Differential Effects of Phosphodiesterase-Sensitive and -Resistant Analogs of cAMP on Initiation of Contraction in Cardiac Ventricular Myocytes

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ABSTRACT
Amplitudes of cardiac contractions initiated by Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) are proportional to the magnitude of Ca\(^{2+}\) current (I\(_{\text{Ca-L}}\)). However, large contractions accompanied by little inward current have been reported in some but not all studies in which cells were dialyzed with different analogs of cAMP. This study compares the effects of different phosphodiesterase (PDE)-resistant and PDE-sensitive analogs of cAMP on CICR, and investigates whether cAMP sensitizes CICR so that small currents induce large contractions. Experiments were conducted in voltage-clamped guinea pig ventricular myocytes at 37°C, with different analogs of cAMP added to patch pipette solutions. With PDE sensitive Tris-cAMP, contraction-voltage relations were bell-shaped and proportional to I\(_{\text{Ca-L}}\). In contrast, dialysis with PDE-resistant dibutyryl-cAMP resulted in sigmoidal contraction-voltage relations and large responses with little inward current. Similarly, in cells loaded with fura-2, large Ca\(^{2+}\) transients were elicited with little inward current in cells dialyzed with PDE-resistant but not PDE-sensitive cAMP. However, large transients were observed with PDE-sensitive cAMP when PDE was inhibited with 3-isobutyl-1-methylxanthine. When the amplitude of I\(_{\text{Ca-L}}\) was varied by partial block with Cd\(^{2+}\), or by partial inactivation, CICR remained proportional to the amplitude of I\(_{\text{Ca-L}}\). Thus, cAMP altered the relationship between Ca\(^{2+}\) transients and membrane potential but did not sensitize conventional CICR coupled to I\(_{\text{Ca-L}}\). Our results show that effects of different analogs of cAMP on contraction depend on the PDE resistance of the analog tested. Furthermore, PDE can play a major role in modulating cardiac contraction by altering the relationship between membrane potential and Ca\(^{2+}\) release.

Cardiac contraction is strongly affected by drugs and transmitters that activate adenylyl cyclase and cAMP production. For example, it is well known that agents that activate \(\beta\)-adrenergic receptors have profound effects on contraction (Reuter, 1974), mediated by phosphorylation of various protein targets (Movsesian, 1999). Furthermore, defects in phosphorylation of proteins involved in initiation of contraction may contribute substantially to contractile dysfunction in heart failure (Sjaastad et al., 2003).

Elevation of cAMP activates protein kinase A and promotes phosphorylation of a number of protein targets important in regulation of strength of contraction, including L-type Ca\(^{2+}\) channels, phospholamban, ryanodine receptors, and troponin I (Movsesian, 1999). Phosphorylation of L-type Ca\(^{2+}\) channels increases the magnitude of L-type Ca\(^{2+}\) current (I\(_{\text{Ca-L}}\)) (McDonald et al., 1994), which may increase contraction by increasing sarcoplasmic reticulum (SR) stores of Ca\(^{2+}\) available for release. Also, increased I\(_{\text{Ca-L}}\) may provide a stronger trigger for SR Ca\(^{2+}\) release through a process called Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) (Fabiato, 1983).

In CICR a small influx of Ca\(^{2+}\) triggers a large release of Ca\(^{2+}\) from the SR. The influx of Ca\(^{2+}\) that triggers CICR in heart is believed to enter the cell primarily as ICa-L (London and Krueger, 1986; Barcenas-Ruiz and Wier, 1987; Beuckelmann and Wier, 1988; duBell and Houser, 1989; Cleemann and Morad, 1991). Thus, ICa-L not only triggers CICR but also grades the release of SR Ca\(^{2+}\) so that contractions and transients are large when the peak amplitude of ICa-L is large, and small when the magnitude of ICa-L is small. Therefore, one would predict that agents that increase ICa-L would increase the amount of Ca\(^{2+}\) released from the SR.

