\(\alpha\)-Adrenoceptor-Mediated Phosphoinositide Breakdown and Inotropic Response in Rat Left Ventricular Papillary Muscles

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\(\alpha\),-Adrenoceptor stimulation of rat left ventricular papillary muscles by phenylephrine in the presence of propranolol resulted in rapid breakdown of phosphatidylinositol 4,5-bisphosphate (PI-4,5-P<sub>2</sub>) and a triphasic inotropic response in a concentration-dependent manner. The release of inositol trisphosphate (IP<sub>3</sub>) was maximum within 30 seconds and remained high for at least 30 minutes. The IP<sub>3</sub> formation was associated with a rapid, but small, increase in contractile force followed by a transient decline in the contractility prior to the development of a sustained and more pronounced positive inotropic response. Inhibition of PI-4,5-P<sub>2</sub> hydrolysis by the \(\alpha\),,-adrenergic antagonist prazosin or the PI-4,5-P<sub>2</sub> phosphodiesterase inhibitor neomycin blocked all components of the inotropic responses. Combined addition of 2,3-diphasphoglyceric acid, a competitive inhibitor of IP<sub>3</sub>, phosphatase, with phenylephrine doubled the IP<sub>3</sub> formation and potentiated the initial phases of inotropic responses but had no effect on the sustained positive inotropic response. Nifedipine and Mn<sup>2+</sup> did not block the transient inotropic responses but inhibited the sustained positive inotropic response. \(\alpha\),,-Adrenoceptor stimulation resulted in restoration of slow responses in the high K<sup>+</sup>-depolarized muscles in the time course similar to that of the development in the sustained positive inotropic response. Addition of phorbol-12,13-dibutyrate alone or in combination with caffeine or A23187 failed to produce a sustained positive inotropic effect, but pretreatment with this phorbol ester (1-100 nM) for 30 minutes resulted in dose-dependent potentiation of \(\alpha\),,-adrenoceptor-mediated sustained positive inotropic effect associated with enhanced slow responses. These results suggest that the inotropic effects mediated by cardiac \(\alpha\),,-adrenoceptor stimulation occur through the phosphodiesteric cleavage of PI-4,5-P<sub>2</sub>, such that IP<sub>3</sub> may produce transient inotropic effects by mobilizing intracellular Ca<sup>2+</sup>, while diacylglycerol, along with cofactors that are also generated on \(\alpha\),,-adrenoceptor stimulation, may provoke a sustained positive inotropic effect by potentiating slow Ca<sup>2+</sup> channels through activation of protein kinase C. (Circulation Research 1988;62:8-17)

Since the discovery by Ahlquist of \(\alpha\),,-adrenoceptors in cardiac muscle, \(\alpha\),,-adrenoceptor-mediated inotropic responses have been extensively investigated in myocardial preparations from various mammalian species. The previous studies have demonstrated that \(\alpha\),,-adrenoceptor stimulation causes positive inotropic effect (PIE) but in a manner qualitatively different from that mediated by \(\beta\),,-adrenoceptor stimulation. Nevertheless, characterization of \(\alpha\),,-adrenoceptor-mediated inotropic responses has been hampered because of the lack of relevant molecular mechanisms involved in this receptor stimulation. It is generally accepted that PIE mediated by \(\beta\),,-adrenoceptor stimulation is provoked via cAMP, whereas that mediated by \(\alpha\),,-adrenoceptor stimulation is independent of cAMP. In contrast to monophasic PIE mediated by \(\beta\),,-adrenoceptor stimulation, \(\alpha\),,-adrenoceptor stimulation has been reported to produce a transient negative inotropic effect (NIE), resulting in a triphasic inotropic response. Although inhibitory postsynaptic \(\alpha\),,-adrenoceptors were implicated in the transient NIE, generation of separate intracellular pathways can also explain such a triphasic time-course of \(\alpha\),,-adrenoceptor-mediated inotropic responses. It is now known that the interaction of Ca<sup>2+</sup>-mobilizing agonist with its receptor results in rapid breakdown of phosphatidylinositol 4,5-bisphosphate (PI-4,5-P<sub>2</sub>) leading to the formation of inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DG), the putative intracellular second messengers. They act through different signal transduction pathways, but act synergistically, causing full physiologic responses in a number of different cell types. Although very little is known about the relation between the receptor-linked phosphoinositide breakdown and the regulation of contractile activity in cardiac muscle, several recent studies have provided evidence that this response may also take place in cardiac muscle. Brown et al have demonstrated that both \(\alpha\),,-adrenergic and muscarinic stimulants cause the phosphoinositide breakdown in rat ventricular myocytes. Muscarinic stimulation was found to produce a PIE in the presence of a pertussis toxin in chick atrial muscle. Our preliminary studies have documented that the phosphoinositide breakdown is not involved in the normal excitation-contraction coupling process but is caused by \(\alpha\),,-adrenoceptor stimulation in isolated rat papillary mus-
Labelling receptor-mediated alteration of cardiac contractile function. The results of the present study suggest that IP, and DG generated through hydrolysis of PI-4,5-P₂ are possible molecular links between α₁-adrenoceptor stimulation and inotropic responses in cardiac muscle.

