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Protein Phosphatase Inhibitor-1 Augments a Protein Kinase A-Dependent Increase in the Ca^{2+} Loading of the Sarcoplasmic Reticulum Without Changing Its Ca^{2+} Release

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Background: An increase in cytosolic protein phosphatases (PPs) de-phosphorylates phospholamban, decreasing the Ca^{2+} uptake of the sarcoplasmic reticulum (SR). The effects of PP inhibitors on cellular Ca^{2+} handling were investigated.

Methods and Results: Twitch Ca^{2+} transients (CaTs) and cell shortening were measured in intact rat cardiac myocytes, and caffeine-induced Ca^{2+} transients (CaffCaTs) and Ca^{2+} sparks were studied in saponin-permeabilized cells. Calyculin A augmented isoproterenol-induced increases in CaTs and cell shortening without altering the diastolic $[Ca^{2+}]_i$ and twitch $[Ca^{2+}]_i$ decay. The protein kinase A catalytic subunit (PKA_{cat}) increased the peak of CaffCaTs between 5 and 50 U/ml, and the addition of inhibitor-1 (I-1) augmented the increase. PKA_{cat} increased Ca^{2+} spark frequency and the addition of I-1 increased it further. PKA_{cat} at 50 U/ml amplified the peak and prolonged the duration of Ca^{2+} sparks, whereas the addition of I-1 did not alter them. An abrupt inhibition of SR Ca^{2+} uptake following exposure to PKA_{cat} caused a gradual decrease in Ca^{2+} spark frequency, but the addition of I-1 did not accelerate the decline of Ca^{2+} spark frequency or CaffCaTs.

Conclusions: Inhibition of PPs augmented the inotropic effect of isoproterenol. Specific inhibition of PP1 could stimulate the Ca^{2+} uptake of the SR with less significant effects on the Ca^{2+} release. (*Circ J* 2009; **73:** 1133–1140)

Key Words: Calyculin A; Inhibitor-1; Phospholamban; Protein phosphatases; Sarcoplasmic reticulum

Altered cellular Ca^{2+} handling plays a key role in the pathophysiology of heart failure. A typical aspect of failing heart cells is a decrease in the ability to load Ca^{2+} in the sarcoplasmic reticulum (SR), which results in a decreased amplitude and slowed rate of decay of Ca^{2+} transients (CaTs), and in some cases, an increased diastolic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[1,2] The reduction in SR Ca^{2+} loading could be ascribed to a decrease in Ca^{2+} re-uptake by the SR Ca^{2+} ATPase (SERCA), and increases in the SR Ca^{2+} leak and the Ca^{2+} extrusion via Na^+/Ca^{2+} exchanger[3]

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In the heart, the activity of SERCA is inhibited by the associated membrane protein, phospholamban (PLB), in its un-phosphorylated state. However, once Ser-16 and/or Thr-17 of PLB is phosphorylated, this inhibition is alleviated. Ser-16 and Thr-17 are phosphorylated by protein kinase A (PKA) in response to stimulation of the β-adrenergic receptors and by Ca^{2+}/calmodulin-dependent kinase II (CaMKII), respectively[4] In heart failure, PLB is hypo-phosphorylated because of an increase in cytosolic protein phosphatases (PPs)[5–7] Two phosphorylation sites of PLB are substrates for type 1 and 2A PPs[8] On the other hand, the SR Ca^{2+} release channels (ryanodine receptors; RyRs) are hyper-phosphorylated by PKA and CaMKII, resulting in a depletion of FK506-binding proteins (FKBPs), which in turn causes an increased sensitivity to Ca^{2+}-induced activation and defects in the channel function[9–13] To improve the impaired Ca^{2+} handling in heart failure, therefore, the development of novel agents that can selectively enhance phosphorylation of PLB with less significant effects on RyRs is needed.

