one log unit greater than the IC50 of nifedipine for inhibition of potassium-induced contractions in the presence of extracellular calcium. If, indeed, each of these effects are due to the inhibition of influx of calcium through the plasma membrane, it is implied that nifedipine inhibits the voltage-dependent (potassium-stimulated) calcium channel with a greater potency than the alpha receptor-operated (NE-stimulated) calcium channel.

A similar analysis of the effects of verapamil on the voltage-dependent and alpha receptor-operated calcium channels is not possible, since at concentrations which inhibit NE-induced contractions in the presence of extracellular calcium, verapamil also produces some inhibition of NE-induced contractions in the absence of extracellular calcium (figure 22). At these concentrations, verapamil
may therefore have intracellular effects, and so its effects on NE-induced contraction in the presence of bath calcium cannot be presumed to be caused entirely by inhibition of the alpha receptor-operated calcium channel. The selectivity of verapamil (as well as nifedipine and SKF24260) for inhibiting calcium channels opened by potassium and NE can best be studied by determining the concentration-effect relationship for blockade of NE- and potassium-induced $^{45}$Ca influx in this tissue.

The demonstration that bu-MDI and pr-MDI each inhibit contractions induced by NE in the absence of extracellular calcium at concentrations which are similar to those that inhibit NE-induced contractions in the presence of extracellular calcium (figures 23 and 24, Table 2) is consistent with a proposed intracellular site of action for these compounds (see: Rahwan and Witak, 1979; Rahwan et al., 1981; Rahwan, 1982). Evidence for an intracellular site of action for the MDIs has been presented previously for a variety of muscle tissues. Bu-MDI depresses activation heat in stimulated skeletal muscle, thus indicating an inhibition of intracellular calcium release from the sarcoplasmic reticulum (Burchfield et al., 1982). Pr-MDI inhibits caffeine-induced contractions of skeletal muscle in the presence or absence of extracellular calcium (Rahwan and Gerald, 1981), as well as barium-induced contractions of nonvascular smooth muscle (Rahwan et al., 1977), each of which are proposed to be mediated through the release of intracellular calcium. In addition, recent evidence indicates that the negative inotropic effects induced by pr-MDI on isolated guinea-pig left atrium are likely to be due to
inhibition of the effects of calcium at a non-sarcolemma (i.e., intracellular) site (Lynch and Rahwan, 1982). The results shown in figures 23 and 24 indicate that in vascular smooth muscle as well, bu-MDI and pr-MDI are each able to inhibit the effects of calcium. Since these compounds were potent inhibitors of contractions (phasic and sustained) produced by NE-induced mobilization of intracellular calcium, these results also suggest that in this tissue bu-MDI and pr-MDI may have an intracellular site of action.

Bu-MDI and pr-MDI differ from db-cAMP, papaverine, 8-Br-cGMP and nitroprusside (discussed in section C.1 of this Chapter), however, in their relative potencies against NE- and potassium-induced contractions. While db-cAMP, papaverine, 8-Br-cGMP and nitroprusside each inhibit NE-induced contractions (either in the presence or absence of extracellular calcium) with a potency equal to or greater than their respective potencies against potassium-induced contraction, bu-MDI and pr-MDI are each more potent inhibitors of potassium-induced contractions than of those induced by NE (Table 2). The reason for such a difference between these MDI compounds and other vasodilatory agents which are presumed to have an intracellular site of action is not yet clear. However, these results suggest that in addition to their intracellular vasodilatory activity, bu-MDI and pr-MDI may also have an inhibitory effect on the potassium-stimulated voltage-dependent calcium channels of the rat aorta. In fact, at high concentrations (>10^-4 M) bu-MDI and pr-MDI have been suggested to inhibit calcium influx and electrical excitability of the plasma membrane of cardiac muscle cells (Rahwan et al., 1982; Lynch and
Rahwan, 1982). Further experimentation will be necessary in order to
determine whether these agents inhibit calcium influx through
voltage-dependent channels at lower concentrations ($10^{-5}$M to $10^{-4}$M) in
vascular smooth muscle in contrast to their established inability to
do so in cardiac muscle (Lynch and Rahwan, 1982).

