Ca$^{2+}$ Dependence of $\alpha$-Adrenergic Effects on the Contractile Properties and Ca$^{2+}$ Homeostasis of Cardiac Myocytes

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$\alpha$-Adrenergic stimulation is known to enhance myocardial contractility. Adult rat left ventricular myocytes bathed in 1 mM [Ca$^{2+}$] (Ca$_o$) and electrically stimulated at 0.2 Hz responded to $\alpha$-adrenergic stimulation with 50 $\mu$M phenylephrine and 1 $\mu$M propranolol with an increase in twitch amplitude to 177.1±25.6% of control (mean±SEM). In contrast, when cell Ca$^{2+}$ loading was increased by bathing cells in 5 mM Ca$_o$, $\alpha$-adrenergic stimulation decreased twitch amplitude to 68.6±8.2% of control. Time-averaged cytosolic [Ca$^{2+}$] of cells in 1.0 mM Ca$_o$ is enhanced via an increase in the frequency of electrical stimulation. When myocytes were stimulated at 2 Hz in 1 mM Ca$_o$, $\alpha$-adrenergic stimulation did not increase twitch amplitude (103.8±12.4% of control). In myocytes loaded with the Ca$^{2+}$ probe indo-1, $\alpha$-adrenergic effects during stimulation at 0.2 Hz (an increase in twitch amplitude in 1 mM Ca$_o$, and a decrease in twitch amplitude in 5 mM Ca$_o$) were associated with similar changes in the indo-1 transient. In 5 mM Ca$_o$, spontaneous Ca$^{2+}$ releases from the sarcoplasmic reticulum (SR) occurred in the diastolic interval between twitches (2.9±1.4 spontaneous SR Ca$^{2+}$ oscillations/min; n=7); $\alpha$-adrenergic stimulation abolished these oscillations in six of seven cells. Thus, an increase in the frequency of spontaneous diastolic SR Ca$^{2+}$ release (i.e., Ca$^{2+}$ overload) is not the mechanism for the negative inotropic effect of $\alpha$-adrenergic stimulation in 5 mM Ca$_o$. In experiments with unstimulated myocytes, we determined whether the effect of $\alpha$-adrenergic stimulation on cell Ca$^{2+}$ homeostasis and oscillatory SR Ca$^{2+}$ release observed in 5 mM Ca$_o$ occurs only during electrical stimulation, when voltage-dependent currents are operative, or also at rest. Unstimulated rat ventricular myocytes in 5 mM Ca$_o$ exhibit oscillatory SR Ca$^{2+}$ release; $\alpha$-adrenergic stimulation decreased the frequency of these oscillations to 53.9±8.9% of control, and this effect was blocked by 1 $\mu$M prazosin. In unstimulated indo-1-loaded myocytes $\alpha$-adrenergic stimulation decreased the resting indo-1 fluorescence ratio in 5 mM Ca$_o$, whereas it had no effect in 1 mM Ca$_o$. Additional experiments were aimed at defining a role for Ca$^{2+}$-activated, phospholipid-dependent protein kinase C (PKC) for the negative inotropic effect of $\alpha$-adrenergic stimulation in 5 mM Ca$_o$. Short-term preexposure to 0.1 $\mu$M 4$\beta$-phorbol 12-myristate 13-acetate (PMA) has been shown to maximally activate PKC. PMA, in 5 mM Ca$_o$, decreased contraction and indo-1 transient amplitudes and prevented any additional negative inotropic action by $\alpha$-adrenergic stimulation. Additionally, staurosporine (5 nM), a PKC inhibitor, abolished the $\alpha$-adrenergic-stimulated decrease in twitch amplitude in 5 mM Ca$_o$. Thus, an increase in cytosolic [Ca$^{2+}$] achieved via an increase in Ca$_o$ or by rapid pacing leads to effects of $\alpha$-adrenergic stimulation on the contractile properties and Ca$^{2+}$ homeostasis of rat ventricular myocytes that are different from those observed in lower Ca$_o$. These results may be explained with a [Ca$^{2+}$]-dependent enhancement of the activity of PKC, an enzyme that is activated during $\alpha$-adrenergic stimulation of the myocardium. (Circulation Research 1991;69:540–550)

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It is now well established that $\alpha$-adrenergic receptors are present in the mammalian myocardium and that, on binding of an agonist, they mediate an increase in contractility that is usually preceded by a short-lived decrease in twitch amplitude. Additionally, a persistent, rather than a transient, negative inotropic action of $\alpha$-adrenergic agonists has been described in isolated perfused rat hearts and in

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Received August 8, 1990; accepted April 29, 1991.

