In Contrast to Forskolin and 3-Isobutyl-1-methylxanthine, Amrinone Stimulates the Cardiac Voltage-Sensitive Release Mechanism without Increasing Calcium-Induced Calcium Release

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ABSTRACT

The objective of this study was to determine whether the voltage-sensitive release mechanism (VSRM) can be stimulated independently from Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) by drugs that elevate intracellular cAMP. Contractions were measured in voltage-clamped guinea pig ventricular myocytes at 37°C. Na\(^+\) current was blocked. We compared effects of agents that elevate cAMP through activation of adenylyl cyclase (1 \(\mu\)M forskolin), nonspecific inhibition of phosphodiesterases (PDEs) [100 \(\mu\)M 3-isobutyl-1-methylxanthine (IBMX)], and selective inhibition of PDE III (100–500 \(\mu\)M amrinone) on contractions initiated by the VSRM and CICR. Forskolin and IBMX significantly increased peak Ca\(^{2+}\) current and CICR. In addition, these agents also markedly increased contractions elicited by test steps from −65 to −40 mV, which activate the VSRM. However, because these steps also induced inward current in the presence of forskolin or IBMX, CICR could not be excluded. In contrast, amrinone caused a large, concentration-dependent increase in VSRM contractions but had no effect on CICR contractions or Ca\(^{2+}\) current. Sarcoplasmic reticulum Ca\(^{2+}\), assessed by rapid application of caffeine (10 mM), was increased only modestly by all three drugs. Normalization of contractions to caffeine contractures indicated that amrinone increased fractional release by the VSRM, but not CICR. Forskolin and IBMX increased fractional release elicited by steps to −40 mV. Increases in CICR induced by forskolin and IBMX were proportional to caffeine contractures. Thus, positive inotropic effects of cAMP on VSRM contractions may be compartmentalized separately from effects on Ca\(^{2+}\) current and CICR.

The strength of cardiac contraction is regulated by several mechanisms, one of which is the adenylyl cyclase (AC)-protein kinase A (PKA) cascade (Sugden and Bogoyevitch, 1995; Rapundalo, 1998). Activation of \(\beta\)-adrenergic receptors activates AC, which leads to increased production of cAMP. cAMP promotes phosphorylation of several key proteins by PKA, which affects force development in cardiac muscle. Levels of cAMP also are affected by degradation of cAMP by phosphodiesterases (PDEs) (Burns et al., 1996; Houslay and Milligan, 1997). Therefore, drugs that affect either synthesis or degradation of cAMP can alter protein phosphorylation by the AC/PKA pathway.

Phosphorylation of a number of proteins involved in excitation-contraction coupling by PKA may increase cardiac contraction. Phosphorylation of L-type Ca\(^{2+}\) channels increases L-type Ca\(^{2+}\) current (\(I_{\text{Ca-L}}\)) (McDonald et al., 1994), which could increase contraction through several routes. First, an increase in \(I_{\text{Ca-L}}\) provides a larger trigger for Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR), a mechanism for sarcoplasmic reticulum (SR) Ca\(^{2+}\) release (Fabiato, 1983). In CICR a small amount of Ca\(^{2+}\) entering the cell as \(I_{\text{Ca-L}}\) binds to and opens Ca\(^{2+}\) release channels (ryanodine receptors) in the SR. The amount of SR Ca\(^{2+}\) released by CICR can be graded by the magnitude of \(I_{\text{Ca-L}}\) (Beuckelmann and Wier, 1988; Bassani et al., 1995). Therefore, an increase in the magnitude of \(I_{\text{Ca-L}}\) may trigger larger CICR contractions. Second, increased influx of Ca\(^{2+}\) may increase SR Ca\(^{2+}\) stores and thereby increase Ca\(^{2+}\) available for release (Bassani et al., 1995; Janczewski et al., 1995; Spencer and Berlin, 1995).

ABBREVIATIONS: AC, adenylyl cyclase; PKA, protein kinase A; PDE, phosphodiesterase; \(I_{\text{Ca-L}}\), L-type Ca\(^{2+}\) current; CICR, calcium-induced calcium release; SR, sarcoplasmic reticulum; VSRM, voltage-sensitive release mechanism; IBMX, 3-isobutyl-1-methylxanthine; \(V_{\text{PC}}\), postconditioning potential; \(V_I\), current-voltage.