Although Ca\(^{2+}\) release is believed to be proportional to ICa-L, several studies have reported that this proportionality...
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is lost under specific experimental conditions (Hussain and Orchard, 1997; Mackiewicz et al., 2000; Emanuel et al., 2001; Ferrier and Howlett, 2001; Viatchenko-Karpinski and Gyorke, 2001). For example, in cells exposed to the nonselective β-adrenergic agonist isoproterenol, contraction-voltage relations are sigmoidal, although current-voltage (I-V) relations for ICa-L remain bell-shaped (Hussain and Orchard, 1997; Viatchenko-Karpinski and Gyorke, 2001). It is likely that this effect is mediated through stimulation of cAMP production, because ventricular myocytes diazoyzed with intracellular patch pipette solutions containing 8-bromo-cAMP at 37°C also exhibit sigmoidal contraction-voltage relationships (Ferrier et al., 1998; Mackiewicz et al., 2000). One explanation that has been advanced to explain this deviation from proportionality to ICa-L is that elevation of intracellular cAMP sensitizes CICR so that even very small Ca2+ currents trigger large contractions and Ca2+ transients (Hussain and Orchard, 1997; Wier and Balke, 1999; Piacentino et al., 2000; Viatchenko-Karpinski and Gyorke, 2001). Thus, it is possible that stimulatory agents such as β-agonists might alter the proportionality between amplitude of ICa-L and magnitude of SR Ca2+ release so that small currents elicit large responses.

Sigmoidal contraction-voltage relations have been reported in several studies that included 8-bromo-cAMP in patch pipette solutions (Ferrier et al., 1998; Mackiewicz et al., 2000). However, another study that used Tris-cAMP, reported broadening of the conventional relation, although the curve remained bell-shaped (Piacentino et al., 2000). These observations suggest that these two analogs of cAMP are not equally effective in altering the sensitivity of CICR. We hypothesize that this disparity might reflect a difference in degradation of these analogs, because 8-bromo-cAMP is resistant to degradation by phosphodiesterase (PDE), whereas Tris-cAMP is not (Meyer and Miller, 1974). Therefore, the objectives of this study were to compare the effects of different PDE-resistant and PDE-sensitive analogs of cAMP on CICR and to determine whether elevation of intracellular cAMP alters CICR so that small currents elicit large Ca2+ transients and contractions.

Materials and Methods

Animals and Myocyte Isolation. Experiments were conducted according to the guidelines published by the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Animal Care. Isolated guinea pig ventricular myocytes were prepared by enzymatic dissociation of hearts with collagenase and protease as described in detail previously (Xiong et al., 2001). Myocytes were placed in an experimental chamber on the stage of an inverted microscope (Eclipse TE2000; Nikon, Tokyo, Japan) and superfused at 3 ml/min with a solution at 37°C containing 45 mM NaCl, 100 mM choline Cl, 2 mM CaCl2, 4 mM KCl, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES (pH 7.4 with NaOH), and 300 mM lidocaine to block Na+ current. In some experiments, switches either to nominally Ca2+-free or Cd2+-containing solutions at 37°C were made within 300 ms by a computer-controlled rapid solution changer (Ferrier et al., 2000).

Voltage Clamp. Discontinuous single electrode voltage-clamp recordings (sample rate 7–8 kHz) were made with an Axoclamp 2A voltage-clamp amplifier (Axon Instruments, Inc., Foster City, CA). Experiments were conducted with 1 to 3 MΩ pipette resistances filled with Na+-free solution to inhibit Ca2+ influx by way of Na+-Ca2+ exchange. The composition of this solution was 70 mM KCl, 70 mM K aspartate, 4 mM MgATP, 1 mM MgCl2, 2.5 mM KH2PO4, 0.12 mM CaCl2, 0.5 mM EGTA, and 10 mM HEPES, pH 7.2, with KOH. The pipette solutions were made to contain approximately 46 mM free Ca2+ (Ecal for Windows version 1.1, 1996; Biosoft, Ferguson, MO). Pipette-to-bath liquid junction potentials were measured (−8 to −10 mV) and compensated before data acquisition. An agar bridge (2.7 M KCl) was used as a bath ground to minimize changes in bath-to-ground liquid junction potentials with solution changes.

CAMP Analogs. Various PDE-resistant and PDE-sensitive analogs of cAMP were added to the patch pipette solutions for specific protocols. The following procedures were followed rigorously to minimize degradation of cAMP. Stock solutions of 0.1 mM cAMP were prepared in deionized water. Stock cAMP was added to aliquots of intracellular solution to give a final concentration of 50 μM cAMP. The aliquots of intracellular solution were frozen at −70°C for no longer than 2 weeks. Aliquots of intracellular solution were thawed on the day of use. CAMP containing solutions were never subjected to more than one cycle of freezing and thawing. Once thawed, intracellular solutions were kept on ice throughout experiments. Pipettes were filled with intracellular solutions by way of plastic syringes that also were kept on ice.