**Materials and Methods**

**Papillary Muscle Preparation and Phosphoinositide Labelling**

Male Sprague-Dawley rats weighing 300–500 g were anesthetized with an intraperitoneal injection of sodium pentobarbital, and the heart was quickly removed. The left ventricular papillary muscles in diameters between 0.8 and 1.0 mm were suspended in an organ bath of Tyrode’s solution containing (mM) NaCl 122.5, KCl 5.4, CaCl₂ 1.8, MgSO₄ 1.1, NaHCO₃ 24, and glucose 10, pH 7.4, when equilibrated with 95% O₂-5% CO₂ gas mixture at 32°C. All experimental procedures, including the labelling study with [³H]inositol, were begun after an equilibration period of 50 minutes in Tyrode’s buffer, during which contractile force was monitored to check the viability of the preparations. In other words, the preparations used for further experiments did not show significant decline of systolic contractile force or increase in resting force during this period. After the equilibration period, the muscles were preincubated with 20 μCi myo-[2-³H]inositol (specific radioactivity, 16.5 Ci/mmol; New England Nuclear, Boston, Mass.) in 5 ml Tyrode’s solution for 90 minutes. After this incubation, the muscles were rinsed with fresh Tyrode’s solution without the labelled inositol and were incubated in the same buffer for a further period of 30 minutes to minimize a labelled inositol pool size. The effects of various treatments on phosphoinositide breakdown were evaluated in these preparations in the presence of 5 mM unlabelled inositol and 10 mM LiCl, an inhibitor of inositol 1-phosphatase.

**Determination of [³H]Phosphoinositides and [³H]Inositol Phosphates**

At the end of each experiment, the muscles were quickly blotted and frozen in liquid nitrogen. The frozen tissues were weighed and homogenized in a glass homogenizer to which 1.0 ml of chloroform-methanol (1:2, vol/vol) solution containing 0.005% butylated hydroxytoluene was added. The homogenate was transferred to a glass vial, and the homogenizer was rinsed three times with 1.0 ml of chloroform-methanol-water (1:1, vol/vol). The aqueous phase was aspirated, and the organic phase was evaporated under a stream of nitrogen. The lipid residues were resolved in 100 μl of chloroform-methanol mixture (2:1, vol/vol). A 50-μl portion of the resolved lipids was applied to a potassium oxalate (1%) impregnated silica gel H plate (Analtech, Newark, Del.), which had been activated for 60 minutes at 110°C. Phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI-4-P), and PI-4,5-P₂ were separated on this plate in a solvent system of chloroform/acetone/methanol/glacial acetic acid/water (40:15:13:12:8, vol/vol).

The spots of the phosphoinositides identified by cochromatography with authentic lipid standards were visualized by brief exposure to iodine vapor, scraped off scintillation vials, and counted for radioactivity.

**Measurements of Contractile Force**

The papillary muscles suspended in an organ bath in Tyrode’s buffer were loaded with 500 mg and were driven electrically by rectangular pulses with a frequency of 1 Hz, a duration of 10 msec, and a voltage of twice the threshold. The isometric tension was measured using a force displacement transducer and recorded through an amplifier (Grass Instrument Co., Quincy, Mass.). In an attempt to study the role of slow Ca²⁺ channels in the contractile response, the papillary muscles were exposed to high K⁺ buffer solution containing 25 mM KCl with an equimolar reduction of sodium chloride to inactivate inward Na⁺ currents. When the preparations became inexcitable, the stimulation rate was reduced to 0.3 Hz, and the stimulus intensity was increased severalfold above the threshold with the stimulus duration being maintained at 10 msec.