0022-3565/01/2983-954–963$3.00

Received January 25, 2001; accepted May 11, 2001

This paper is available online at http://jpet.aspetjournals.org

Biophys J

Vol. 74, No. 3

Printed in U.S.A.
The AC/PKA pathway also may increase contraction by promoting Ca\(^{2+}\) uptake into the SR by the SR Ca\(^{2+}\) ATPase. This is caused by phosphorylation of the regulatory protein phospholamban (Tada et al., 1979). Phosphorylation causes phospholamban to dissociate from the Ca\(^{2+}\) ATPase, thus relieving inhibition of this pump (Rapundalo, 1998). The resulting increase in SR Ca\(^{2+}\) stores leads to increased release of Ca\(^{2+}\) and an increase in the magnitude of contraction. In addition, ryanodine receptors also can be phosphorylated (Takasago et al., 1989). A recent report suggests that increased phosphorylation of ryanodine receptors by PKA displaces FKBP12.6, a regulatory protein bound to these receptors (Marx et al., 2000). Displacement of FKBP12.6 leads to an increase in open probability of the SR ryanodine receptors and may increase SR Ca\(^{2+}\) release.

Phosphorylation of these protein targets stimulates excitation-contraction coupling mediated through CICR. However, SR Ca\(^{2+}\) also can be released by a voltage-sensitive release mechanism (VSRM), which couples release of SR Ca\(^{2+}\) to depolarization, independently of the magnitude of IC\(_{\text{a,L}}\), as shown by us and others (Ferrier and Howlett, 1995; Hobai et al., 1997; Howlett et al., 1998; Mackiewicz et al., 2000; Ferrier and Howlett, 2000). Activation of the VSRM is markedly sensitive to phosphorylation by PKA (Ferrier et al., 1998) as well as by Ca\(^{2+}\)-calmodulin-dependent kinase (Zhu and Ferrier, 2000). Thus, the AC/PKA pathway might also regulate cardiac contraction by effects on the VSRM. Because release of SR Ca\(^{2+}\) by the VSRM is not proportional to the magnitude of IC\(_{\text{a,L}}\), increases in magnitude of IC\(_{\text{a,L}}\) in response to phosphorylation should not affect the VSRM directly. However, SR Ca\(^{2+}\) release initiated by the VSRM might be affected by increases in SR Ca\(^{2+}\) load or changes in ryanodine receptor activation. It also is possible that the VSRM might have one or more phosphorylation sites in addition to those that influence CICR. In this case, the VSRM might be regulated separately from CICR.

The goal of this study is to determine whether the VSRM can be stimulated independently from CICR through the AC/PKA pathway. This question is investigated with agents that stimulate AC (forskolin, Metzger and Lindner, 1981; Seamon et al., 1981), decrease degradation of cAMP by inhibiting PDEs nonselectively [3-isobutyl-1-methylxanthine (IBMX), Shahid and Nicholson, 1990], or selectively inhibit PDE III (amrinone, Harrison et al., 1986; Weisshaar et al., 1986). The specific objectives of this study are to 1) determine whether contractions initiated by CICR and the VSRM are increased by drugs that increase cAMP by these different actions in isolated cardiac myocytes; 2) determine whether increased contraction mediated by these drugs requires stimulation of IC\(_{\text{a,L}}\); 3) determine whether stimulation of the VSRM by agents that increase cAMP always is accompanied by stimulation of CICR; and 4) determine whether agents that increase cAMP act to increase the fraction of SR Ca\(^{2+}\) stores that are released.

### Materials and Methods

Experiments were conducted on isolated guinea pig ventricular myocytes. All experiments were performed in accordance with the guidelines published by the Canadian Council on Animal Care and this investigation was approved by the Dalhousie University Committee on Animal Care. Male guinea pigs (325–400 g; Charles River, Montreal, QC, Canada) were anesthetized with pentobarbital sodium (120 mg/kg i.p.). Animals also were administered heparin (3300 IU/kg i.p.). The heart was removed and perfused retrogradely through the aorta for 7 min (10–12 ml/min) with Ca\(^{2+}\)-free solution (37°C, pH 7.2) with the following composition: 120 mM NaCl, 4 mM KCl, 22 mM NaHCO\(_3\), 4 mM NaH\(_2\)PO\(_4\), 1 mM MgSO\(_4\), 5.5 mM glucose bubbled with 95% O\(_2\), 5% CO\(_2\). Collagenase [1 mg/ml, Worthington I (202 U/mg), Freehold, NJ] and protease [0.1 mg/ml, Sigma type XIV (5.2 U/mg), St. Louis, MO] were then included in the perfusate for about 3 min. Then the ventricles were minced and washed in substrate-enriched solution with the following composition: 80 mM KOH, 50 mM glutamic acid, 30 mM KCl, 30 mM KH\(_2\)PO\(_4\), 20 mM taurine, 10 mM HEPES, 10 mM glucose, 3 mM MgSO\(_4\), 0.5 mM EGTA, pH 7.4, with KOH.