Inhibition of PPs is expected to activate SR Ca^{2+} uptake and to recover myocardial contractility in the failing heart. Cell membrane-permeant PP inhibitors, such as okadaic acid, cantharidin, and calyculin A (Caly A), increase the phosphorylation of PLB without increasing cAMP content[6,14,15] However, because the effects of these PP inhibitors are not selective for PLB, non-specific phosphorylation of other Ca^{2+} regulatory proteins (eg, L-type Ca^{2+} channels and troponin I) may induce other adverse effects. Inhibitor-1 (I-1) is a potent and specific inhibitor of PP1 only when it was phosphorylated by PKA at the phosphorylation site, Thr-35[16] In the failing heart, downregulation of β-adrenergic receptors and decreased PKA activity result in decreased phosphorylation of Thr-35 and inactivation of I-1, leading to increased PP1 activity and de-phosphorylation of PLB[17–19]

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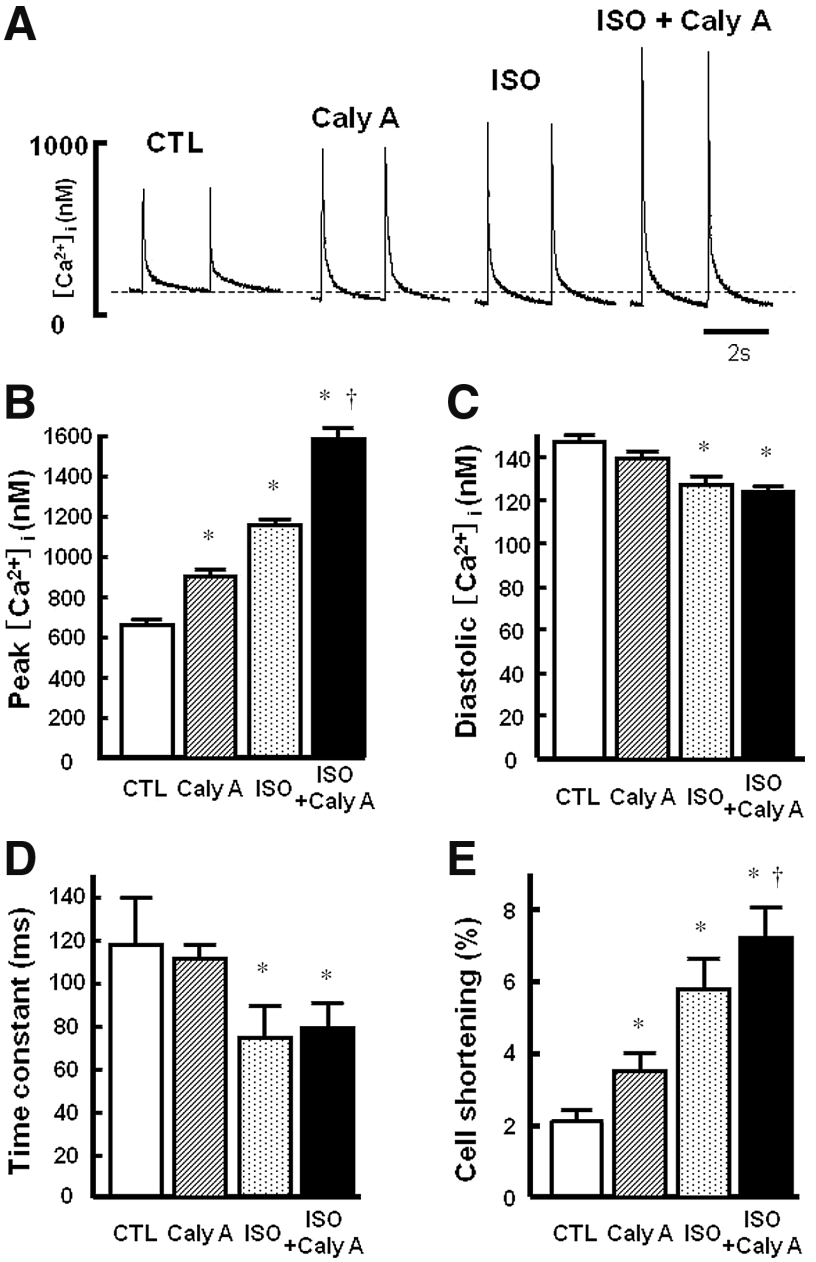

Figure 1. Effects of isoproterenol (ISO) and calyculin A (Caly A) on the twitch Ca^{2+} transients (CaTs) and cell shortening in intact cardiac myocytes. (**A**) Representative recordings of CaTs in the control perfusion (CTL), in the presence of Caly A (100 nmol/L), ISO (10 nmol/L) and ISO and Caly A (ISO + Caly A). (**B**–**E**) ISO increased the peak CaTs (**B**), decreased the diastolic $[Ca^{2+}]_i$ (**C**) and the time constant of $[Ca^{2+}]_i$ decay (**D**), and as a result increased cell shortening (**E**). Caly A by itself increases the peak of CaTs and cell shortening. Following the addition of Caly A to ISO further increased the peak of CaTs and cell shortening, although Caly A did not alter the diastolic $[Ca^{2+}]_i$ or the time constant of $[Ca^{2+}]_i$ decay in the presence or absence of ISO. Data are mean ± SEM. *P<0.05 vs CTL, †P<0.05 vs ISO by 1-way repeated ANOVA.