**Materials**

When phenylephrine (Sigma) was used as an α₁-adrenoceptor agonist, 0.3 μM propranolol was always included 10 minutes before the exposure to phenylephrine to prevent a β-adrenoceptor stimulating effect of phenylephrine at high concentrations. Neomycin, 2,3-diphosphoglyceric acid (DPG; pentacyclohexylammonium salt), and caffeine were obtained from Sigma and were diluted in distilled deionized water to appropriate stock concentrations. Nifedipine, phorbol-12,13-dibutyrate (PDBu), 4α-phorbol, 4α-phorbol-12,13-didecanoate, and A23187 were obtained from Sigma and were diluted in dimethyl sulfoxide. Prazosin was used as an α₁-adrenoceptor antagonist and was a gift from Pfizer.
Inc., New York. Student's t test was used for statistical analysis.

Results

Phosphoinositide Breakdown by \( \alpha \)-Adrenoceptor Stimulation

The pulse-chase experiments employed in this study (see "Materials and Methods") demonstrated the loss of phosphoinositides from the labelled phosphoinositide pool (Figure 1). When the papillary muscles were incubated with \([H]\)inositol, the label was incorporated into all three of the phosphoinositides. Most of the label (85.8%) was recovered as PI, whereas 7.6% of the label appeared as PI-4,5-P\(_2\), and the remaining 6.6% was PI-4-P before the exposure to phenylephrine. Control preparations that were stimulated electrically without phenylephrine for up to 30 minutes did not reduce the labelling of any of these phosphoinositides significantly. A significant decline in the labelling of PI-4,5-P\(_2\) was observed 1 minute after \( \alpha \)-adrenoceptor stimulation. The labelling of PI-4,5-P\(_2\) then tended to increase toward the baseline. The loss of radioactivity from PI-4-P was insignificant over 30 minutes of the stimulation, while the labelling of PI continued to decrease slowly but significantly for 30 minutes.

Formation of Inositol Phosphates by \( \alpha \)-Adrenoceptor Stimulation

Stimulation of \( \alpha \)-adrenoceptors in the papillary muscles resulted in a significant increase in the formation of \([H]\)inositol phosphates (Figure 2). Analysis of individual phosphates showed that the level of \([H]\)IP, and \([H]\)inositol bisphosphate (IP\(_2\)) reached maximum within 30 seconds after the stimulation and remained unchanged or gradually declined thereafter (Figure 2A). The level of \([H]\)inositol phosphate (IP) did not change significantly for the initial 30 seconds after \( \alpha \)-adrenoceptor stimulation but increased with the duration of the receptor stimulation (Figure 2B). The levels of \([H]\)glycerophosphoinositol and \([H]\)inositol were not significantly different from those without the receptor stimulation (not shown). Since tissue accumulation of total inositol phosphates (IP + IP\(_2\) + IP\(_3\)) in the presence of Li\(^+\) is known to reflect agonist-dependent breakdown of phosphoinositides,\(^4\) this measurement was used as an indicator of the receptor-mediated phosphoinositide breakdown in the following study.

Inotropic Effects Mediated by \( \alpha \)-Adrenoceptor Stimulation

Stimulation of \( \alpha \)-adrenoceptors in the papillary muscles resulted in a triphasic inotropic response (Figure 3). The initial increase in contractile force appears immediately after the stimulation, reaching a maximum level within 30 seconds (termed phase 1 inotropic response). The contractile force then declines below the baseline, producing a negative inotropic phase that reaches a maximum level at 80–90 seconds after the stimulation (termed phase 2 inotropic response). The second increase in contractile force was more pronounced than that of phase 1 inotropic response, reaching a maximum level at 5–6 minutes and persisting for at least 20 minutes (termed phase 3 inotropic response).

Concentration-Response Curves for \( \alpha \)-Adrenoceptor-Mediated \([H]\)Inositol Phosphate Formation and Inotropic Responses

The concentration-response curve for the effect of \( \alpha \)-adrenoceptor stimulation on the formation of total \([H]\)inositol phosphates is shown in Figure 4A. A significant increase in the formation of total \([H]\)inositol phosphates occurred at phenylephrine concentrations above 0.1 \( \mu \)M. A near-maximal formation was observed at a concentration of 0.3 mM. Thus, the concentration of phenylephrine for a half-maximal formation of \([H]\)inositol phosphates was approximately 3 \( \mu \)M. Phenylephrine at a concentration of 0.1 \( \mu \)M produced appreciable inotropic effects only on phase 3 inotropic response because of difficulty in determining a small change in the contractile force during phase 1 and phase 2 inotropic responses at this phenylephrine concentration. However, concentration-response curves for phase 1 and phase 2 inotropic responses also followed patterns similar to those observed for phase 3 inotropic response at phenyleph-
rine concentrations higher than 1 µM. Consequently, concentration-response curves were similar among the [3H]inositol phosphate formation and the inotropic responses when each inotropic response was plotted as a function of concentrations of phenylephrine (Figure 4B).