Measurements and Analysis. Unloaded cell shortening was recorded at 120 Hz with a television camera and video edge detector (Crescent Electronics, Sandy, Utah). Ca2+ transients were detected with a DeltaRAM system (Photon Technology International, Brunswick, NJ). Myocytes were loaded with 2.0 μM Fura-2/acetoxymethyl ester (Fura-2/AM) for 20 min in the dark at room temperature. After loading, extracellular dye was washed from the myocytes with extracellular buffer solution for 20 min. Fluorescence signals were collected from a field slightly smaller than the size of a single myocyte. The field was adjusted for each cell with an adjustable aperture. Dye was excited at wavelengths of 340 and 380 nm, and fluorescence emission ratio was measured at 510 nm. Background fluorescence was subtracted before ratios were determined. Recording times were kept as brief as possible to minimize washout and photobleaching of fura-2.

Currents and contractions were acquired and analyzed with pClamp (version 6.0 or 8.0). Recordings were digitized with either a Labmaster TL-125 or a Digidata 1322A A/D interface (Axon Instruments, Inc.) at sample rates up to 50 kHz and stored on a computer for later analysis. Emission ratios were acquired at 100 Hz and analyzed with Felix software (Photon Technology International). Emission ratios were converted to Ca2+ concentrations with a calibration curve determined in vitro with the same optical path used for experiments. Voltage-clamp test steps were preceded by trains of 10 conditioning pulses at 2 Hz, to provide a consistent activation history. Conditioning pulses were 200-ms-long pulses to 0 mV from the holding potential of −80 mV. Test steps were made from a postconditioning potential that followed the conditioning pulse train. Additional details of voltage-clamp protocols are provided in specific results sections.

Peak inward ICa-L was measured as the difference between the maximum inward deflection and a reference point 200 ms after the beginning of the step. When Ca2+ tail currents were measured, ICa-L was determined as the difference between currents recorded in the presence and absence of extracellular Ca2+. Switches between 2 and 0 mM Ca2+ were made with the rapid solution changer. Peak cell shortening was measured as the difference between peak shortening and a reference point immediately preceding the onset of cell shortening. The amplitudes of Ca2+ transients were measured, after conversion to Ca2+ concentration, as the difference between peak and immediately preceding Ca2+ concentrations.

Statistics. Data are presented as means ± S.E.M. Differences between means were assessed either with a Student’s t test, or with a one-way repeated measures analysis of variance. Differences were considered significant for values of p < 0.05. Curve fitting and statistical analyses were performed with SigmaPlot (version 5.0) and SigmaStat (version 2.03) (SPSS Science, Chicago, IL).
Fig. 1. Contraction-voltage relations are sigmoidal in cells dialyzed with dibutyryl-cAMP, a PDE-resistant analog of cAMP. The voltage protocol is shown at the top. 
A, representative recordings of contraction (top) and current (bottom) activated by a test step to 0 mV. Steps to 0 mV elicited both inward current and contraction. 
B, representative recordings from the same myocyte depolarized to +70 mV. This step elicited a large contraction with little if any inward current. 
C, mean contraction-voltage relation. 
D, mean current-voltage relation was bell-shaped in cells dialyzed with dibutyryl-cAMP. n = 9 myocytes.

Fig. 2. Contraction-voltage relations are bell-shaped and parallel to current-voltage relations for I_{Ca-L} when myocytes are dialyzed with PDE-sensitive Tris-cAMP. 
A, representative recordings show that a step to 0 mV elicited both inward current and contraction. 
B, with Tris-cAMP, a step to +70 mV did not elicit either inward current or contraction. 
C and D, mean contraction-voltage and current-voltage relations were bell-shaped in myocytes dialyzed with Tris-cAMP. n = 6 myocytes.
Chemicals. Lidocaine, choline chloride, HEPES buffer, EGTA, MgCl₂, and analogs of cAMP (Na cAMP, Tris-cAMP, 8-bromo-cAMP, and dibutyryl cAMP) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Sigma-Aldrich Canada Ltd. Fura-2/AM was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from BDH Inc. (Toronto, ON, Canada). A stock solution of Fura-2/AM was prepared by dissolving 50 µg of Fura-2/AM in 20 µl of anhydrous dimethyl sulfoxide (Sigma-Aldrich Canada Ltd.). All other chemicals were dissolved in deionized water.

Results

The first series of experiments examined the relationship between contraction and voltage in myocytes dialyzed with dibutyryl-cAMP, a PDE-resistant analog of cAMP (Meyer and Miller, 1974). In these experiments, test steps were preceded by 10 conditioning pulses from the holding potential of −80 to 0 mV. Conditioning pulses were followed by 200-ms test steps from a postconditioning potential of −70 mV to more depolarized membrane potentials. Figure 1, A and B, shows representative records of contractions and currents from a cell dialyzed with dibutyryl-cAMP. A test step to −40 mV elicited a large Ca²⁺ transient with little inward current. B, recordings of Ca²⁺ transients and currents from a cell dialyzed with Tris-cAMP. The step to −40 mV did not elicit a Ca²⁺ transient.
contraction-voltage relations were sigmoidal in the cells dia-
lized with dibutyryl-cAMP. Thus, large contractions were
observed with either large or small inward currents when
dibutyryl-cAMP was added to patch pipette solutions.