Conversely, in failing human cardiomyocytes adenoviral over-expression of constitutively active I-1 is associated with salvage of the β-adrenergic response![9,20]

To examine the possibility of inhibiting PPs to improve cellular Ca^{2+} handling and contractility in heart failure, we examined the effects of Caly A on CaTs and twitch cell shortening in intact rat ventricular myocytes, and then investigated the changes in SR Ca^{2+} loading and Ca^{2+} sparks with inhibition of PP1 by I-1 in permeabilized myocytes.

Methods

Cardiac Myocyte Isolation and Permeabilization

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Isolated myocytes from male Sprague-Dawley rats (250–300 g) were obtained by enzymatic dissociation and kept in a modified Kraft-Brühe solution containing (mmol/L) 70 KOH, 40 KCl, 20 KH_2PO_4, 3 $MgCl_2$, 50 glutamic acid, 10 glucose, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 3 ethyleneglycol-bis (b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (pH 7.4 with KOH). All experiments were conducted at room temperature within 6 h of cell isolation. In the experiments using intact myocytes, the cells were placed in a chamber and perfused with a normal Tyrode solution, composed of (mmol/L) 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5.6 glucose and 5 HEPES (pH 7.4 with NaOH). Cells were electrically stimulated with 2 ms voltage pulses of 1.5× threshold amplitude at 0.5 Hz, delivered through a pair of platinum electrodes placed on either side of the experimental chamber.

For the permeabilization of the sarcolemmal membrane, the cells were perfused with saponin (0.05 mg/L) in a Ca^{2+}-free internal solution, which contained (mmol/L) 50 KCl, 80 K-asparate, 2 Na-pyruvate, 20 HEPES, 3 $MgCl_2-6H_2O$, 2