Effects of Prazosin and Neomycin

If the hydrolysis of PI-4,5-P2 is an essential link in the pharmacomechanical coupling mediated by the cardiac α₁-adrenoceptor stimulation, inhibition of the hydrolysis by antagonizing the receptors or by inhibiting the PI-4,5-P2 phosphodiesterase should block the inotropic responses. To test this hypothesis, we used prazosin as an α₁-adrenergic antagonist and neomycin, which has been shown to bind tightly with PI-4,5-P2, thereby preventing its enzymatic degradation.20 When the papillary muscles labelled with [3H]inositol were exposed to 0.1 µM prazosin or 0.1 mM neomycin, the formation of total [3H]inositol phosphates induced by 10 µM phenylephrine was significantly inhibited but to a larger extent by prazosin (Table 1). This inhibition was associated with abolition (by prazosin) or diminution (by neomycin) of all components of inotropic responses mediated by α₁-adrenoceptor stimulation.

Effects of DPG

Among the products of inositol lipid metabolism, IP₃ (probably only D-1,4,5 isomer and not D- or L-1,3,4 isomer)21 is believed to be the only molecule capable of mobilizing Ca²⁺ from nonmitochondrial intracellular compartments. Therefore, DPG, a competitive inhibitor of IP₃ phosphatase,22,23 was used to characterize the effects of this molecule on the inotropic responses mediated by α₁-adrenoceptor stimulation. When the papillary muscles were exposed to 10 µM phenylephrine together with 1 mM DPG, the formation of [3H]IP₃ increased twofold within 1 minute compared with that without DPG, and this higher level was maintained for 30 minutes (Figure 5A). The levels of [3H]IP₂ and [3H]IP in the tissues treated with DPG tended to be lower than those without DPG (Figure 5A and B), indicating that DPG increased IP₃ accumulation by inhibiting the conversion of IP₃ into IP₂ and not by stimulating PI-4,5-P₂ breakdown. The effects of DPG on the phenylephrine-induced inotropic responses were also tested. The combined addition of DPG and phenylephrine significantly increased phase 1 and phase 2 inotropic responses but had no effect on phase 3 inotropic response (Table 2). The above findings suggest that the biologically active IP₃ isomer may be generated on α₁-adrenoceptor stimulation, and this isomer may produce the transient inotropic responses.

Effects of Nifedipine and Mn²⁺

If phase 1 inotropic response is due to Ca²⁺ mobilization from the intracellular compartments, this response should not be blocked by Ca²⁺ antagonists. Treatment with 0.1 µM nifedipine or 0.3 mM MnCl₂, for 30 minutes reduced the contractility to 70.6 ± 3.7% (mean ± SEM; n = 5) or 66.2 ± 2.6% (mean ± SEM; n = 5), respectively. When 10 µM phenylephrine was included after these treatments and the subsequent changes in the contractility were compared with the basal contractile force obtained just prior to addition of phenylephrine, phase 1 and phase 2 inotropic responses were significantly greater (Figure 5B). The above findings suggest that the biologically active IP₃ isomer may be generated on α₁-adrenoceptor stimulation, and this isomer may produce the transient inotropic responses.
were not significantly different from those of control preparations, but phase 3 inotropic response was significantly diminished (Table 3). These data indicate that the transient PIE is relatively unaffected by inhibition of slow Ca\(^{2+}\) channels.

**Effects of Phorbol Esters**

We then tested the hypothesis that DG derived from the phosphodiesteratic cleavage of PI-4,5-P\(_2\) may play a key role in the sustained PIE through the stimulation of protein kinase C activity. To study this signal transfer pathway, we used tumor-promoting phorbol esters, which appear to mimic endogenously produced DG in activating protein kinase C.\(^{26,28}\) Phorbol diester receptors have already been identified and characterized in isolated rat cardiac myocytes.\(^{29}\) PDBu at concentrations ranging from 1 to 100 nM failed to produce PIE over 60 minutes (Figure 7A). Since Ca\(^{2+}\) mobilization was found to be a prerequisite to elicit a phorbol-induced sustained and maximal contractile response in smooth muscles,\(^{30}\) Ca\(^{2+}\)-mobilizing agents, caffeine and a calcium ionophore A23187, were used. Caffeine (5 mM) was added 30 minutes after addition of PDBu. Thirty minutes’ preincubation with PDBu was chosen to ensure the association of this hydrophobic compound with the associated signaling pathway.