After 1 to 2 h of incubation at room temperature, myocytes were placed in an experimental chamber (approximate volume = 0.75 ml) mounted on the stage of an inverted microscope. Cells were allowed to adhere to the bottom of the chamber for 15 to 20 min and were then superfused at 37°C with a HEPES-buffered solution with the following composition: 145 mM NaCl, 4 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, 10 mM HEPES, pH 7.4, with NaOH. Sodium currents were blocked with 200 to 250 µM lidocaine. In a previous study we have shown that the VSRM can be elicited regardless of whether sodium current is eliminated with lidocaine, tetrodotoxin, or by substitution of extracellular sodium with choline or sucrose (Ferrier and Howlett, 1995). In most experiments, solutions were pumped through the chamber at a rate of 3 ml/min and were changed by switching the inlet to the pump between solutions. Changeover of bath solution was complete in approximately 90 s. In some experiments, caffeine (10 mM) was applied in extracellular solution at 37°C with a computer-controlled solution switching device. The switcher allowed complete change of the solution bathing the myocyte in less than 0.5 s.

Recordings were made with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Discontinuous single-electrode voltage-clamp (sample rate 7–10 kHz) techniques were used with high-resistance microelectrodes (16–25 MΩ), filled with 2.7 M KCl) to reduce cell dialysis. A 2.7 M KCl-agar bridge was used as a bath ground. pCLAMP software (Axon Instruments) was used to generate voltage-clamp protocols and to acquire and analyze data. Electrode settling was continuously monitored to ensure accurate voltage measurement.

Unloaded cell shortening was sampled at 120 Hz with a video edge detector (Crescent Electronics, Sandy, UT) coupled to a television camera (model 1-GP-CD60; Panasonic, Osaka, Japan). All voltage-clamp protocols included 10 conditioning pulses before voltage steps to test potentials. Conditioning pulses were 200-ms depolarizations from the holding potential of −80 to 0 mV, delivered at a constant frequency of either 2 or 3 Hz. Conditioning trains were followed by a step to a postconditioning potential (V\(_{\text{pc}}\)) from which activation steps were made. Additional details of specific voltage-clamp protocols can be found in the appropriate sections under Results. Current, voltage, and contractions were digitized with a Labmaster A/D interface at sample rates up to 50 kHz (TL1-125; Axon Instruments) and stored on computer for subsequent analysis.

IC\(_{\text{a,L}}\) was measured as the difference between the peak inward current and a reference point at which IC\(_{\text{a,L}}\) approached zero. The time required for IC\(_{\text{a,L}}\) to decay was determined by rapid solution switches to either zero extracellular Ca\(^{2+}\) or 100 µM Cd\(^{2+}\). Magnitude of SR stores of Ca\(^{2+}\) was estimated by rapid application of 10 mM caffeine for 4 s with the rapid solution switching device. The peak of caffeine contractures is used frequently as a measure of SR Ca\(^{2+}\) (Bassani et al., 1995).

Differences between means for control and drug treatment were determined using a Student’s paired t test. Two-way analysis of variance with repeated measures was used to determine treatment differences in contraction-voltage and current-voltage (I-V) relationships. Differences were considered statistically significant when p was less than 0.05. All statistical analyses were performed with Sigma Stat (version 2.03; Jandel Scientific, San Rafael, CA). Data
are expressed as mean ± S.E.M. The value of n represents the number of myocytes sampled. No more than two replicates were collected from the same heart.

Lidocaine, amrinone, forskolin, caffeine, HEPES, L-glutamic acid, taurine, EGTA, and MgCl₂ were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), and IBMX was purchased from Calbiochem (La Jolla, CA). All other chemicals were purchased from BDH Inc. (Toronto, ON, Canada). Amrinone (0.05 M) was dissolved in 0.2 M HCl to make a stock solution. Stock solution was diluted in the extracellular solution to achieve the final concentration and the pH of the final solution was adjusted to 7.4 with NaOH. IBMX and forskolin were dissolved in dimethyl sulfoxide (final concentrations 0.03 and 0.003%, respectively). Control solutions for all IBMX and forskolin experiments contained dimethyl sulfoxide to control for solvent effects. Other drugs (lidocaine and caffeine) were dissolved in deionized water or directly in extracellular solution.