The next series of experiments examined contraction-volt-
age and I-V relations in myocytes dialyzed with intracellular
solution containing the PDE-sensitive analog Tris-cAMP
(Meyer and Miller, 1974). Representative traces are shown in
Fig. 2, A and B. Currents and contractions were maximal
with a step to 0 mV, whereas both current and contractions
were small with a step to −70 mV. Figure 2, C and D, shows
mean contraction-voltage and I-V relations for myocytes di-
alized with 50 μM Tris-cAMP. Contraction-voltage relations
were bell-shaped and roughly proportional to I_{Ca,L} with this
analog.

The next series of experiments was designed to determine
with the ability of small currents to elicited large contrac-
tions was a function of the PDE resistance of the cAMP
analog added to the patch pipette solution. To test this, cells
were activated with a voltage-clamp protocol consisting of
sequential test steps from −65 to −40 mV and then −40 to 0
mV. This protocol was used because the step to −40 mV
elicits very little inward current, whereas the step to 0 mV
elicits near maximal I_{Ca,L}. In addition, to determine whether
changes in contraction reflected changes in Ca^{2+} release,
cells were loaded with fura-2 and Ca^{2+} transients were mea-
sured. Figure 3 shows representative recordings of Ca^{2+}
transients and currents in cells dialyzed with either dibu-
yryl-cAMP or Tris-cAMP. In the presence of PDE-resistant
d dibutyryl-cAMP, prominent Ca^{2+} transients were observed
with the step to −40 mV, as well as the step to 0 mV (Fig. 3A).
In contrast, when myocytes were dialyzed with PDE-sensi-
tive Tris-cAMP, the first activation step did not initiate a
transient, although the step to 0 mV initiated a Ca^{2+} tran-
sient and I_{Ca,L} (Fig. 3B).

Figure 4 compares the mean amplitudes of Ca^{2+} transients
and currents recorded in myocytes dialyzed with patch pi-
pettes containing either no cAMP or selected PDE-resistant
or PDE-sensitive analogs of cAMP. Figure 4A shows mean
Ca^{2+} transients initiated by the step from −65 to −40 mV. In
the absence of cAMP in the patch pipette solution, there was
minimal release of Ca^{2+} with the step to −40 mV. However,
in the presence of PDE-resistant 8-bromo- or dibutyryl-cAMP,
large transients were elicited by this step. In contrast, nei-
ther Tris- nor Na cAMP, both of which are PDE-sensitive,
supported activation of Ca^{2+} transients by the step to −40
mV. Interestingly, inward currents elicited by this step were
small with all analogs of cAMP tested (Fig. 4B). Figure 4, C
and D, shows effects of different cAMP analogs on the mag-
nitudes of Ca^{2+} transients and currents initiated by a step
from −40 to 0 mV, which activates large inward I_{Ca,L}. This
step initiated Ca^{2+} transients of similar magnitude regard-
less of whether cells were dialyzed with cAMP, or whether
the cAMP was PDE-sensitive or PDE-resistant. It is possible
that the absence of significant effects on transients initiated
by steps to 0 mV, even by the PDE-resistant analogs of
cAMP, may reflect the order of activation. The large increase
in the transient at −40 mV observed with 8-bromo- and
dibutyryl-cAMP may have partially depleted the SR and reduced the effect on contractions initiated by the subsequent step to 0 mV. However, the amplitude of $I_{\text{Ca,L}}$ was increased slightly by all cAMP analogs except Tris-cAMP.

As shown in Fig. 4, A and B, large Ca$^{2+}$ transients were elicited by steps to $-40$ mV, which activated small inward currents only in cells dialyzed with PDE-resistant analogs of cAMP. If PDE resistance accounts for the differences between analogs of cAMP, inhibition of PDE should eliminate this difference. Therefore, we examined the effect of Na cAMP in the presence of IBMX, a nonselective PDE inhibitor. Figure 5, A and B, shows representative recordings of Ca$^{2+}$ transients and currents activated by steps to $-40$ and 0 mV in cells dialyzed with Na cAMP. Figure 5A was recorded in the absence of IBMX and shows that the step to $-40$ mV elicited little release of Ca$^{2+}$. B, exposure of the same cell to 100 $\mu$M IBMX resulted in the appearance of a large Ca$^{2+}$ transient with the step to $-40$ mV. C and D, similar results were observed with IBMX in the absence of exogenous cAMP in the patch pipette.