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Published as a preliminary report in abstract form (Circulation 1987;76[suppl IV]:IV-64).
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rabbit papillary muscles.6,7 The mechanism for the persistent negative effect of α-adrenergic stimulation has not been investigated, but different possibilities should be considered: 1) α-Adrenergic agonists are powerful vasoconstrictors, and in isolated hearts a decrease in coronary flow would lead to myocardial hypoxia and decrease the amplitude of the contraction via this mechanism. 2) Alternatively, it is possible that the negative effect of α-adrenergic stimulation could be mediated by Ca²⁺ overload.9-11 In cardiac muscle, interventions that increase cell Ca²⁺ loading have a positive inotropic effect; however, once the maximal contractile response has been achieved, a further increase in cell Ca²⁺ does not cause stronger contractions but leads to a plateau and then to a decrease in twitch strength. 3) Finally, it is possible that neither of these mechanisms is responsible for the negative effect of α-adrenergic stimulation and that, according to the experimental conditions, α-receptor agonists might have different effects on myocardial Ca²⁺ homeostasis and either increase or decrease the cytosolic [Ca²⁺] (Ca,) transient that causes the twitch contraction.

The purpose of this study was to identify a condition under which α-adrenergic stimulation would have a sustained negative inotropic action and to investigate the mechanism of such an effect. We used enzymatically dissociated left ventricular myocytes from the adult rat because this preparation is devoid of coronary vascularization and because in single cardiac cells Ca²⁺ overload can be readily identified by the appearance of spontaneous Ca²⁺ oscillations in the interval between electrically stimulated twitches.9 These Ca²⁺ oscillations are due to Ca²⁺ release from the sarcoplasmic reticulum (SR)12-14 and also occur in cells devoid of sarcolemma.12,13 In isolated myocardial cells, spontaneous SR Ca²⁺ release is manifest either as a localized band of contracted sarcomeres that slowly propagates as a contractile wave along the length of the myocyte15 or as a spontaneous increase in Ca in cells loaded with a Ca²⁺ indicator.16 The frequency of spontaneous SR Ca²⁺ release is enhanced by an increase in cell Ca²⁺ loading, regardless of how this is achieved,9,14 and is abolished by depletion of SR Ca²⁺.14 In myocardial cells, the de novo appearance of this phenomenon causes the development of alternans in contractility9,17 and saturation of the positive inotropic response to interventions that increase cell Ca²⁺ loading.9

Materials and Methods

Isolation Procedure

Left ventricular myocytes were enzymatically dissociated as previously described.18 Briefly, 2−4-month-old male Wistar rats from the Gerontology Research Center Colony were decapitated and the heart was quickly removed and retrogradely perfused with 25 ml nominally Ca²⁺-free bicarbonate buffer at 36±1°C continuously gassed with 95% O₂−5% CO₂ to keep the pH at 7.40±0.05. The perfusate was then switched to a similar solution to which collagenase and CaCl₂ had been added to achieve a final concentration of 160 units/ml and 60 μM, respectively. After ~20 minutes of perfusion with this medium, the left ventricle was isolated, and single cardiac myocytes were mechanically disaggregated and resuspended in a bicarbonate buffer with 1.8 mM bathing [Ca²⁺] (Ca₅).

Contractile Measurements

The contractile characteristics of individual cells were determined as previously described.18 Myocytes were transferred to a lucite chamber on the stage of an inverted microscope equipped with phase contrast and were continuously superfused with a buffer of the following composition (mM): NaCl 137, MgSO₄ 1.2, KCl 5, NH₄PO₄ 1.2, HEPES 20, d-glucose 16, and CaCl₂ 1−5; pH was 7.4. Solutions were preheated to keep the temperature in the bath at 35°C. Two platinum electrodes placed in the bathing fluid and connected to a stimulator (model SD9, Grass Instrument Co., Quincy, Mass.) were used to field-stimulate the myocyte to twitch with pulses of 2−4 msec. The image of the individual cell under study was projected through a TV camera (Panasonic, Matsushita Communication Industrial Co., Ltd., Yokohama, Japan) to a monitor (model WV-5200, Panasonic), and changes in cell length were quantified via edge-motion detection with a video dimension analyzer (model 303, Instrumentation for Physiology & Medicine, Inc., San Diego). The signal was then transmitted to a chart recorder (Brush model 220, Gould, Inc., Cleveland, Ohio) and to a computer (Vax 11/730) for on-line analysis. Peak twitch amplitude was expressed as percent of the resting cell length (ES%).