Results

Effects of forskolin, an activator of AC, on currents and contractions were examined in the first series of experiments. Contractions initiated by the VSRM and CICR were activated separately by voltage-clamp steps from −65 to −40 mV, and from −40 to 0 mV, respectively. We have validated the use of this protocol for separation of the VSRM and CICR in previous studies that have been reviewed recently (Ferrier and Howlett, 2001). Briefly, we showed that specific inhibitors of the VSRM and CICR eliminated the contractions initiated by the steps to −40 and 0 mV, respectively.

Representative traces recorded before exposure to forskolin are shown in Fig. 1A. The step to −40 mV activated a small VSRM contraction, which was accompanied by little current. The step to 0 mV initiated a larger CICR contraction and ICa-L. When cells were superfused with 1 µM forskolin, contractions initiated by both test steps were increased in magnitude (Fig. 1B). Forskolin also increased inward currents observed with both test steps. Figure 1C shows mean data for amplitudes of contraction in the absence and presence of forskolin. Forskolin significantly increased contractions initiated by steps to both test voltages. Forskolin caused a relatively greater increase in the amplitudes of contractions observed with steps to −40 mV, compared with CICR contractions initiated with steps to 0 mV. However, forskolin also significantly increased the magnitudes of inward currents observed with both steps (Fig. 1D). Because forskolin increased current elicited by the step to −40 mV, it is not clear whether stimulation of the corresponding contraction represents stimulation of the VSRM, or whether CICR also participates.

The effects of forskolin on SR Ca²⁺ were estimated by rapid application of 10 mM caffeine. Representative traces of caffeine-induced contractures are shown in Fig. 1E. Forskolin increased

![Fig. 1. Inotropic effects of forskolin, a stimulator of AC, are accompanied by increases in inward currents. The voltage-clamp protocol is shown at top left. A, sequential voltage steps from −65 to −40 and 0 mV activated VSRM and CICR contractions, respectively (top). Corresponding currents are shown below. B, superfusion of cells with 1 µM forskolin increased the magnitude of contractions and inward currents elicited by steps to −40 and 0 mV. C, mean data demonstrate that forskolin significantly increased the magnitudes of contractions initiated by test steps to −40 and 0 mV (n = 11). D, forskolin also significantly increased the magnitudes of inward currents initiated by steps to −40 and 0 mV. E, protocol for caffeine application is shown at top. Traces show caffeine contractures recorded from a cell exposed to caffeine (10 mM) for 4 s, before and after exposure to 1 µM forskolin. The peak of the caffeine contracture increased slightly in the presence of forskolin. F, mean data illustrates that the effect of forskolin on caffeine contractures was not statistically significant (n = 7). *p < 0.05; **p < 0.01.](http://jpet.aspetjournals.org/doi/abs/10.1124/jpet.110.175655)
the peak contraction induced by caffeine application. Mean amplitudes of peak caffeine responses measured before and after exposure to forskolin are shown in Fig. 1F. Forskolin caused a modest increase in the mean peak amplitude of contraction, although this was not statistically significant.

Effects of forskolin on contraction-voltage and I-V relationships also were determined, with the voltage-clamp protocol shown in Fig. 2A, right. Following conditioning pulses, activation steps were made to different membrane potentials from a V_{PC} of either −70 or −40 mV. These V_{PC} were tested because phasic contractions initiated by the VSRM are inactivated when steps are made from −40 mV but are available from −70 mV (Ferrier et al., 1998; Howlett et al., 1998). Representative recordings of contractions and currents elicited by a step from −70 to −20 mV are shown in Fig. 2A, left. Forskolin markedly increased both contraction and inward current. Figure 2B shows mean contraction-voltage relationships determined with depolarizing steps from a V_{PC} of −70 mV, before and after exposure to 1 μM forskolin. Contraction-voltage relationships from this V_{PC} were sigmoidal, which reflects the availability of the VSRM (Ferrier and Howlett, 1995; Howlett et al., 1998). Forskolin caused a significant increase in the maximum of the sigmoidal contraction-voltage relationship and shifted the potential at which contraction was first observed to the left. In contrast, when the V_{PC} was −40 mV, and only CICR contributed to initiation of contraction, contraction-voltage relationships were bell-shaped before and after exposure to forskolin (Fig. 2C). Forskolin increased the peak of the contraction-voltage relationship. Figure 2, D and E, shows mean data for I-V relationships. Forskolin significantly increased the peaks of the I-V relationships determined from both V_{PC}.