To determine whether IBMX also would allow endogenous cAMP to activate Ca$^{2+}$ release at negative potentials we examined the effects of IBMX when cAMP was absent from the pipette solution. Figure 5, C and D, shows recordings of Ca$^{2+}$ transients and currents from a myocyte dialyzed with intracellular solution with no cAMP added. Figure 5C shows that the step to $-40$ mV did not elicit a Ca$^{2+}$ transient; however, the step to 0 mV resulted in a large Ca$^{2+}$ transient. When the same myocyte was exposed to 100 $\mu$M IBMX (Fig. 5D), both steps initiated large Ca$^{2+}$ transients.

Figure 6 shows mean data for transients and currents with and without IBMX. The mean amplitudes of Ca$^{2+}$ transients elicited by steps to $-40$ mV were very small in cells dialyzed either with Na cAMP or with no cAMP in the absence of IBMX (Fig. 6A). However, IBMX caused large increases in the magnitude of Ca$^{2+}$ transients initiated by steps to $-40$ mV in cells with Na cAMP or no cAMP. IBMX had no effect on inward current elicited by steps to $-40$ mV (Fig. 6B), but caused moderate increases in $I_{\text{Ca,L}}$ activated by steps to 0 mV (Fig. 6D). IBMX had little effect on Ca$^{2+}$ transients initiated...
by the step to 0 mV (Fig. 6C). These observations suggest that the absence of Ca\(^{2+}\) transients at negative potentials in cells dialyzed with PDE-sensitive cAMP or no cAMP likely reflects degradation of cAMP by PDE.

When degradation of cAMP by PDE is minimized, cAMP alters excitation-contraction coupling so that large Ca\(^{2+}\) transients can be elicited with little inward current. It is possible that PDE-resistant analogs of cAMP increase the sensitivity of CICR so that small currents initiate large Ca\(^{2+}\) transients. To determine whether PDE-resistant 8-bromo-cAMP sensitizes CICR, we examined the effects of decreasing the magnitude of IC\(_{\text{Ca-L}}\) through partial block with Cd\(^{2+}\).

Figure 7 shows the effects of different concentrations of Cd\(^{2+}\) on currents and contractions elicited by sequential test steps to -40 and 0 mV in a cell dialyzed with 8-bromo-cAMP. The voltage protocol, shown at the top of Fig. 7, incorporated a 4-s-long postconditioning potential to allow rapid application of Cd\(^{2+}\) after conditioning pulses but before the test steps. Figure 7A shows that Cd\(^{2+}\) caused a concentration-dependent inhibition of IC\(_{\text{Ca-L}}\) elicited by the step to 0 mV. Progressive inhibition of IC\(_{\text{Ca-L}}\) was accompanied by a parallel depression of contractions elicited by this step. Thus, CICR clearly was graded by the amplitude of IC\(_{\text{Ca-L}}\) in cells dialyzed with PDE-resistant cAMP. However, contractions elicited by the step to -40 mV were not affected by any of the concentrations of Cd\(^{2+}\) tested.

Figure 7B shows mean amplitudes of contractions and inward currents plotted as a function of Cd\(^{2+}\) concentration. Increasing concentrations of Cd\(^{2+}\) caused progressive inhibition of peak IC\(_{\text{Ca-L}}\) elicited by steps to 0 mV. The mean amplitude of contractions initiated by the same steps decreased in parallel with the amplitudes of IC\(_{\text{Ca-L}}\). Thus, small currents only elicited small contractions. In contrast, the amplitudes of contractions initiated by the steps to -40 mV were unaffected by Cd\(^{2+}\). It is unclear whether the small current associated with this step was blocked by Cd\(^{2+}\), because of the small size of the current and because Cd\(^{2+}\) blockade is known to be voltage-dependent (Lansman et al., 1986).

It is possible that the different effects of Cd\(^{2+}\) on the contractions elicited by the steps to -40 and 0 mV might reflect the order of activation. The first step to -40 mV may partially deplete SR stores of Ca\(^{2+}\) and alter the sensitivity of CICR during the subsequent step to 0 mV. We tested this possibility directly by performing similar experiments with a protocol modified to omit the first step to -40 mV as shown at the top of Fig. 8. Figure 8A shows that increasing concentrations of Cd\(^{2+}\) still caused parallel inhibition of IC\(_{\text{Ca-L}}\) and contraction with this protocol. Figure 8B presents mean data showing that IC\(_{\text{Ca-L}}\) and contraction exhibited essentially identical concentration-response curves to increasing concentrations of Cd\(^{2+}\). These results indicate that PDE-resistant cAMP does not sensitize CICR so that small currents give rise to large contractions or transients at 0 mV.