It should be noted, however, that if indeed bu-MDI and pr-MDI are
capable of inhibiting smooth muscle contractility via membrane as well
as intracellular sites of action, they do so with much less
selectivity for membrane sites than the calcium entry blocking agents
previously discussed in this section. The concentrations of verapamil
which dose-dependently inhibited potassium-induced contractions are
completely distinguishable from those concentrations which exhibit
intracellular vasodilatory activity (i.e. inhibitory activity against
NE-induced contractions in calcium-free buffer), and neither
nifedipine nor SKF24260 were found to markedly inhibit contractions
which were dependent upon intracellular calcium. In contrast, the
concentrations of bu-MDI and pr-MDI which inhibit potassium-induced
contractions are close to those which inhibit contractions which are
induced via intracellular calcium (compare figures 20-22 to figures 23
and 24). Thus, if bu-MDI and pr-MDI inhibit calcium influx through
voltage-dependent channels, this inhibition probably represents only
one of a number of vasodilatory mechanisms utilized by these compounds
in these concentrations.

The quaternary butyl MDI (Q-bu-MDI) was originally synthesized in
our laboratories (Witiak et al., 1982) in anticipation that, since it
is a permanently charged molecule, it would be incapable of entering
the cell, and would therefore be devoid of intracellular effects. Subsequent experimentation demonstrated that Q-bu-MDI was indeed a less potent negative inotropic agent than either bu-MDI or pr-MDI in the isolated guinea-pig atrium (Lynch et al., 1982). In the rat aorta, as well, Q-bu-MDI was generally found to be a less potent inhibitor of contractions than either bu-MDI or pr-MDI (Table 2; Witiak et al., 1982). Contrary to what might be expected from an agent which is presumably devoid of intracellular activity, however, Q-bu-MDI was found to inhibit in a concentration-dependent manner the NE-stimulated contractions which were mediated by intracellular calcium mobilization (figure 25). In fact, the difference in potency between Q-bu-MDI and bu-MDI for inhibition of NE-induced contractions in the absence of extracellular calcium was relatively small: Q-bu-MDI was only 0.2 to 0.3 log units less potent than bu-MDI, as judged by their respective IC50 values (Table 2). If we are to assume that, as a quaternary salt, Q-bu-MDI is itself not able to penetrate the cell membrane, the potency of this compound against NE-induced contractions in calcium-free buffer may be due to membrane perturbation resulting in an impeded interaction of NE with its alpha receptor or to a diminished response (intracellular calcium mobilization) triggered by the interaction of NE with its alpha receptor. Either of these effects could be mediated by an "extracellular" interaction of Q-bu-MDI with the smooth muscle plasma membrane. Preliminary experiments subsequently described in Chapter III do not support an intracellular site of action for Q-bu-MDI.

The nature of pr-MDI-induced inhibition of the NE cumulative
concentration-response curve after the elimination of the phasic component of contraction (figure 26) further highlights the differences between the type of inhibition produced by this agent and that produced by the cyclic nucleotide analogues, db-cAMP and 8-Br-cGMP (figures 14 and 15). While db-cAMP and 8-Br-cGMP each inhibit the NE concentration-response curve according to the classic noncompetitive model, pr-MDI-induced inhibition of this curve resembles a mixed competitive and noncompetitive interaction (Ariens et al., 1964): increasing concentrations of pr-MDI not only cause a progressive diminution of the maximum NE-inducible contraction in calcium-free buffer, but also result in a progressive shift in the ED50 of the remaining NE concentration-response relationship (figure 26). At present it is not clear why pr-MDI should shift the ED50 of the NE concentration-response curve. Since the degree of shift induced is not proportional to the log of the pr-MDI concentration, it is unlikely the pr-MDI is acting in part as a classical competitive antagonist, which obeys the principles of mass action (Ariens and Simonis, 1964). One possibility is that by interacting with the plasma membrane, pr-MDI may alter the environment of the alpha receptor in such a way that it alters the affinity of NE-alpha receptor binding.