We next determined the effects of IBMX, a nonspecific inhibitor of PDE, which is expected to increase cAMP by inhibiting its degradation. Representative records of contractions and currents before and after application of 100 μM IBMX are shown in Fig. 3, A and B, respectively. IBMX caused a large increase in contractions initiated by the test step to −40 mV and a more modest increase in contractions initiated by the step to 0 mV. Currents elicited by both test steps also were increased by IBMX. Figure 3C shows mean data for the effects of IBMX on contractions. IBMX caused a dramatic and significant increase in the magnitude of contractions at −40 mV, with only a modest increase in mean amplitudes of contractions at 0 mV. Figure 3D demonstrates that IBMX significantly increased the mean peak amplitudes of inward current elicited by steps to −40 and 0 mV.

Figure 3E shows representative recordings of caffeine contractions initiated before and after exposure of a myocyte to IBMX. Rapid application of caffeine caused a greater peak contracture in the presence of IBMX than in control. Figure 3F demonstrates that IBMX significantly increased mean amplitudes of caffeine contractures.

Effects of IBMX on contraction-voltage and I-V relationships are shown in Fig. 4. The voltage-clamp protocol and representative traces of contractions and currents are shown in Fig. 4A. The representative example shows responses initiated by a step from −60 mV to +10 mV in the absence and presence of 100 μM IBMX. IBMX increased the peak amplitudes of both contraction and inward current. Figure 4B shows mean contraction-voltage relationships determined with depolarizing steps from a V_{PC} of −60 mV, which allows activation of the VSRM. IBMX dramatically increased the maximum amplitude of the contraction-voltage relationship and contractions were observed at more negative potentials. When the V_{PC} was −40 mV, IBMX also caused a significant increase in maximum contraction, however, the contraction-voltage relationship remained bell-shaped (Fig. 4C). Figure 4, D and E, shows that IBMX also increased the peak amplitudes of the corresponding I-V relationships determined from either V_{PC}.

Our results demonstrate that both forskolin and IBMX enhance contractions elicited by steps to −40 mV. However, both drugs also increase current elicited by this test step, and also clearly enhanced CICR with the test step to 0 mV. Thus, it is not clear whether stimulation of contractions elicited by steps to −40 mV represents an effect on the VSRM or whether it includes recruitment of CICR.

Both IBMX and forskolin are expected to increase levels of cAMP throughout the cell, however, specific PDEs may regulate cAMP levels at different sites (Kauffman et al., 1989; Lugnier et al., 1993). Therefore, we next examined the effects of amrinone, a selective PDE III inhibitor, on contractions and currents. Figure 5, A and B, shows representative recordings of currents
and contractions elicited by steps to \(-40\) and \(0\) mV, in the absence and presence of \(500\) \(\mu\)M amrinone. Amrinone caused a large increase in the amplitude of the contraction initiated by the step to \(-40\) mV with little effect on the amplitudes of the CICR contraction or inward currents. Figure 5, C and D, shows mean data for contractions and currents elicited with this protocol. Amrinone caused a large and significant increase in the amplitude of contractions elicited by the step to \(-40\) mV. However, amrinone did not significantly affect the amplitudes of CICR contractions or inward currents. Thus, unlike forskolin or IBMX, amrinone stimulated contractions elicited by the step to \(-40\) mV with virtually no effect on current or CICR. Therefore, the increase in the contraction initiated by the step to \(-40\) mV likely represents selective stimulation of the VSRM by amrinone.

We also examined the effects of amrinone on the amplitudes of caffeine contractures, to assess changes in SR \(Ca^{2+}\). Representative recordings of caffeine contractures are shown in Fig. 5E. Amrinone caused a small increase in the peak amplitude of the caffeine contracture. Mean results, shown in Fig. 5F, confirm that a modest increase in caffeine contractures accompanied exposure to amrinone (<0.05).

Results shown in Fig. 5 suggest that \(500\) \(\mu\)M amrinone affects contractions initiated by the VSRM without stimulating CICR or \(I_{\text{Ca-L}}\). If this is correct, one would predict that amrinone should enhance contractions initiated by steps to positive potentials where \(I_{\text{Ca-L}}\) is minimal. Figure 6 shows recordings from an experiment to test this. Fig. 6A was recorded before exposure of the cell to amrinone. Because the VSRM was available for activation, a step to \(+80\) mV initiated a phasic contraction although no inward current deflection was observed. Exposure of the same myocyte to \(200\) or \(500\) \(\mu\)M amrinone caused a large increase in the amplitude of the contraction initiated by this step. The increase in contraction was not accompanied by any change in the current recording. Similar results were observed in 14 experiments. These observations further indicate that amrinone increases contraction by an effect on the VSRM, which is independent of CICR and \(I_{\text{Ca-L}}\).