Simultaneous Measurement of Length and Indo-1 Fluorescence

In other experiments, cell length and indo-1 fluorescence were measured simultaneously as recently described.16 Briefly, single myocytes bathed in HEPES-buffered medium were loaded with the ester derivative (AM form) of the fluorescent Ca²⁺ probe indo-1. Fluorescence was excited by epi-illumination with 10-μsec flashes of 350±5 nm light. Paired photomultipliers collected indo-1 emission by simultaneously measuring spectral windows of 391−434 and 457−507 nm selected by band-pass interference filters. The ratio of indo-1 emission at the two wavelengths was calculated using a pair of fast integrator sample-and-hold circuits under the control of a Vax 11/730 computer, and it was taken as a measure of Ca₅. Cell length was simultaneously monitored using red light (650−750 nm) to form a bright field image of the cell, which was projected onto a photodiode array with a 5-msec scan rate. Both loading of the Ca²⁺ probe into the cells and the experiments were performed at 25°C because a more rapid loss of the indicator from the cell has been observed at 37°C than at room temperature.16
was followed by a significant positive inotropic effect. The addition of prazosin, a specific \( \alpha_1 \)-blocker, reversed the effect of \( \alpha \)-adrenergic stimulation on the contraction, which then recurred after removal of the antagonist.

Figure 2 shows tracings from a representative experiment in which cell length and indo-1 fluorescence were monitored. The lower tracings of the simultaneously recorded indo-1 transient and twitch were obtained at the times indicated by the letters in the upper continuous recording of cell length. From this figure it is apparent that both the transient decrease in the amplitude of the contraction as well as the positive inotropic effect of phenylephrine were associated with similar directional changes in the amplitude of the indo-1 transient. This result is in agreement with another report on the effect of \( \alpha \)-adrenergic stimulation on the Ca\(_2\) transient and does not exclude a role for increased myofilament responsiveness to Ca\(_{\text{+}}\), which also appears to contribute to the positive inotropic action of \( \alpha \)-agonists.5,21,22

Figure 3A shows average data on the time course of the effect of \( \alpha \)-adrenergic stimulation on the twitch. Cells exposed to the \( \alpha \)-agonist in 1 mM Ca\(_0\) showed a significant increase in twitch amplitude, which saturated within 5 minutes of exposure to the drug.

**Negative Inotropic Effect of \( \alpha \)-Adrenergic Stimulation**

A markedly different effect of \( \alpha \)-adrenergic stimulation was observed in cells bathed in 5 mM Ca\(_0\) (Figure 3A). In high Ca\(_0\), phenylephrine decreased twitch amplitude, and this response saturated between 5 and 15 minutes of exposure to the drug. Figure 3B shows the effect of phenylephrine at the 20-minute time point in cells bathed in 1 and 5 mM Ca\(_0\) compared with their respective time controls. It is apparent that \( \alpha \)-adrenergic stimulation enhanced contraction amplitude in 1 mM Ca\(_0\), whereas it had a negative inotropic action in 5 mM Ca\(_0\). Control tracings did not show any significant change in twitch amplitude for the duration of this experiment. In indo-1-loaded rat myocytes, the negative inotropic action of \( \alpha \)-adrenergic stimulation was associated with a decrease in the amplitude of the indo-1 transient (Figure 4), and this effect was completely reversible with washout of the \( \alpha \)-agonist.