Interestingly, at high concentrations and in the absence of extracellular calcium, verapamil also inhibits the cumulative NE concentration-response curve in a manner which resembles a mixed competitive and non-competitive interaction (figure 27). Verapamil and its analogue, D600, have been reported to displace alpha receptor
ligand binding in a number of isolated membrane preparations (Blackmore et al., 1979; Fairhurst et al., 1980; Glossman and Hornung, 1980; Barnathan et al., 1982; Karliner et al., 1982). This effect resembles a competitive interaction, since in membranes isolated from the rat heart, rat brain and human platelets, D600 and verapamil reduce the affinity of alpha receptor radioligand binding without changing the total number of binding sites (Glossman and Hornung, 1980; Barnathan et al., 1982; Karliner et al., 1982). It is not clear, however, whether this change in affinity is caused by direct competition of verapamil for the radioligand binding site, or by an alteration of the binding properties of the receptor which may be due to the interaction of verapamil with other components of the membrane. Ariens et al. (1964) have pointed out that the binding of an agent to a membrane component may alter the affinity of another agent for its membrane binding site if the two membrane binding sites are in some way coupled. Thus it is possible that by interacting with the alpha receptor-operated calcium channel, verapamil may affect ligand binding to the alpha receptor, since these two are presumably "coupled" in some way (Bolton, 1979). The demonstration by Flaim and Craven (1981) that verapamil inhibits NE-stimulated $^{45}$Ca uptake in the rabbit aorta supports the notion that verapamil inhibits the alpha receptor-operated channel, as does our demonstration that in micromolar concentrations verapamil inhibits rat aortic contractions induced by NE in the presence of extracellular calcium (figure 22).

At this time the mechanisms by which verapamil and pr-MDI induce both a shift and a depression of the NE concentration-response
relationship in the absence of extracellular calcium remain unclear. Since calcium has many intracellular regulatory functions in addition to its actomyosin activating properties, the complex effects of these two compounds may also be the result of inhibition of a number of intracellular calcium-dependent processes brought about by their binding to calmodulin (Piascik et al., 1981; Johnson et al., 1981).

In summary, the results described in this section indicate that:

1.) In the rat aorta, at concentrations which completely inhibit potassium-induced contraction, and thus, presumably, calcium influx through the voltage-dependent channels, nifedipine, SKF24260 and verapamil do not appear to have intracellular vasodilatory activity;

2.) While high concentrations of verapamil (10^{-4} M) may have intracellular vasodilatory effects, neither nifedipine nor SKF24260 exhibit marked intracellular vasodilatory activity even at concentrations as high as 10^{-4} M;

3.) Nifedipine, SKF24260 and verapamil each appear to inhibit the NE-stimulated alpha receptor-operated calcium channel as well as the potassium-stimulated voltage-dependent calcium channel; however, their relative potencies for each of these channel may differ, nifedipine being more selective for the voltage-operated channels than SKF24260;

4.) While both bu-MDI and pr-MDI appear to have intracellular sites of action in the rat aorta, it is possible that they act at more than one sight, and that one of those sites is the voltage-dependent channel of the plasma membrane (even though a blocking effect on myocardial membrane calcium channels has previously been ruled out); and

5.) Q-bu-MDI exhibits vasodilatory activity (which is unexpected) against NE-induced contractions in calcium-free
medium, and so its mechanism of action requires further investigation (see next Chapter).
CHAPTER III

Examination of the Mechanism of Action of Vasodilators on Non-adrenergically Mediated Aortic Contractions Induced by U44069.

A. Introduction

Evidence presented in the previous chapters demonstrates that NE induces a biphasic contraction of the rat aorta in calcium-free buffer containing 1 mM EGTA, and that each phase of this contraction can be inhibited by vasodilatory agents which have intracellular sites of action. The assignment of an intracellular site of action of a vasodilatory agent derives its strongest support from the demonstration that such a vasodilator can inhibit NE-induced contractions in the absence of extracellular calcium at concentrations similar to those that inhibit contractions induced by NE or potassium in the presence of extracellular calcium (Chapter II, section C.1). However, it should be noted that an agent which inhibits NE activation of the alpha receptor (i.e. a competitive or noncompetitive alpha receptor antagonist), or an agent which alters the plasma membrane in such a way as to inhibit the ability of the alpha receptor to stimulate intracellular calcium mobilization, would also inhibit NE-induced contractions of the aortic tissue in calcium-free medium. For this reason it would be beneficial to identify additional mechanisms,
which are independent of the alpha receptor, whereby contractions of
the rat aorta could be similarly induced by mobilizing intracellular
calcium.