Although Cd\(^{2+}\) caused a graded block of CICR at 0 mV, large contractions persisted at -40 mV in the presence of cAMP. The gain of CICR has been reported to increase at negative membrane potentials (Lopez-Lopez et al., 1995; Santana et al., 1996; Adachi-Akahane et al., 1999). Thus, it is
possible that PDE-resistant cAMP sensitizes CICR at negative potentials where gain is already high. Beuckelmann and Wier (1988) reported that repolarization to negative potentials from positive potentials induces contractions attributable to CICR. The magnitude of I_{Ca-L} elicited by repolarization can be graded by the degree of inactivation caused by the initial depolarization. We used this approach to examine the relationship between amplitude of I_{Ca-L} and contraction at \(-40\) mV in cells dialyzed with PDE-resistant cAMP. Figure 9 shows the voltage-clamp protocol plus representative recordings of currents and contractions. Cells were depolarized from \(-65\) mV to a potential between 0 and \(+100\) mV, followed by repolarization to \(-40\) mV. The amplitudes of contractions initiated by repolarization varied with the membrane potential during the preceding depolarization. Contractions were large when the preceding depolarization was to \(+100\) mV, but decreased to negligible levels as depolarization approached 0 mV. Inward currents elicited by repolarization were obscured by the capacitive transient caused by this voltage step. To measure inward Ca^{2+} currents initiated by repolarization, the same protocols were repeated in extracellular solution containing 0 mM Ca^{2+}. Recordings in 0 mM Ca^{2+} were subtracted from recordings made in 2 mM Ca^{2+} to provide the recordings of I_{Ca-L} shown in Fig. 10A. The amplitude of peak inward Ca^{2+} current was maximal when the preceding depolarization was to \(+100\) mV and decreased to negligible levels as depolarization approached 0 mV. Figure 10B shows records of contraction accompanying these inward currents. Figure 10C shows a plot of contraction amplitude as a function of the magnitude of inward current for the traces shown in Fig. 10, A and B. Contraction was directly proportional to the amplitude of I_{Ca-L}.

Figure 11, A and B, shows mean amplitudes of contractions and currents as functions of prepulse voltage, for five replicates of the experiment illustrated in Fig. 10. Both contraction and current increased with the voltage of the prepulse. Figure 12A shows a plot of mean amplitudes of contraction as a function of mean peak inward I_{Ca-L} from the mean data shown in Fig. 11. The mean data were well fitted by a re-
gression line ($r^2 = 0.99$). Figure 12A also shows 95% confidence limits for this regression. These data clearly show that contractions remained proportional to $I_{Ca-L}$ at $-40 \text{ mV}$ in the presence of PDE-resistant 8-bromo-cAMP. Thus, in protocols designed to examine CICR directly small currents elicited only small contractions, even at negative potentials where the gain of CICR is high.

Figure 12B shows a similar plot for mean amplitudes of contraction and peak inward $I_{Ca-L}$ for experiments in which the magnitude of $I_{Ca-L}$ was graded by increasing concentrations of Cd$^{2+}$ (Fig. 7). Figure 12B shows that contraction also was directly proportional to peak $I_{Ca-L}$ in protocols in which $I_{Ca-L}$ was elicited by depolarization rather than repolarization. Figure 12, A and B, also shows data for mean contractions and currents elicited by depolarizing steps from $-65$ to $-40 \text{ mV}$ in the same experiments. These steps elicited large contractions with little inward current. These data clearly do not fall within the confidence limits for the relationships between contraction and current for conventional CICR in Fig. 12, A and B. Our observations demonstrate that PDE-resistant cAMP does not sensitize conventional CICR so that small currents trigger large contractions.