Rat myocardial preparations studied under these conditions of high cell Ca\(_{\text{+}}\) loading may exhibit diastolic spontaneous SR Ca\(_{\text{+}}\) oscillations.9,17,23 In single cardiac cells, the occurrence of this phenomenon in the interval between stimulated twitches indicates that average twitch amplitude cannot be increased via interventions that further enhance cell Ca\(_{\text{+}}\) loading.9 Such perturbations would increase the frequency of spontaneous SR Ca\(_{\text{+}}\) release, and because of its negative effect on action potential–mediated Ca\(_{\text{+}}\) release, average twitch amplitude would diminish.9,17,23 In myocytes not loaded with indo-1 and stimulated at 0.2 Hz in 5 mM Ca\(_0\), diastolic contractile waves (i.e., the mechanical expression of spontaneous SR Ca\(_{\text{+}}\) release) occurred

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Materials

Phenylephrine hydrochloride, propanolol hydrochloride, and 4\(\beta\)-phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co., St. Louis, Mo. Prazosin was a gift of Pfizer Laboratories Division, New York. Indo-1 AM and staurosporine were purchased from Calbiochem Corp., San Diego. The \( \alpha \)-agonist, phenylephrine hydrochloride, was used at a concentration of 50 \( \mu \)M, which had been shown to elicit a maximal \( \alpha \)-adrenergic effect.3,5 Propanolol (1 \( \mu \)M) had no effect on the contractile properties of individual myocytes (results not shown) and was in the bathing medium throughout the course of all experiments described in the present study to prevent any \( \beta \)-adrenergic action of phenylephrine.

Statistical Methods

The data are expressed as mean±SEM. Comparison of the time courses was made on the data expressed as percent of control using a two-way repeated-measures analysis of variance (ANOVA) with the Bonferroni procedure for multiple comparisons. The value for each cell at time 0 was taken as its own control, and time 0 was omitted from the analyses.19 In addition, an unpaired Student’s t test was made at the time point of the last data collection. A value of \( p<0.05 \) was taken to indicate statistical significance. To determine when a plateau was reached within each drug group, a one-way repeated-measures ANOVA was done with Bonferroni tests for differences of means among time points on the observed data.20

Results

**Positive Inotropic Effect of \( \alpha \)-Adrenergic Stimulation**

Our first aim was to determine whether enzymatically dissociated rat myocytes bathed in physiological Ca\(_0\), exhibit the expected increase in contractility in response to \( \alpha \)-adrenergic stimulation. Figure 1 shows the original tracing from a representative myocyte continuously stimulated at 0.2 Hz in 1 mM Ca\(_0\). Shortly after the addition of phenylephrine, there was a transient decrease in twitch amplitude, which

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**Figure 1.** Positive inotropic effect of \( \alpha \)-adrenergic stimulation in 1 mM Ca\(_0\). A representative tracing of the effect of \( \alpha \)-adrenergic stimulation with 50 \( \mu \)M phenylephrine on a rat myocyte field stimulated at 0.2 Hz is shown. The increase in contractility is preceded by a small decrease in twitch amplitude, and it is reversibly antagonized by the \( \alpha_1 \)-blocker prazosin (1 \( \mu \)M). The tracing is continuous.
at a frequency of 2.9±1.4/min (n=7). Phenylephrine abolished spontaneous Ca\(^{2+}\) release within 15 minutes of exposure in six of seven cells, and in one cell, wave frequency decreased from 11/min to 3/min. The representative example in Figure 5 shows that in high Ca\(_0\) the \(\alpha\)-adrenergic agonist not only reversibly decreased the amplitude of the twitch but that it also suppressed spontaneous diastolic waves and alternans in twitch amplitude that were present in control.

The results presented so far demonstrate that in high Ca\(_0\), \(\alpha\)-adrenergic stimulation has a negative inotropic effect. The decrease in twitch contraction is related to a decrease in cell Ca\(^{2+}\) loading and is not the consequence of Ca\(^{2+}\) overload, which would have been associated with an increase in the frequency of spontaneous SR Ca\(^{2+}\) oscillations\(^9\) rather than with the suppression of this phenomenon. These results suggest that the increase in cell Ca\(^{2+}\) loading that is achieved via an increase in Ca\(_0\) modifies the contractile response to \(\alpha\)-adrenergic stimulation. At a high stimulation rate, the more frequent occurrence of Ca\(_i\) transients raises the time-averaged Ca\(_i\) above what it is at a lower stimulation rate. Thus, if the effect of \(\alpha\)-adrenergic stimulation is modulated by the extent of cell Ca\(^{2+}\) loading, it may be possible that the response to phenylephrine in 1 mM Ca\(_i\) during electrical stimulation at 2 Hz may differ from that at 0.2 Hz, and this would help to define more clearly the role of \(\alpha\)-adrenergic stimulation under physiological conditions. Figure 6 shows a representative example of the effect of phenylephrine on a non-indo-1–loaded rat myocyte stimulated at 2 Hz. Under these conditions, in contrast to the result depicted in Figure 1, there was no positive inotropic effect of \(\alpha\)-adrenergic stimulation. Average data for myocytes studied with this protocol show that, after 20 minutes of superfusion with phenylephrine, twitch amplitude was 103.8±12.4% of that at time 0 (n=6; ES% at time 0 was 5.5±0.6). This result was not significantly different from time controls, which at 20 minutes had a twitch amplitude that was 86.9±7.6% of that at time 0 (n=3; ES% at time 0 was 3.5±0.6).