Recently it has been reported that U44069, a stable analogue of
PGH$_2$, produces a contraction of the rabbit aorta in the absence of
extracellular calcium which is sustained for many hours, and it has
been proposed that this U44069-mediated contraction is the result of
mobilization of calcium from an intracellular pool (van Breemen et
al., 1980; Loutzenhiser and van Breemen, 1981). This effect of U44069
is not blocked by the alpha receptor antagonist, phentolamine, and is
therefore not mediated through the activation of the smooth muscle
alpha receptor, nor through the release of neuronal NE (Loutzenhiser

The present investigation was designed to assess the
responsiveness of the rat aorta to U44069 in the presence and in the
absence of extracellular calcium, and to determine whether
contractions induced by U44069 in the absence of extracellular calcium
could be useful in determining the mechanisms of action of
vasodilatory agents.

B. Methods

Rat aortic tissue segments were isolated and mounted for the
measurement of isometric tension generation as described in the
Methods section of Chapter I. Normal (calcium-containing) buffer was
also prepared as described in Chapter I. Calcium-free solutions were
made by excluding calcium and including the appropriate amount of EGTA
as indicated below. High (100mM) potassium buffer was made by substituting KCl for NaCl on an equimolar basis to maintain tonicity.

The response of the aorta to U44069 (Upjohn Company, Kalamazoo, MI) or potassium in calcium-free medium was determined according to the protocol described in Chapter I (Methods section 1). Briefly, aortic strips which had been equilibrated for 1 to 1 1/2 hours in normal (calcium-containing) buffer solution were rinsed four times at 3-minute intervals with 10 ml volumes of calcium-free buffer containing 0.0, 0.1, 1.0 and 10.0 mM EGTA, and equilibrated in the respective buffer for a total of 20 mins. Thereafter, the tissues were exposed to either U44069 (3 x 10^-6M) or KCl (100mM) in the calcium-free medium, and the resultant contractions were monitored for 30 minutes. The tissues were then washed in calcium-free buffer containing the same amount of EGTA, and the baseline resting tension was again obtained. Where necessary, tension generation was corrected for slight shifts in tissue resting tension. Finally, the tissues were reincubated for 30 minutes in normal (calcium-containing) physiological solution and exposed again to U44069 (3 x 10^-6M) or KCl (100mM) in order to ascertain maximal contractions of the tissues. Contractions in the absence of extracellular calcium were expressed as a percentage of the maximum contraction induced by the respective agonist in the presence of 2.5 mM bath calcium.

The effects of nifedipine, bu-MDI or Q-bu-MDI on contractions induced by U44069 were determined according to the following protocol. Aortic strips which had been equilibrated for 1 to 1 1/2 hours in normal (calcium-containing) buffer solution were rinsed 4 times over a
10-minute period with 10 ml volumes of calcium-free buffer containing 1.0 mM EGTA. Tissues were then incubated for 20 minutes in 1.0 mM EGTA buffer alone, or in 1.0 mM EGTA buffer containing one of the following pharmacological agents: nifedipine (3 x 10^-7 M), bu-MDI (10^-4 M), or the quaternary analogue of bu-MDI (Q-bu-MDI, 10^-4 M). U44069 (3 x 10^-6 M) was then introduced to each of the above preparations, and the contractile activity was monitored. The tissues were subsequently washed in 1.0 mM EGTA-calcium-free buffer, re-equilibrated in normal (calcium-containing) buffer, and contracted maximally to U44069 (3 x 10^-6 M). Contractions induced by U44069 in the absence of extracellular calcium (and in the absence or the presence of nifedipine, bu-MDI, or Q-bu-MDI) were expressed as a percentage of the maximum contraction induced by U44069 alone in the presence of 2.5 mM bath calcium.