**Table 1. Effects of Prazosin and Neomycin on α\(_1\)-Adrenoceptor-Mediated Phosphoinositide Breakdown and Inotropic Responses**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>([^{3}H])inositol phosphates (cpm/mg wet wt)</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.5 ± 1.8‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>54.5 ± 3.5</td>
<td>107.0 ± 1.5</td>
<td>84.3 ± 2.0</td>
<td>151.4 ± 7.2</td>
</tr>
<tr>
<td>Prazosin + PE</td>
<td>20.9 ± 2.6‡</td>
<td>ND*</td>
<td>97.5 ± 1.5</td>
<td>106.0 ± 2.5‡</td>
</tr>
<tr>
<td>Neomycin + PE</td>
<td>35.3 ± 3.1‡</td>
<td>102.8 ± 0.6‡</td>
<td>92.0 ± 2.3‡</td>
<td>131.2 ± 4.4‡</td>
</tr>
</tbody>
</table>

Rat left ventricular papillary muscles labelled with \([^{3}H]\)inositol were treated with prazosin or neomycin for 10 minutes in the presence of 0.3 μM propranolol, 10 mM LiCl, and 5 mM inositol and exposed to 10 μM phenylephrine (PE) for 10 minutes. Control preparations were treated with 0.3 μM propranolol, 10 mM LiCl, and 5 mM inositol for 20 minutes. The muscles were frozen in liquid nitrogen and \([^{3}H]\)inositol phosphates were determined as described in "Materials and Methods." Effects of 0.1 μM prazosin or 0.1 mM neomycin on α\(_1\)-adrenoceptor-mediated inotropic responses were also measured in these preparations. The maximum inotropic response in each inotropic phase was compared with the basal contractile force obtained just prior to addition of phenylephrine. Results are expressed as mean ± SEM of 4 or 6 preparations.

*Not detectable; ‡p<0.05, §p<0.01 compared with PE.
with an inner leaflet of the sarcolemmal membrane. Without PDBu, caffeine increased contractile force rapidly by 10–20% over the basal contractility and then reduced it to a sub-basal level within 2 minutes, as was observed in the transient inotropic responses mediated by α₁-adrenoceptor stimulation (Figure 7B). However, the caffeine-induced NIE was not reversed by the observed in the transient inotropic responses mediated by propranolol. The PDBu solvent presence of 0.3 μM concentration of α₁-agonist by promoting up-regulation of the protein kinase C binding sites. 3' The cofactors that are also generated on α₁-adrenoceptor stimulation, producing a monophasic inotropic response (Figure 8D). The PIE mediated by PDBu and phenylephrine was abolished by pretreatment with 0.3 μM prazosin (Figure 8E). Pretreatment with 4α-phorbol and 4α-phorbol-12,13-didecanoate, biologically inactive phorbols, for 30 minutes at concentrations ranging from 0.01 to 1 μM, were without an effect on α₁-adrenoceptor-mediated inotropic responses (not shown). The dose-response effect on PDBu on α₁-adrenoceptor-mediated PIE was analyzed by comparing the maximum contractile force during the sustained PIE (Figure 9). The data demonstrate that at concentrations ranging from 1 to 100 nM, PDBu produced dose-dependent potentiation of α₁-adrenoceptor-mediated PIE. Furthermore, the pretreatment with 100 nM PDBu for 30 minutes resulted in a significant enhancement of slow responses mediated by α₁-adrenoceptor stimulation (Table 4), indicating that enhanced the sustained PIE at a concentration as low as 1 nM, although it had little effect on the initial PIE (Figure 8B). PDBu at a concentration of 10 nM increased the sustained PIE while dissipating the NIE, producing a biphasic inotropic response (Figure 8C). At a concentration of 100 nM, the phorbol ester continued to increase the contractile force following α₁-adrenoceptor stimulation, producing a monophasic inotropic response (Figure 8C). The PIE mediated by PDBu and phenylephrine was abolished by pretreatment with 0.3 μM prazosin (Figure 8E). Pretreatment with 4α-phorbol and 4α-phorbol-12,13-didecanoate, biologically inactive phorbols, for 30 minutes at concentrations ranging from 0.01 to 1 μM, were without an effect on α₁-adrenoceptor-mediated inotropic responses (not shown). The dose-response effect on PDBu on α₁-adrenoceptor-mediated PIE was analyzed by comparing the maximum contractile force during the sustained PIE (Figure 9). The data demonstrate that at concentrations ranging from 1 to 100 nM, PDBu produced dose-dependent potentiation of α₁-adrenoceptor-mediated PIE. Furthermore, the pretreatment with 100 nM PDBu for 30 minutes resulted in a significant enhancement of slow responses mediated by α₁-adrenoceptor stimulation (Table 4), indicating that