We also examined the effects of three different concentrations of amrinone on contraction-voltage and I-V relationships. We first examined the effects of amrinone on currents and contractions initiated by depolarizing steps from a VPC of \(-60\) mV, where the VSRM is available to contribute to contraction. Figure 7A shows the effects of \(100\) \(\mu\)M amrinone on contraction-voltage relationships (top) and corresponding I-V relationships (bottom). Amrinone significantly increased the amplitude of the sigmoidal contraction-voltage relationship, but had no effect on the I-V relationship. Increasing the concentration to \(200\) \(\mu\)M (Fig. 7B) and \(500\) \(\mu\)M (Fig. 7C) demonstrated a concentration-dependent increase in the amplitude of the contraction-voltage relationship. A slight increase in \(I_{\text{Ca-L}}\) only was observed at the
highest concentration of amrinone, and only occurred over a limited range of test potentials.

The effects of 100 to 500 \textmu{}M amrinone also were determined on contraction-voltage and I-V relationships when the \( V_{PC} \) was \(-40 \) mV to inactivate the VSRM. Figure 8, A–C, shows that amrinone had no effect on the bell-shaped contraction-voltage relationship at any of the concentrations tested. Amrinone also had virtually no effect on the corresponding I-V relationships. These observations demonstrate that amrinone did not affect contractions initiated by CICR.

Amrinone strongly increased VSRM contractions with little effect on CICR. Therefore, it is unlikely that a change in SR stores accounts for the effect on VSRM contractions. Alternatively, it is possible that amrinone affects the ability of the VSRM to release SR Ca\(^{2+}\). This possibility was examined by normalizing VSRM and CICR contractions to the magnitudes of caffeine contractures, as an index of the fraction of SR stores released by each mechanism. VSRM and CICR contractions initiated by sequential steps to \(-40 \) and \(0 \) mV were normalized to caffeine contractures elicited in the same cells (data from Fig. 5). Figure 9A shows that fractional release initiated by the VSRM was greatly enhanced by amrinone. In contrast, fractional release by CICR was not affected by amrinone.

Effects of forskolin and IBMX on fractional release also were determined. Figure 9B demonstrates that forskolin caused a marked increase in fractional release by steps to \(-40 \) mV, and only a slight, nonsignificant increase in fractional release with steps to \(0 \) mV. The effects of IBMX were essentially identical to those of forskolin (Fig. 9C). Thus, all three agents that increase cAMP markedly increased fractional release of SR Ca\(^{2+}\) by the step to \(-40 \) mV, but not the step to \(0 \) mV.

**Discussion**

Our study demonstrates that drugs that stimulate the AC/PKA pathway can increase cardiac contractions initiated by CICR and/or the VSRM. Forskolin or IBMX caused a nonselective increase in contractions and inward currents. In contrast, amrinone, which selectively inhibits PDE III, increased the amplitude of VSRM contractions with virtually no effect on CICR or \( I_{Ca,L} \). All three agents increased the fraction of SR Ca\(^{2+}\) stores released by depolarizations typically used to activate the VSRM. However, none of these drugs increased the fraction of Ca\(^{2+}\) released from the SR by CICR. These observations demonstrate that drugs that stimulate the AC/PKA pathway can modulate the VSRM, and that the VSRM can be modulated independently of effects on CICR and \( I_{Ca,L} \).

The AC/PKA pathway phosphorylates several protein targets important in excitation-contraction coupling. These targets include L-type Ca\(^{2+}\) channels (McDonald et al., 1994), ryanodine receptors (Takasago et al., 1989), phospholamban (Tada et al., 1979; Rapundalo, 1998), and myofilaments (Roos, 1987). Forskolin, which activates AC, would result in a generalized increase in cAMP, which might increase phosphorylation of all of these proteins. Indeed, forskolin clearly stimulated \( I_{Ca,L} \) as reported previously (Hartzell and Fischmeister, 1987; Yuan and Bers, 1995). Furthermore, forskolin stimulated CICR contractions initiated by voltage steps to \(0 \) mV in addition to contractions initiated by steps to \(-40 \) mV. Because forskolin also increased inward current with steps to \(-40 \) mV, one cannot tell whether enhancement of this contraction was mediated only through the VSRM or whether CICR also was recruited.