The nifedipine concentration chosen for this study represents three times that determined in figure 20 (Chapter II) to produce complete relaxation of aortic strips contracted by 40 mM KCl in the presence of 2.5 mM bath calcium. The concentrations of bu-MDI and Q-bu-MDI used in this study were found to significantly inhibit contractions of the rat aorta induced by potassium in the presence of bath calcium (2.5 mM) or by NE in either the presence or absence of bath calcium (Chapter 2, Section 3, figures 23 and 25). The concentration of U44069 used was that determined in separate preliminary experiments to produce a maximal contraction of the aortic strips in calcium-free (1.0 mM EGTA) buffer.
C. Results

The contractility of the rat aortic strips to either high potassium or to U44069 in each calcium-free buffer system, and a typical tracing of a tissue contraction induced by U44069 in calcium-free-EGTA buffer are presented in Figure 28. As demonstrated previously (figure 2, Chapter 1, section C.1), tissues which had been washed in calcium-free buffer containing 0.1, 1.0 or 10.0 mM EGTA were refractory to stimulation with 100 mM potassium. In contrast, tissues bathed in calcium-free-EGTA buffers remained significantly responsive to U44069, and appeared to respond with an initial rapid phasic contraction, which was then followed by a more slowly developing sustained contraction (figure 28B). The magnitudes of these contractions induced by U44069 in calcium-free-EGTA buffers averaged between 17 and 22 percent of the control response (determined in presence of extracellular calcium) for the phasic component of contraction, and between 22 and 25 percent of the control response (determined in presence of extracellular calcium) for the sustained component (figure 28A).

Figure 29 shows the effects of nifedipine, bu-MDI and Q-bu-MDI pretreatment on U44069-mediated aortic contractions in the absence of extracellular calcium. Whereas neither nifedipine nor Q-bu-MDI interfered with the contractile action of U44069 in calcium-free 1 mM EGTA buffer, the spasmogenic action of U44069 was significantly inhibited by bu-MDI (P<0.001 by Student t-test).
Figure 28. Contractile response of the rat aorta to U44069 and KCl in the absence of extracellular calcium. Each bar represents the mean ± S.E.M. Values are expressed as a percentage of the maximum contractile force induced during control contractions by the respective agonists in 2.5 mM bath calcium. Maximum tension generation during these monophasic control contractions to U44069 (3 x 10^-6 M) and KCl (100 mM) were 1.12 ± 0.03 g and 1.19 ± 0.05 g, respectively. Inset shows a typical tracing of a biphasic contraction induced by U44069 (3 x 10^-6 M) of the aorta in calcium-free (1.0 mM EGTA) medium.
Figure 28.
Figure 29. Effects of nifedipine, Q-bu-MDI and bu-MDI on the contractile response of the rat aorta to U44069 in the absence of extracellular calcium. Each bar represents the mean ± S.E.M. Values are expressed as a percentage of the maximum force induced during control contractions by U44069 (3 x 10^-6 M) alone in 2.5 mM bath calcium. Maximum tension generation during these monophasic control contractions was 1.07 ± 0.03 g.
Figure 29.

PERCENT OF CONTROL RESPONSE TO U44069 OBTAINED IN PRESENCE OF 2.5 mM CALCIUM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phasic</th>
<th>Sustained</th>
</tr>
</thead>
<tbody>
<tr>
<td>U44069 (3x10^-6 M)</td>
<td>(2)</td>
<td>(2)</td>
</tr>
<tr>
<td>NIFEDIPINE (3x10^-7 M)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>U44069 (3x10^-6 M) + Q-bu-MDI (10^-4 M)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>U44069 (3x10^-6 M) + bu-MDI (10^-4 M)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
</tbody>
</table>
D. Discussion

The demonstration that the rat aorta remains responsive to U44069 in calcium-free-EGTA buffer, but is refractory to potassium under these conditions indicates that, similar to its activity on the rabbit aorta (Loutzenhiser and van Breemen, 1981), U44069 stimulates the mobilization of intracellular calcium in the rat aorta (see Chapter 1). This conclusion is further supported by the inability of nifedipine to inhibit the U44069-induced contractions in calcium-free-EGTA buffer (figure 29). While nifedipine is a potent inhibitor of calcium influx through the smooth muscle plasma membrane calcium channels, it has little effect on contractions which are induced by the mobilization of intracellular calcium (figure 20). Moreover, it is interesting to note that the response induced by U44069 in calcium-free buffer resembles that induced by NE under the same conditions: it is a biphasic contraction consisting of an initial rapidly-developing but short lived phasic component, which is immediately followed by a more slowly developing component that is maintained for a prolonged period of time (compare figure 3 of Chapter I with figure 28B).