![Figure 5. Effects of 2,3-di-phosphoglyceric acid (DPG) on α₁-adrenoceptor-mediated [3H]inositol phosphate formation. Rat left ventricular papillary muscles labelled with [3H]inositol were treated with phenylephrine (PE) as described in the legend to Figure 1 with (open symbols) or without (filled symbols) DPG for various periods. O—O, IP (Panel A); △—△, IP (Panel A); O—O, IP (Panel B). Each point represents the mean±SEM of 4–6 preparations.](http://circres.ahajournals.org/figure/5)

### Table 2. Effects of 2,3-Diphosphoglyceric Acid (DPG) on α₁-Adrenoceptor-Mediated Inotropic Responses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>106.3±1.4</td>
<td>85.2±1.4</td>
<td>148.0±7.9</td>
</tr>
<tr>
<td>DPG + PE</td>
<td>112.3±1.4*</td>
<td>76.2±2.9*</td>
<td>144.6±8.7</td>
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</tbody>
</table>

Rat left ventricular papillary muscles were treated with 0.3 μM propranolol for 10 minutes and exposed to 10 μM phenylephrine (PE) with or without 1 mM DPG. The maximum inotropic response in each inotropic phase was compared with the basal contractile force obtained just prior to addition of phenylephrine. Results are expressed as mean±SEM of 6 preparations.

* p<0.05 compared with PE.

### Table 3. Effects of Nifedipine and Mn²⁺ on α₁-Adrenoceptor-Mediated Inotropic Responses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>107.2±0.8</td>
<td>83.6±4.1</td>
<td>148.5±6.0</td>
</tr>
<tr>
<td>Nifedipine + PE</td>
<td>106.1±0.5</td>
<td>78.9±3.9</td>
<td>127.6±6.5*</td>
</tr>
<tr>
<td>MnCl₂ + PE</td>
<td>108.8±1.1</td>
<td>86.9±2.5</td>
<td>127.5±7.0*</td>
</tr>
</tbody>
</table>

Rat left ventricular papillary muscles were treated with 0.1 μM nifedipine or 0.3 mM MnCl₂ for 30 minutes and with 0.3 μM propranolol for the last 10 minutes and exposed to 10 μM phenylephrine (PE). The maximum inotropic response in each inotropic phase was compared with the basal contractile force obtained just prior to addition of phenylephrine. Results are expressed as mean±SEM of 5 preparations.

* p<0.05 compared with PE.
FIGURE 6. Effects of α₁-adrenoceptor stimulation on K⁺-depolarized rat left ventricular papillary muscles. Rat left ventricular papillary muscle was rendered inexcitable by exposure to high K⁺ (25 mM) buffer solution, and the mode of electrical stimulation was changed as described in "Materials and Methods." When the muscle was treated with 0.3 μM propranolol (PROP) for 10 minutes and exposed to 10 μM phenylephrine (PE), the preparation restored the contractile activity.