Forskolin also affected I-V and contraction-voltage relationships. When the VSRM was inactivated, forskolin increased the peak of the I-V relationship and corresponding I-V relationships elicited by voltage steps from \(V_{PC}\) values of \(-60 \) and \(-40 \) mV (\(n=5\)). * * *, significantly different from control, \(p<0.05\).

Agents that increase cAMP by inhibiting its degradation also affected excitation-contraction coupling. PDE isozymes I through V have been identified in cardiac muscle (Shahid and Nicholson, 1990; Bode et al., 1991; Burns et al., 1996). IBMX, which inhibits all PDEs (Shahid and Nicholson, 1990; Bode et al., 1991; Burns et al., 1996), should cause a generalized increase in cAMP much like forskolin. Indeed, IBMX increased \( I_{Ca,L} \) as reported previously (Mubagwa et al., 1993; Verde et al., 1999) and exerted effects on contraction-voltage and I-V relationships similar to those of forskolin. IBMX also had very similar effects to...
forskolin on contractions and currents initiated by steps to negative potentials.

Because forskolin and IBMX both result in the appearance of substantial current with negative test steps used to activate the VSRM, it is difficult to evaluate whether these agents stimulate the VSRM independently of CICR. A recent study reported that stimulation of β-adrenergic receptors with isoproterenol also caused current to appear at negative potentials, and concluded that CICR might account for contractions at these potentials in the presence of adrenergic stimulation (Piacentino et al., 2000). Isoproterenol, like forskolin or IBMX, might cause a generalized increase in cAMP and phosphorylate multiple protein targets involved in cardiac excitation-contraction coupling. Because of the generalized increase in phosphorylation produced by isoproterenol, forskolin, and IBMX, these agents do not permit one to distinguish between contractions resulting from enhancement of the VSRM or CICR.

Interestingly, the specific PDE III inhibitor amrinone selectively increased contractions initiated by steps to positive potentials where \( I_{Ca-L} \) is minimal. The voltage-clamp protocol is shown at top. A, records of contraction and current recorded in response to a 200-ms step to +80 mV in the absence of amrinone. The test step initiated a phasic contraction without measurable inward current. B, traces recorded from the same myocyte in the presence of 200 μM amrinone. Contraction was increased with no effect on the current recording. C, similar results were obtained with 500 μM amrinone.

![Fig. 5. PDE III inhibitor amrinone causes a selective increase in the magnitude of VSRM contractions. The voltage-clamp protocol is shown at the top. A, sequential voltage steps from -60 to -40 and 0 mV were used to activate VSRM and CICR contractions (top) and currents (bottom). B, amrinone (500 μM) increased the magnitude of the VSRM contraction but did not affect CICR contraction or inward currents. C, mean data show that amrinone significantly increased the magnitude of VSRM contractions, with no effect on mean amplitudes of CICR contractions (n = 5). D, amrinone also had no significant effect on currents elicited by either activation step. E, representative traces illustrating effects of amrinone on caffeine contractures. Amrinone caused an increase in the peak of the caffeine contracture. F, mean data demonstrate that the effect of amrinone was significant (n = 5). *, significantly different from control, p < 0.05.](https://www.aspetjournals.org/doi/abs/10.1124/jpet.106.099818)
cannot be explained by stimulation of I_{Ca-L} as suggested previously for isoproterenol by Piacentino et al. (2000).

Previous studies of PDE III inhibitors, including amrinone, have provided conflicting reports of both positive and negative inotropic effects and variable effects on Ca^{2+} current (Rosenthal and Ferrier, 1982; Kondo et al., 1983; Malecot et al., 1985; Matsui et al., 1999; Verde et al., 1999). These studies used different cardiac tissues, drug concentrations, and experimental conditions. The variability in published observations may reflect differences in availability of the VSRM under different conditions.

Amrinone also caused a small increase in caffeine contractions, indicative of a small increase in SR stores of Ca^{2+}.

However, amrinone did not increase CICR contractions, which largely depend on SR Ca^{2+} release. Therefore, it is unlikely the dramatic increase in VSRRM contractions caused by amrinone can be explained by this small increase in SR Ca^{2+} stores. Alternatively, amrinone might affect the release process of the VSRM. Evidence supporting this possibility comes from plots of VSRM and CICR contractions normalized to caffeine contractures, to estimate the fraction of SR Ca^{2+} released. Amrinone markedly increased fractional release of SR Ca^{2+} by the VSRM, but not CICR. In contrast, increases in CICR contractions caused by the latter agents were proportional to the increase in SR Ca^{2+} stores.