The demonstration that U44069 induces a contraction of the rat aorta in calcium-free medium which appears to be analogous to that induced by NE under similar conditions adds a new dimension to the use of calcium-free buffers for the identification of sites and mechanisms of action for vasodilatory agents. It has been shown in Chapter II that intracellularly acting vasodilatory agents can be expected to inhibit contractions which are mediated by the mobilization of
intracellular calcium. However, it was also pointed out that agents which inhibit NE activation of the alpha receptor (either by interacting with the receptor directly, or by perturbing the membrane so as to compromise receptor function) would also be expected to inhibit NE-induced contractions in the absence of extracellular calcium. By developing an additional alternate (i.e., non-alpha receptor-mediated) means of mobilizing intracellular calcium to induce muscle contraction, it becomes possible to distinguish between vasodilators acting through alpha receptor blockade and those acting through non-adrenergic intracellular mechanisms.

As previously discussed (Introduction, section IV B; Chapter II, section C.3), bu-MDI is a calcium antagonistic compound which has been demonstrated in a variety of pharmacological experiments to exert its actions intracellularly (Rahwan and Witiak, 1979; Rahwan et al., 1981; Rahwan, 1982). Consistent with its proposed intracellular site of action, (and also evidence against an alpha blocking activity), bu-MDI potently inhibits NE-induced contractions of the rat aorta which are dependent upon intracellular calcium (Chapter II, section C.3) and also inhibits U44069-mediated contractions in calcium-free medium.

Q-bu-MDI, the quaternary analogue of bu-MDI, was synthesized in our laboratories (Witiak et al., 1982) with the assumption that, as a permanently charged molecule, it would not enter the cell and would therefore be devoid of intracellular activity. However, it was subsequently found (figure 25) that Q-bu-MDI exhibited significant activity against NE-induced contractions in calcium-free medium (which are dependent solely upon intracellular calcium), and the possibility that
this agent may perturb the plasma membrane to inhibit alpha receptor function was discussed (Chapter 2, section 3). This latter interpretation is supported by the absence of an inhibitory action of Q-bu-MDI on U44069-mediated contractions in calcium-free medium (figure 29).

IV. Summary

The studies described herein were designed to address a number of questions concerning the regulation of intracellular calcium in the rat aortic smooth muscle by vasoconstrictor and vasodilator agents. The work presented in the first chapter addressed the question of whether, in the rat aorta, intracellular calcium pools could be mobilized to mediate muscle contraction induced by the vasoconstrictor agent, norepinephrine. This chapter also characterized and discussed the nature of the calcium pools which were mobilized. In the second chapter, the ability of vasodilatory agents to regulate contractions which are mediated through the mobilization of intracellular calcium was investigated, and the results were compared with the effects of these agents upon contractions mediated by extracellular calcium. Since the ability of a vasodilatory agent to regulate smooth muscle contraction depends both upon the mechanism of activation of the muscle (the agonist used and its source of calcium) and upon the mechanism of regulation by the vasodilator, these studies yielded valuable information about the possible existence of intracellular regulatory processes which are activated by vasodilatory agents. Finally, the third chapter assessed the ability of vasodilators to antagonize the non-adrenergically-mediated contractions of the rat
aorta induced by U44069—an agent which mobilizes intracellular calcium without interacting with the membrane alpha adrenergic receptor—thus providing a useful model for further characterizing the mechanisms of action of vasodilators.