Discussion

α₁-Adrenoceptor stimulation of rat left ventricular papillary muscles resulted in a significant breakdown of PI-4,5-P₂ within 1 minute concomitant with a maximum increase in IP₃ formation within 30 seconds without a significant loss of other phosphoinositides. This finding indicates that the initial event in α₁-adrenoceptor-mediated inositol lipid metabolism in the papillary muscle is the phosphodiesteratic cleavage of PI-4,5-P₂. The labelling of PI-4,5-P₂ after 1 minute of the receptor stimulation tended to increase toward the baseline. Similarly, the formation of IP₃ remained unchanged or attenuated gradually after 1 minute of the receptor stimulation. On the other hand, the loss of radioactivity from PI and the accumulation of IP were progressive during the receptor stimulation. These data can be interpreted as follows: 1) the degradation of PI-4,5-P₂ promotes sequential phosphorylation of PI to PI-4-P and to PI-4,5-P₂ by kinases, resulting in a significant decrease in the labelling of PI 30 minutes after the receptor stimulation without an appreciable loss of radioactivity from the intermediary phosphoinositol PI-4-P; and 2) IP₃ is converted rapidly into IP₂ and into IP by phosphatases. However, it is possible that phospholipase C, which hydrolyzes PI-4,5-P₂, also attacked PI directly during the progressive decline in the labelling of PI, as has been documented in angiotensin-stimulated vascular smooth muscle cells.12
The present study demonstrated a close correlation between phosphoinositide breakdown and inotropic responses mediated by \( \alpha \)-adrenoceptor stimulation in the papillary muscles. \( \alpha \)-Adrenoceptor stimulation resulted in the formation of inositol phosphates in a concentration-dependent manner similar to that of inotropic responses. Inhibition of phosphoinositide breakdown by the \( \alpha \)-adrenoceptor antagonist or the PI-4,5-P\(_2\) phosphodiesterase inhibitor blocked all components of the inotropic responses. A growing body of evidence suggests that the breakdown of PI-4,5-P\(_2\) promotes \( \mathrm{Ca}^{2+}\)-mediated physiologic responses through the generation of two second messengers, IP\(_3\) and DG.\(^8\)

The ability of IP\(_3\) to mobilize \( \mathrm{Ca}^{2+}\) from the cardiac sarcoplasmic reticulum is a controversial issue. The studies using skinned cardiac fibers or isolated cardiac sarcoplasmic reticulum have shown that IP\(_3\) mobilizes \( \mathrm{Ca}^{2+}\) from nonmitochondrial intracellular compartments.\(^{39-35}\) Movsesian et al.\(^{36}\) however, reported that addition of IP\(_3\) failed to cause \( \mathrm{Ca}^{2+}\) release from permeabilized cardiac myocytes and sarcoplasmic reticulum. The evidence shown in the present study that there was rapid formation of IP\(_3\), associated with a transient PIE following \( \alpha \)-adrenoceptor stimulation and that this PIE was relatively insensitive to \( \mathrm{Ca}^{2+}\) antagonists supports the role of IP\(_3\) as a potential mediator of intracellular \( \mathrm{Ca}^{2+}\) mobilization. Although anion exchange chromatography employed in this study cannot preclude the possibility that increased IP\(_3\) levels are accounted for by an accumulation of inositol 1,3,4-trisphosphate isomer after a prolonged agonist stimulation,\(^{21,23}\) enhanced transient inotropic responses associated with increased IP\(_3\) levels by the treatment with phenylephrine and DPG suggest that the biologically active inositol 1,4,5-trisphosphate isomer may be generated at least during the early period of \( \alpha \)-adrenoceptor stimulation.

The correlation between tissue IP\(_3\) levels and the extent of the transient NIE as documented by the present study suggests that the transient NIE may also be triggered by IP\(_3\) formation. One possible mechanism by which IP\(_3\) formation causes NIE is \( \mathrm{Ca}^{2+}\) overload induced by intracellular \( \mathrm{Ca}^{2+}\) mobilization. In this regard, our present study and others\(^{37,39}\) have documented that caffeine, \( \mathrm{Ca}^{2+}\) ionophore, or high concentrations of strophanthidin, all of which could cause random diastolic release of intracellular \( \mathrm{Ca}^{2+}\), produce NIE following a transient PIE. Alternatively, \( \alpha \)-adrenoceptor-mediated reduction of cardiac cAMP levels\(^{40}\) may be associated with the transient NIE. The existence of inhibitory \( \alpha \)-adrenoceptors would also be attributable to NIE,\(^7\) although such receptors have not yet been identified. Further research will be required to elucidate the exact mechanisms of the transient NIE, as well as to obtain direct evidence concerning the effects of IP\(_3\) on cardiac contractile force in relation to intracellular \( \mathrm{Ca}^{2+}\) metabolism.