These experiments do not determine whether the negative inotropic action of phenylephrine in 5 mM Ca\(_i\) or the lack of an effect of the \(\alpha\)-adrenergic agonist during electrical stimulation at 2 Hz in 1 mM Ca\(_i\) is due to changes in the action potential and voltage-dependent currents or whether an effect of \(\alpha\)-adrenergic stimulation on cell Ca\(^{2+}\) homeostasis would also occur in the absence of electrical stimulation, when the action potential does not modulate the extent of cell Ca\(^{2+}\) loading. This issue will be addressed in the next section.

**Effect of \(\alpha\)-Adrenergic Stimulation on Myocytes at Rest**

In the absence of electrical stimulation, rat ventricular myocytes bathed in 5 mM Ca\(_i\) exhibit spontaneous SR Ca\(^{2+}\) oscillations that occur with a relatively periodic interval at a frequency of ~0.2–0.5 Hz.\(^{14,18}\) In contrast to the experiments previously described, under these conditions the extent of cell Ca\(^{2+}\) loading and the frequency of spontaneous SR Ca\(^{2+}\) oscillations are not modulated by the occurrence of action
Effect of α-Adrenergic Stimulation on Myocytes Pretreated With PMA or Staurosporine

The experiments to be described in this section aimed at further characterizing the mechanism for the effects of phenylephrine in 5 mM Ca\textsubscript{0} and during pacing at 2 Hz.

α-Adrenergic stimulation leads to the activation of a Ca\textsuperscript{2+}-activated, phospholipid-dependent protein kinase C (PKC).\textsuperscript{24,25} It is also known that activation of PKC by PMA has effects on the Ca\textsubscript{1} transient that causes the twitch and on spontaneous SR Ca\textsuperscript{2+} oscillations, which are similar to those of phenylephrine in high Ca\textsubscript{0} (Figures 3, 4, and 7). Additionally, PKC activity is highly sensitive to [Ca\textsuperscript{2+}]\textsubscript{i} in the range of what is expected to be found in the cytosol of myocardial cells (i.e., 0.1–1 μM).\textsuperscript{23} Thus, we have hypothesized that an increase in cell Ca\textsuperscript{2+} loading may enhance α-adrenergic–induced PKC activation and lead to an effect of the α-agonist similar to that observed in response to PMA.

If this hypothesis were correct, preexposure to 10\textsuperscript{–7} M PMA, which has previously been shown to maximally and rapidly activate PKC in adult rat cardiac myocytes,\textsuperscript{26} would be expected to prevent the effects of phenylephrine in 5 mM Ca\textsubscript{0}. Figure 9 shows a representative example of the action of PMA (10\textsuperscript{–7} M) in 5 mM Ca\textsubscript{0}. PMA had a negative inotropic effect, which is likely to result from PKC activation. This result confirms that PMA decreases both peak systolic and diastolic indo-1 ratios and that these effects are associated with a diminution in twitch amplitude and an increase in diastolic cell length.\textsuperscript{25,26} In the continuing presence of PMA, there was no effect of phenylephrine either on the amplitude of the Ca\textsubscript{1} transient or of the twitch. In myocytes studied with this protocol, PMA decreased twitch amplitude to 67.4±8.67% of control (n=3; ES% in control was 14.5±2.4) and phenylephrine had no further effect on twitch amplitude, which was 95.1±4.8% of the contraction in response to PMA alone.