We have shown previously that activation of the VSRM by exogenous cAMP is mediated through PKA (Ferrier et al., 1998). The present results suggest phosphorylation of the VSRM by PKA is functionally compartmentalized by PDE III. Some PDEs may be associated with or anchored to specific targets within the cell (Houslay and Milligan, 1997), as shown schematically in Fig. 10. A subpopulation of PDE III is anchored to t-tubular SR junctional structures, whereas PDE IV has been associated with sarcolemma (Kaufman et al., 1989; Lugnier et al., 1993). The regulatory subunit of PKA (RII) also is believed to be anchored to phosphorylation targets by anchoring proteins (A-kinase anchor proteins), and thus to direct phosphorylation to specific targets (McCartney et al., 1995; Houslay and Milligan, 1997). For example, A-kinase anchor protein 100 colocalizes with ryanodine receptors in t-tubule/junctional SR of cardiac myocytes (McCartney et al., 1995; Yang et al., 1998). An anchored PDE may reduce cAMP levels locally and influence cAMP available for phosphorylation of adjacent proteins by PKA. Thus, drugs such as amrinone might cause localized changes in cAMP levels and phosphorylation by inhibiting anchored PDE III near specific targets (Houslay and Milligan, 1997). This may explain how amrinone, unlike forskolin or IBMX, specifically enhanced

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**Fig. 7.** Amrinone increases the amplitudes of contraction-voltage relationships when the VSRM is available, with little effect on Ca^{2+} current. The experiments were conducted with the voltage-clamp protocol illustrated in Fig. 4. A–C, increasing concentrations of amrinone significantly increased the amplitudes of contraction-voltage relationships when the VSRM was available (V_{PC} of -60 mV) (top panels). Amrinone had no effect on the amplitudes of I-V relationships at 100 and 200 µM and only caused a minimal increase at 500 µM (bottom panels) (n = 4–14 cells/group). Open symbols indicate data for myocytes in the absence of amrinone (control). Filled symbols represent data recorded in the presence of amrinone. *, significantly different from control, p < 0.05.
contractions initiated by the VSRM with little or no effect on CICR or inward currents.

In Fig. 10, we depict the VSRM and CICR as separate mechanisms because they have very different properties, some of which are mutually exclusive (Ferrier and Howlett, 2001). The observation that the VSRM can be modulated independently of CICR adds to the evidence that these are separate mechanisms. In Fig. 10 we have indicated putative phosphorylation sites for PKA on L-type Ca\(^{2+}\) channels, ryanodine receptors, phospholamban-SR Ca\(^{2+}\) ATPase, and on...
a component of the VSRM. Stimulation of AC by forskolin probably would cause a generalized increase in cAMP, although regional concentration differences may occur in the vicinity of different PDEs. A nonselective PDE inhibitor such as IBMX would cause a generalized decrease in degradation of cAMP by all PDEs. This probably would increase phosphorylation by cAMP at all sites and would stimulate both mechanisms of excitation-contraction coupling. In contrast, amrinone would be expected to increase cAMP levels primarily in the vicinity of PDE III, which would allow a selective enhancement of the VSRM. In Fig. 10 we have situated PDE III on or near SR Ca$^{2+}$-release channels associated with the VSRM because PDE III has been localized to the junctional SR (Kauffman et al., 1989; Lugnier et al., 1993) and because amrinone facilitated the VSRM. However, identification of the specific component of the VSRM that is phosphorylated will require additional investigation.

Our results suggest that the VSRM may serve as a major regulatory site for cardiac contraction by the AC/PKA pathway. Furthermore, the VSRM can be regulated independently of CICR and independently of effects on $I_{\text{CaL}}$. Thus, our study provides evidence that the AC/PKA cascade has divergent paths that regulate CICR and the VSRM separately. Our results also indicate that stimulation of the VSRM by the AC/PKA pathway results in release of a greater fraction of SR Ca$^{2+}$. Furthermore, the phosphorylation site(s) for the VSRM are regulated by PDE III, and local changes in cAMP caused by inhibition of PDE III may affect the VSRM without affecting CICR or $I_{\text{CaL}}$. In contrast, most of the inotropic effect of forskolin and IBMX on CICR could be accounted for on the basis of increased SR load under the conditions of our study. Thus, pharmacological agents that affect the AC/PKA cascade at different points may have very different effects on cardiac excitation-contraction coupling.

Acknowledgments
We are grateful for excellent technical assistance provided by Peter Nicholl, Claire Guyette, Cindy Mapplebeck, Steve Foster, Jiequan Zhen, and Isabel Redondo.

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