**Discussion**

The objectives of this study were to compare the effects of different PDE-resistant and PDE-sensitive analogs of cAMP on CICR and to determine whether elevation of intracellular cAMP alters CICR so that small currents elicit large Ca$^{2+}$ transients and contractions. We found that contraction-voltage relations were sigmoidal when cells were dialyzed with dibutyryl-cAMP, a PDE-resistant analog. However, when cells were dialyzed with Tris-cAMP, a PDE-sensitive analog of cAMP, contraction-voltage relations were bell-shaped. In contrast, the current-voltage relation for $I_{Ca-L}$ remained bell-shaped in the presence of either analog. Differences in the effects of these two types of cAMP analogs were also apparent when we measured Ca$^{2+}$ transients. Large Ca$^{2+}$ transients occurred in the presence of small inward currents when cells were dialyzed with PDE-resistant cAMP, but not when cells...
were dialyzed with PDE-sensitive cAMP. Interestingly, when cells were treated with IBMX to inhibit PDE, large Ca^{2+} transients with little inward current could be elicited in the presence of PDE-sensitive analogs of cAMP. To determine whether CICR was sensitized by cAMP, we graded the magnitude of I_{Ca-L} with Cd^{2+} or inactivation. CICR initiated at either 0 or −40 mV, remained smoothly graded by I_{Ca-L}, even in the presence of PDE-resistant 8-bromo-cAMP. Thus, large contractions initiated with little if any current in the presence of PDE-resistant cAMP were not explained by sensitization of CICR so that large responses occurred with little inward current.

In the present study, all experiments were conducted with 0 mM Na⁺ in the patch pipette solution to inhibit influx of Ca^{2+} by way of Na⁺-Ca^{2+} exchange. Under these conditions, the occurrence of bell-shaped and sigmoidal contraction-voltage relations depended on whether myocytes were dialyzed with PDE-sensitive or PDE-resistant analogs of cAMP. This difference may explain observations made in previous studies. We and others (Ferrier et al., 1998; Mackiewicz et al., 2000) have reported that intracellular dialysis with 8-bromo-cAMP results in sigmoidal contraction-voltage relations. The present study showed the same result with another PDE-resistant analog of cAMP, dibutyryl-cAMP. On the other hand, Piacentino et al. (2000) reported bell-shaped contraction-voltage relations when cells were dialyzed with Tris-cAMP. The present study agrees with this finding and shows that a bell-shaped contraction-voltage relationship would be expected in cells dialyzed with Tris-cAMP, which is readily broken down by PDE. Thus, the occurrence of sigmoidal or bell-shaped contraction-voltage relations likely reflects the use of PDE-resistant or PDE-sensitive cAMP analogs, respectively.

To investigate whether PDE-resistant cAMP modified CICR so that small I_{Ca-L} currents elicited large responses, we used two protocols that clearly activated CICR. First, CICR responses initiated by voltage-clamp steps from −40 to 0 mV were examined. The magnitude of I_{Ca-L} initiated by this step was graded by different concentrations of Cd^{2+}. With this approach, we found that contractions were smoothly graded by the amplitude of I_{Ca-L} and small I_{Ca-L} currents initiated only very small contractions. Large contractions initiated by conventional CICR occurred only in response to large currents. These experiments suggest that sensitization of CICR by cAMP cannot explain the large contractions accompanied by small inward currents at more negative potentials (e.g., −40 mV). However, the gain of CICR is greater at negative potentials than at more depolarized potentials (Lopez-Lopez et al., 1995; Santana et al., 1996; Adachi-Akahane et al., 1999). Thus, it is possible that sensitization of CICR would only be observed at more negative potentials. To test this possibility, I_{Ca-L} was initiated by repolarization to −40 mV (Barcenas-Ruiz and Wier, 1987). The repolarizing step was made from different positive potentials to vary the magnitude of I_{Ca-L} through partial inactivation. These experiments demonstrated that, even at −40 mV, CICR remained graded by magnitude of current so that small I_{Ca-L} currents elicited only very small contractions. Thus, dialysis of cells with PDE-resistant cAMP did not alter CICR so that small I_{Ca-L} currents would initiate large contractions at either 0 or −40 mV.