To further test the hypothesis that the effects of α-adrenergic stimulation in high Ca\textsubscript{0} may be mediated by PKC, we aimed at inhibiting the activity of this enzyme with staurosporine, a known PKC inhibitor.\textsuperscript{29,30} Indo-1–loaded myocardial cells were preincubated with 5 nM staurosporine for 2 hours before the onset of the experiment and were subsequently superfused with a solution containing 5 mM Ca\textsubscript{0} and 5 nM staurosporine. After 20 minutes of exposure to phenylephrine, twitch amplitude was 88.7±2.9% of that at time 0 (n=4; ES% at time 0 was 15.2±1.5). This was not significantly different from time controls that were also exposed to staurosporine. Contraction amplitude of these control cells showed a mild and progressive decrease over the course of the study and at 20 minutes was 79.2±8.8% of that at time 0 (n=3; ES% at time 0 was 11.1±2.3). A representative example of the effect of phenylephrine on the twitch and Ca\textsubscript{1} transient amplitudes of a staurosporinetreated myocyte is shown (Figure 10).
Discussion

The results described in this paper show that, under conditions of high cell Ca\(^{2+}\) loading achieved by increasing Ca, from 1 to 5 mM, \(\alpha\)-adrenergic stimulation of myocardial cells exerts a negative rather than a positive inotropic effect (Figure 3). Additionally, if time-averaged Ca, in 1 mM Ca, is enhanced by increasing stimulation frequency, rather than via an increase in Ca, there is no positive inotropic effect of phenylephrine (Figure 6). The effect of \(\alpha\)-adrenergic stimulation at 2 Hz in 1 mM Ca, is intermediate between those obtained during stimulation at 0.2 Hz in 1 and 5 mM Ca, and suggests that there may be a "continuum" in the Ca\(^{2+}\) dependence of \(\alpha\)-adrenergic effects on the contractile properties and Ca\(^{2+}\) homeostasis of the myocardium. It is noteworthy that the result shown in Figure 6 is in agreement with previous reports that have demonstrated a frequency dependence of the positive inotropic effect of \(\alpha\)-adrenergic stimulation that is less effective at high than at low stimulation rates.\(^6,7\)

A persistent negative inotropic effect of \(\alpha\)-adrenergic stimulation has been previously reported in rabbit papillary muscles\(^6,7\) and in isolated perfused
rat hearts.\(^8\) This response might have been secondary to a decrease in cAMP levels possibly mediated by the activation of phosphodiesterase activity.\(^3\) Another possible mechanism for the persistent negative inotropic effect of \(\alpha\)-adrenergic stimulation would be the development of a condition of Ca\(^{2+}\) overload.\(^9\) However, this hypothesis is not supported by our data. In isolated myocardial cells, Ca\(^{2+}\) overload is characterized by the occurrence of oscillatory SR Ca\(^{2+}\) release in the interval between stimulated twitches. In fact, in single myocytes, the appearance of diastolic SR Ca\(^{2+}\) release coincides with saturation of the positive inotropic response to interventions that act via an increase in cell Ca\(^{2+}\) loading, and as the frequency of spontaneous Ca\(^{2+}\) oscillations increases, the average twitch amplitude of the myocyte diminishes.\(^9\) In multicellular cardiac preparations, the same mechanism appears to underlie the decrease in systolic function.\(^32\) The increase in dia-

**Figure 6.** Representative tracing of the effect of 50 \(\mu M\) phenylephrine on a rat myocyte field-stimulated at 2 Hz in 1 mM Ca\(_{\text{a}}\). In contrast to the result depicted in Figure 1, at a frequency of 2 Hz \(\alpha\)-adrenergic stimulation had no significant positive inotropic effect (see text).

**Figure 7.** Effect of \(\alpha\)-adrenergic stimulation on spontaneous contractile wave frequency in rat myocytes studied in the absence of electrical stimulation in 5 mM Ca\(_{\text{a}}\). Panel A: Tracing from a representative rat myocyte showing spontaneous contractile waves under control conditions and at different times after the addition of phenylephrine (50 \(\mu M\)). Panel B: Tracing showing that the \(\alpha\)-adrenoceptor antagonist prazosin (1 \(\mu M\)) blocked the decrease in the frequency of spontaneous contractile waves produced by phenylephrine (50 \(\mu M\)) in a representative rat myocyte. Panel C: Average results and time course of the effect of \(\alpha\)-adrenergic stimulation for cells studied as in panel A (\(\bullet\), \(n=7\); wave frequency at time 0 was 18.6±2.6 waves/min) and panel B (\(\circ\), \(n=5\); wave frequency at time 0 was 17.0±4.0 waves/min). Phenylephrine produced a statistically significant decrease in the frequency of spontaneous sarcoplasmic reticulum Ca\(^{2+}\) oscillations.
stolic tonus, and the occurrence of arrhythmias associated with conditions of Ca\textsuperscript{2+} overload.