The results of these studies may be summarized as follows: (1) In the absence of extracellular calcium NE induces contraction of the rat aorta by mobilizing calcium from intracellular sources. Potassium, by contrast, is not capable of inducing contractions under these conditions due to the dependence of its action on extracellular calcium. (2) Two functionally distinct intracellular calcium pools mediate the contraction induced by NE, which is biphasic in nature, in the absence of extracellular calcium. The first phase of the NE-induced contraction in the absence of extracellular calcium is a rapidly-induced but short-lived phasic contraction which can only be elicited once when the tissue is in calcium-free buffer, but which can be re-elicited if the tissue is briefly re-exposed to extracellular calcium between NE exposure (presumably allowing reloading of the intracellular calcium pools). The second phase of this NE-induced contraction is a slowly developing but sustained contraction. This contraction persists as long as the tissue is exposed to NE, and can be repeatedly elicited with NE in calcium-free buffer even without intermittent re-exposure of tissue to extracellular calcium (indicating that this effect involves a relatively large intracellular calcium pool or one which can be replenished by intracellular calcium redistribution). (3) The phasic and sustained components of the NE-induced biphasic contraction in the absence of extracellular calcium
are probably mediated by the same mechanism: activation of the aortic alpha receptor by NE. Both the phasic and the sustained contractions are evoked in an identical concentration-dependent manner by NE, and each is similarly inhibited by phentolamine. The pA$_2$ for phentolamine inhibition of the sustained contraction (7.7) is similar to that previously reported for the alpha receptor, as is the K$_A$ for NE (6.0 x 10$^{-8}$M). (4) The phasic and sustained contractions induced by NE in the absence of extracellular calcium are each subject to similar intracellular regulatory mechanisms: each is inhibited in a concentration-dependent manner by similar concentrations of a number of intracellularly-acting vasodilatory agents including papaverine, 8-Br-cGMP, nitroprusside, bu-MDI and pr-MDI. Only db-cAMP showed some selectivity for inhibiting one component of the NE-induced biphasic contraction more than the other: it inhibited the sustained contractions at concentrations 0.41 log units less than those required to similarly inhibit the phasic component of contraction. (5) The NE-induced contractions in calcium-free buffer are not affected by vasodilator agents which normally act by inhibiting calcium influx through the plasma membrane (including nifedipine, SKF24260 and verapamil). Thus, by comparing the efficacy of a vasodilatory agent against contractions which are induced by potassium or NE in the presence of extracellular calcium to the efficacy of that vasodilator against NE-induced contractions in the absence of extracellular calcium, vasodilatory agents which act primarily by blocking the influx of calcium through membrane calcium channels can readily be identified. (6) The demonstration that a vasodilatory agent inhibits
NE-induced contractions which are mediated solely by intracellular calcium at concentrations of the vasodilator similar to those which inhibit NE- or potassium-induced contractions observed in the presence of extracellular calcium suggests that the vasodilatory agent is acting exclusively at an intracellular site of action. This suggestion is further supported by the demonstrations that the vasodilator in question inhibits the NE concentration-response curve in the absence of extracellular calcium in a noncompetitive manner, since this finding excludes the possibility that the vasodilator acts purely as a competitive alpha receptor antagonist. (7) The prostaglandin endoperoxide (PGH₂) analogue, U44069, like NE, is also capable of inducing contraction of the rat aorta in calcium-free medium by mobilizing intracellular calcium. However, U44069 is not an alpha adrenergic agonist nor does it act by releasing neuronal NE. The contraction which is induced by U44069 resembles that induced by NE: it is biphasic in nature, and consists of an initial, rapidly-induced phasic component of contraction which is immediately followed by a more slowly induced and prolonged sustained component. (8) The demonstration that a vasodilatory agent inhibits the contractions induced by U44069 in the absence of extracellular calcium at concentrations similar to those that inhibit NE-induced contractions in the absence of extracellular calcium further supports the proposal that the vasodilator has an intracellular site of action. In fact, since U44069 activates vascular smooth muscle by a mechanism independent of the alpha adrenoreceptor, the demonstration that a vasodilator inhibits U44069-induced contractions may be particularly
important in assigning an intracellular site of action to the vasodilator. In this way the possibility that the vasodilator inhibits NE-induced contractions in the absence of extracellular calcium by noncompetitively inhibiting the alpha receptor on the plasma membrane can be excluded. (9) By comparing the inhibitory efficacy of tertiary bu-MDI and quaternary Q-bu-MDI against contractions induced by NE in the absence of extracellular calcium with their efficacy against U44069-induced contractions under similar experimental conditions, a possible difference in the mechanism of action of these MDIs has been identified. While bu-MDI inhibited equally NE- and U44069-induced contractions—a finding consistent with its proposed intracellular calcium antagonist activity—Q-bu-MDI was only effective against the contraction induced by NE. This finding suggests that Q-bu-MDI may produce its vasodilatory activity at an extracellular site perhaps by interfering with alpha receptor function as a result of some physiochemical membrane perturbation.
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