The present study suggests that the sustained PIE may be dependent on an increase in \( \mathrm{Ca}^{2+}\) influx via slow \( \mathrm{Ca}^{2+}\) channels. The sustained PIE was inhibited by \( \mathrm{Ca}^{2+}\) channel blockers (Table 3), and the slow responses occurred in a time course similar to that of development in the sustained PIE (Figure 6). Miura et al.\(^{24}\) first reported that \( \alpha \)-adrenoceptor stimulation restored \( \mathrm{Ca}^{2+}\)-dependent action potential and tension development in the partially depolarized rabbit papillary muscles. Brückner and Scholz\(^{25}\) also reported \( \alpha \)-adrenoceptor-mediated increase in peak inward \( \mathrm{Ca}^{2+}\) current and prolongation of action potential duration without an alteration of outward current in the bovine ventricular trabeculae. More recently, Lindemann\(^{41}\) found that \( \alpha \)-adrenoceptor-mediated slow responses in the partially depolarized rat ventricles accompanied with phosphorylation of 15-kDa protein in the sarcolemmal membrane, which also occurred during exposure to isoproterenol. He proposed that this protein phosphorylation may be involved in increases in the slow inward \( \mathrm{Ca}^{2+}\) current induced by stimulation of either \( \alpha \)- or \( \beta \)-adrenergic receptors.

The present study has demonstrated that PDBu potentiates a sustained PIE as well as slow responses mediated by \( \alpha \)-adrenoceptor stimulation when it has no effect on the basal contractile force by itself. The

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**Table 4. Effects of Phorbol-12,13-Dibutyrate (PDBu) on \( \alpha \)-Adrenoceptor-Mediated Slow Responses**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Contractile force (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO + PE</td>
<td>133 ± 25.0</td>
</tr>
<tr>
<td>PDBu + PE</td>
<td>311 ± 46.8*</td>
</tr>
</tbody>
</table>

Rat left ventricular papillary muscles treated with 100 nM PDBu or the vehicle, 0.005% dimethyl sulfoxide, for 30 minutes were rendered inexcitable by exposure to high K+ (25 mM) buffer solution as described in "Materials and Methods." Slow responses were induced by addition of 10 \( \mu \)M phenylephrine in the presence of 0.3 \( \mu \)M propranolol. The maximum contractile force was compared with the basal contractile force obtained just prior to the exposure to high K+ buffer solution. Results are expressed as mean ± SEM of 5 preparations.

\*p<0.01 compared with DMSO + PE.

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**Figure 9. Effects of phorbol-12,13-dibutyrate (PDBu) on \( \alpha \)-adrenoceptor-mediated positive inotropic effect.** Rat left ventricular papillary muscles treated with 0.005% dimethyl sulfoxide (DMSO) or 1–100 nM PDBu for 30 minutes were exposed to 10 \( \mu \)M phenylephrine in the presence of 0.3 \( \mu \)M propranolol. The maximum contractile force was compared with the basal contractile force obtained just prior to addition of phenylephrine. Each point represents the mean ± SEM of 6 preparations.
effective concentration range of PDBu in potentiating α₁-adrenoceptor-mediated PIE are consistent with those in stimulating protein kinase C activity in vitro. Biologically inactive phorbols had no effect on α₁-adrenoceptor-mediated PIE, excluding a nonspecific hydrophobic interaction as a possible mechanism for the phorbol action. The PIE mediated by PDBu and phenylephrine was abolished by pretreatment with prazosin. Taken together, these observations support the view that PDBu increases contractile force on α₁-adrenoceptor stimulation by potentiating slow Ca²⁺ channels through activation of protein kinase C. Since PDBu in combination with caffeine or A23187 was found to be ineffective in eliciting a sustained PIE in the papillary muscle preparations, essential cofactors for PDBu potentiation of contractile force may be distinct from Ca²⁺ mobilization induced by these Ca²⁺-mobilizing treatments.

In summary, our results suggest that the inotropic effects mediated by α₁-adrenoceptor stimulation in the rat left ventricular papillary muscles may be provoked by IP₃ and DG generated through the receptor-linked degradation of PI₄,5-P₂. These second messengers can activate separate intracellular pathways at different time scales, thereby producing a triphasic inotropic response. IP₃ may produce a rapid, but small and transient, PIE by mobilizing intracellular Ca²⁺, which could trigger a transient NIE. The sustained PIE appears to be caused by increased Ca²⁺ influx via slow Ca²⁺ channels. Enhancement of α₁-adrenoceptor-mediated sustained PIE as well as slow responses by pretreatment with PDBu indirectly suggests that the sustained PIE may be mediated by DG through activation of protein kinase C, but this inotropic response may require additional intracellular pathways that are also generated on α₁-adrenoceptor stimulation. These cofactors remain to be identified.

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