The experiment described in Figure 5 indicates that the negative effect of \( \alpha \)-adrenergic stimulation is associated with inhibition of the spontaneous diastolic SR Ca\textsuperscript{2+} releases rather than with an increase. This result as well as the decrease in peak systolic and diastolic indo-1 ratio (Figure 4), the reduced frequency of spontaneous SR Ca\textsuperscript{2+} oscillations (Figure 7), and the decrease in the indo-1 ratio in unstimulated myocytes in 5 mM Ca\textsubscript{o} (Figure 8B) show that in high Ca\textsubscript{o} the negative action of phenylephrine is not due to Ca\textsuperscript{2+} overload but to a decrease in Ca\textsubscript{i}.

It is of interest to discuss how the extent of cell Ca\textsuperscript{2+} loading achieved by varying Ca\textsubscript{o} may lead to opposite effects of \( \alpha \)-adrenergic stimulation on Ca\textsubscript{i} homeostasis and contractility of the myocardium. From studies in a variety of cell types, it is known that \( \alpha \)-adrenergic stimulation increases phosphatidylinositol turnover and leads to the formation of at least two second messengers, inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and 1,2-diacylglycerol (DAG). DAG activates PKC, and in cardiac muscle, IP\textsubscript{3} releases Ca\textsuperscript{2+} from the SR. In the myocardium, PKC phosphorylates different proteins in the SR, and on the myofilaments and may also increase cytosolic pH possibly via activation of Na\textsuperscript{+}-H\textsuperscript{+} exchange, as it is known to do in other tissues. Indeed, preliminary results from our laboratory show that cytosolic alkalization, mediated by activation of Na\textsuperscript{+}-H\textsuperscript{+} exchange, also occurs during...

**Figure 9.** Representative tracing of the effect of 50 \( \mu \)M phenylephrine in the presence of 10\textsuperscript{-6} M phosphol myristate acetate (PMA). Stimulation rate was at 0.2 Hz, and Ca\textsubscript{o} was 5 mM. The lower tracings of indo-1 fluorescence and length were obtained at the times indicated by the letters in the upper continuous length recording. The control condition is shown in tracing a. PMA had a negative inotropic effect and increased diastolic cell length. These changes were associated with a decrease in both peak systolic and diastolic indo-1 ratios (tracing b). In the continuing presence of PMA, the \( \alpha \)-adrenergic agonist had no effect on the amplitude of the twitch and of the Ca\textsubscript{i} transient (tracing c). Phenylephrine washout is also shown (tracing d).

**Figure 10.** Representative tracing of the effect of 50 \( \mu \)M phenylephrine in the presence of 5 nM staurosporine. Stimulation rate was at 0.2 Hz, and Ca\textsubscript{o} was 5 mM. The myocyte was preincubated with 5 nM staurosporine in HEPES buffer with 1 mM Ca\textsubscript{o} for 2 hours before the onset of the experiment. The lower tracings of indo-1 fluorescence and length were obtained at the times indicated by the letters in the upper continuous length recording. The control condition is shown in tracing a. Exposure to phenylephrine for 20 minutes had no significant effect on the amplitudes of the twitch and the Ca\textsubscript{i} transient (tracing b). Washout of the \( \alpha \)-adrenergic agonist is also shown (tracing c).
α-adrenergic stimulation of cardiac myocytes and modulates the contractility of these cells.50

Recent experiments with either DAG analogues or tumor-promoting phorbol esters that can substitute for DAG in activating PKC have shown that in cardiac muscle both these agents promote membrane association of PKC26,51 and have a negative inotropic effect.26,51-54 This negative action is due to a decrease in the amplitude of the Ca₂⁺ transient;26; in the absence of electrical stimulation, a DAG analogue decreases the indo-1 fluorescence ratio,26 and both a DAG analogue and a tumor-promoting phorbol ester decrease the frequency of spontaneous SR Ca₂⁺ oscillations.26 Thus, PKC activation is associated with effects on cell Ca₂⁺ homeostasis and contractility that are similar to those described in the present study for α-adrenergic stimulation in high Ca₀.