The mechanism by which large contractions and Ca^{2+} transients can be initiated with little current in cells dialyzed with PDE-resistant cAMP is not clear. High-gain CICR has been suggested as one possibility (Hussain and Orchard, 1997; Wier and Balke, 1999; Piacentino et al., 2000). However, our experiments show that CICR coupled to T-type Ca^{2+} current, which activates at negative membrane potentials (McDonald et al., 1994). However, T-current has been reported to be a weak trigger for CICR (Sipido et al., 1998), and most studies suggest that T-current is insensitive to elevation of cAMP (McDonald et al., 1994). This does not preclude the possibility that the efficacy of T-current to increase SR Ca^{2+} release.
might be increased by phosphorylation of ryanodine receptors, because protein kinase A-dependent phosphorylation has been shown to increase the ligand sensitivity of ryanodine receptors (Uehara et al., 2002). Another possibility is that high-gain CICR is coupled to Ca\(^{2+}\) entry through sodium channels (sli-mode conductance) (Santana et al., 1998). However, contractions initiated at negative potentials persist in the presence of 50 \(\mu\)M tetrodotoxin (Ferrier et al., 2000), which strongly inhibits Ca\(^{2+}\) influx via this means (Santana et al., 1998). Alternatively, after phosphorylation high gain CICR might be coupled to a subpopulation of L-type Ca\(^{2+}\) channels. This subpopulation of channels must have distinct properties, because activation must occur at very negative membrane potentials and little inward current is observed. However, a mechanism coupled to L-type Ca\(^{2+}\) current should be activated by current initiated either by depolarization or by repolarization (Beuckelmann and Wier, 1988), which was not the case. Therefore, if this mechanism is coupled to a subpopulation of L-type Ca\(^{2+}\) channels, these channels must exhibit rapid voltage-inactivation with strong depolarizations, to account for the observation that this mechanism is not activated upon repolarization. Alternatively, because this mechanism is only activated on depolarization and not repolarization, one cannot exclude the possibility of a mechanism that operates independently of Ca\(^{2+}\) current on the basis of the data presented here (Beuckelmann and Wier, 1988). Additional investigations will be needed to explore these possibilities.

Our results demonstrate that dialysis of myocytes with PDE-resistant cAMP changes the configuration of the contraction-voltage relationship. A change in the configuration of the contraction-voltage relation could alter the efficacy of the action potential as a trigger for Ca\(^{2+}\) release. The action potential of mammalian ventricular cells typically reaches approximately +40 mV during the upstroke. This voltage is well beyond the peak of the I-V relationship for I\(_{Ca-L}\). Thus, Ca\(^{2+}\) release and contraction would not be maximal if controlled by the bell-shaped curve observed without cAMP dialysis or PDE inhibitors. However, in the presence of PDE-
resistant cAMP or PDE inhibition, a maximal contraction would be generated at these positive potentials. Thus, the change in the configuration of the contraction-voltage relation may contribute substantially to the positive inotropic effects of agents that increase cAMP, such as β1 agonists.

cAMP only affected the configuration of contraction-voltage relations if the analog was PDE-resistant or if PDE was inhibited. Our results further show that even in the absence of exogenous cAMP, inhibition of PDE by IBMX can modulate Ca\(^{2+}\) release. This observation suggests that basal cAMP synthesis is active even in the absence of β1 agonists. However, much of this cAMP must be hydrolyzed by PDE because SR Ca\(^{2+}\) release is only stimulated if cAMP degradation is inhibited. Interestingly, in a previous study we found that amrinone, a PDE III inhibitor, increased contraction with little effect on I\(_{\text{Ca,L}}\) (Xiong et al., 2001). Collectively, these observations suggest that PDE may serve an important regulatory role in cardiac excitation-contraction coupling.

PDE-resistant cAMP and IBMX caused only modest increases in I\(_{\text{Ca,L}}\) and little, if any, increase in Ca\(^{2+}\) transients initiated by steps to 0 mV. This further indicates that cAMP does not sensitize CICR. A similar result has been observed with the L-channel agonist Bay K 8644. Bay K 8644 increases I\(_{\text{Ca,L}}\) but not contraction (McCall and Bers, 1996; Adachi-Akahane et al., 1999). It is believed that this occurs because Bay K 8644 increases I\(_{\text{Ca,L}}\) by increasing the duration of single channel openings, not the number of channels which open (Bers, 2001). Because a single channel opening may initiate release (Lopez-Lopez et al., 1995; Santana et al., 1996; Wang et al., 2001), increasing the duration of openings would increase current but would not activate any further release. Interestingly, 8-bromo cAMP or stimulation of β-adrenergic receptors both cause a similar prolongation of single channel open times (Yue et al., 1990). This may explain why CICR transients were not increased in the present study despite a modest increase in peak inward I\(_{\text{Ca,L}}\).

In summary, the present study demonstrates that intracellular dialysis with cAMP can result in large contractions and Ca\(^{2+}\) transients occurring with little inward current. However, this only occurs if degradation of cAMP is restricted by use of a PDE-resistant analog or by treating the cells with a PDE inhibitor. Nonetheless, CICR remains smoothly graded by the amplitude of I\(_{\text{Ca,L}}\) with small currents eliciting only small responses. Our results also indicate that PDE may serve an important regulatory role in excitation-contraction coupling by modulating levels of cAMP. Changes in cAMP levels may alter contraction by changing the relationship between Ca release and membrane potential.

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