It is noteworthy that the activity of PKC increases markedly for changes in [Ca²⁺] between 10⁻³ and 10⁻⁵ M,27 which are values similar to those expected to be found in the cytosol of cardiac cells under physiological conditions.26 An increase in Ca₀ from 1 to 5 mM, as described in the present study, would increase Caᵢ within the sensitivity range of PKC and may enhance PKC activation by the α-adrenergic agonist. This is consistent with the finding that in myocardial cells α-adrenergic stimulation causes an increase in PKC activity, which is markedly less than that determined by a tumor-promoting phorbol ester,55 and that an increase in cell Ca²⁺ loading achieved by depolarizing the cells with high [KCl] is associated with an enhancement in the membrane association of PKC induced by α-adrenergic stimulation.25 The results depicted in Figures 9 and 10 show that both PMA, possibly via maximal activation of PKC before the exposure to the α-adrenergic agonist, and the PKC inhibitor staurosporine prevent the negative inotropic effect of phenylephrine in 5 mM Ca₀. This is compatible with the hypothesis that enhanced PKC activation may be the mechanism for the effects of α-adrenergic stimulation in high Ca₀, and the absence of a response to phenylephrine during stimulation at 2 Hz, as described in the present study. However, tumor-promoting phorbol esters have been shown to inhibit phosphoinositide breakdown in cardiomyocytes56 and in other cell types.27 Additionally, staurosporine has been shown to have nonspecific effects independent from PKC inhibition.58 In the absence of IP₃ and PKC measurements, it is not possible to conclusively establish whether the effects of PMA (Figure 9) and staurosporine (Figure 10) are secondary to inhibition of phosphoinositide turnover or whether they are specifically related to maximal activation of PKC by the phorbol ester or PKC inhibition by staurosporine.

Another plausible mechanism for the negative inotropic effect of α-adrenergic stimulation is a decrease in intracellular [Na⁺], which would favor Ca²⁺ extrusion via Na⁺-Ca²⁺ exchange and lead to a decrease in myocardial contractility. This possibility is supported by recent evidence that α-adrenergic stimulation increases the activity of the Na⁺-K⁺ pump59 and that both phorbol esters60 and α-adrenergic agonists61 can decrease intracellular [Na⁺]. However, it is not known whether the effect of α-adrenergic stimulation on intracellular [Na⁺] is affected by Ca²⁺.

In summary, the findings reported in the present study show that the extent of cell Ca²⁺ load modulates the effects of α-adrenergic stimulation on Ca²⁺ homeostasis and the contractility of the myocardial cell. This raises the issue of the effect of α-adrenergic stimulation in the presence of a normal heart rate and in response to the physiological α-adrenergic agonists, that is, norepinephrine and epinephrine, which also have β-adrenergic–stimulating properties that enhance Ca²⁺ entry into the cell. Indeed, a recent report shows that an interaction exists between α- and β-adrenergic effects of norepinephrine and that α₁-adrenergic stimulation diminishes the potentiation of twitch amplitude and depolarization-induced Ca²⁺ entry into the cell, which is seen on β-adrenergic stimulation alone.62 Thus, it appears that, in response to mixed α- and β-adrenoceptor agonists, α-adrenergic stimulation rather than exerting a positive inotropic action may provide a negative feedback loop and counteract the β-adrenergic effect to increase cell Ca²⁺ loading. An enhanced cAMP hydrolysis may be a mechanism for this interaction.63 However, our results suggest that an increase in cell Ca²⁺ in response to β-adrenoceptor stimulation may enhance α-adrenergic–stimulated PKC activity and represent another mechanism for the α- and β-adrenergic antagonism previously reported.62

Acknowledgments

The authors are grateful to Frances O’Connor for her assistance with the statistical analysis and to Miriam Glaser for her excellent secretarial work.

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KEY WORDS • α-adrenoceptors • cardiac inotropism • cardiac myocytes • calcium • protein kinase C
Ca2+ dependence of alpha-adrenergic effects on the contractile properties and Ca2+ homeostasis of cardiac myocytes.

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doi: 10.1161/01.RES.69.2.540

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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