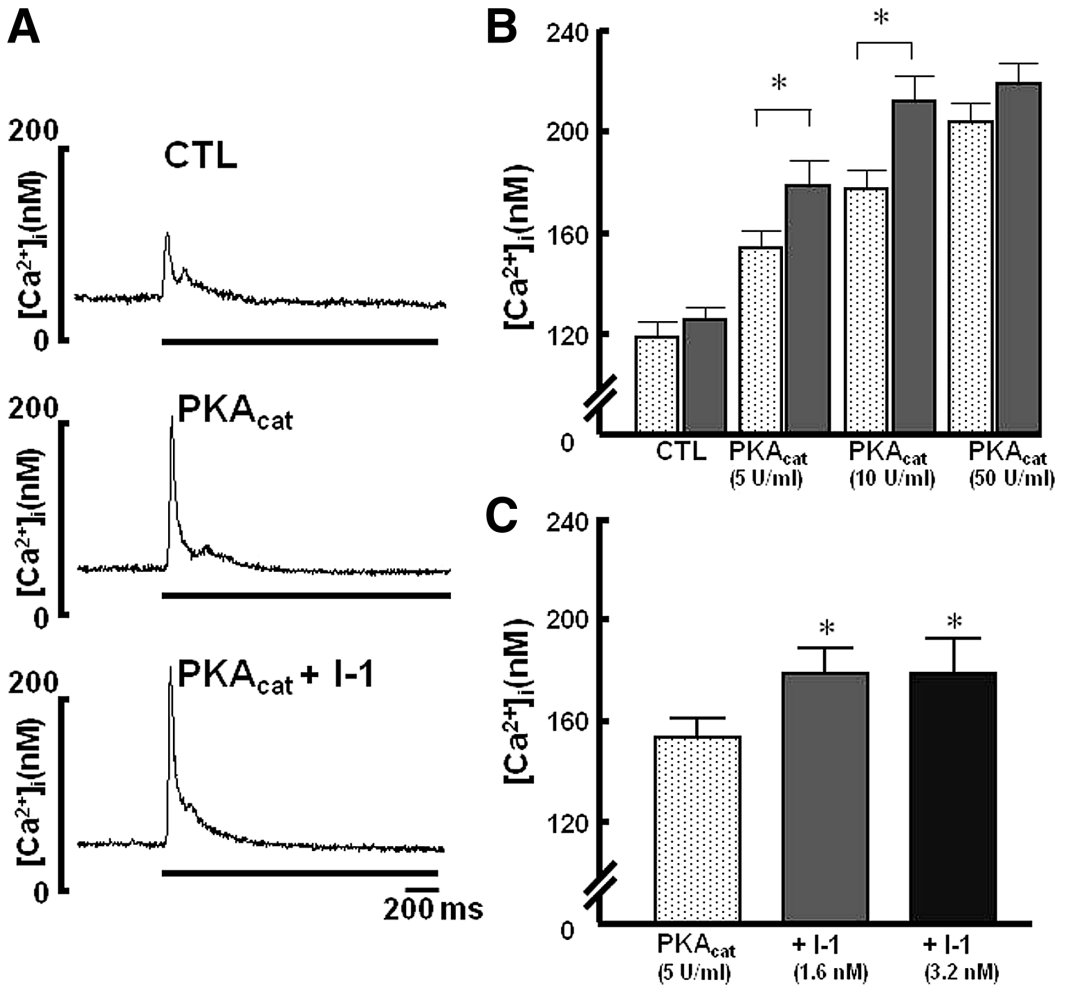

Figure 2. Effects of protein kinase A catalytic subunit (PKA_{cat}) and inhibitor-1 (I-1) on SR Ca^{2+} loading in permeabilized cardiac myocytes. (**A**) Representative recordings of caffeine-induced Ca^{2+} transients (CaffCaTs) in a permeabilized myocyte. The profiles of $[Ca^{2+}]_i$ during the rapid addition of 10 mmol/L caffeine (bars) are shown for the control perfusion (CTL), in the presence of PKA_{cat} (10U/ml), and after the addition of I-1 (1.6 nmol/L; PKA_{cat} + I-1). (**B**) Dose-dependent effects of PKA_{cat} on the amplitude of CaffCaTs between 5 U/ml and 50 U/ml. The perfusion of PKA_{cat} significantly increased the amplitude of the CaffCaTs at 5, 10 and 50 U/ml (dotted columns). After the addition of I-1, the amplitude of the CaffCaTs increased significantly, except at 0 U/ml and 50 U/ml (solid columns). (**C**) Dose-dependent effects of I-1 on the PKA_{cat} (5 U/ml)-induced increase in CaffCaT. The higher (3.6 nmol/L) concentration of I-1 did not further increase the amplitude of CaffCaT. Data are mean ± SEM. *P<0.05 vs PKA_{cat} only, by 2-way repeated ANOVA.

Na_2ATP, and 0.5 EGTA (pH 7.3 with KOH). After the sarcolemmal membrane was permeabilized, the concentration of free Ca^{2+} in the internal solution ($[Ca^{2+}]_c$) was increased to 50 nmol/L, and fluo-3 potassium salt (30 μmol/L) was added. $[Ca^{2+}]_c$ was obtained by mixing EGTA and $CaCl_2$, calculated using a computer program (WINMAXC, provided by Stanford University, Stanford, CA, USA) with consideration of the Mg^{2+} and ATP concentrations.

Ca^{2+} Signal Recording in Intact Myocytes

Isolated rat ventricular myocytes were attached to laminin-coated glass coverslips and loaded with fluo-3/acetoxymethyl ester (20 μmol/L) for 30 min at room temperature. Fluo-3 fluorescence imaging was performed with a laser scanning confocal microscope (LSM 510, Carl Zeiss, Tokyo, Japan) coupled to an inverted microscope (Axiovert S100, Carl Zeiss) with a ×63 water immersion objective (numerical aperture/1.3; Carl Zeiss), an excitation wavelength of 488 nm, and an emission >510 nm. Image acquisition for the quantitative analysis of CaTs was made in the line-scan mode. A single myocyte was scanned repeatedly (250 Hz) along a line parallel to its longitudinal axis. The CaTs were derived from averaged fluorescence intensities along the scanned line. Fluo-3 fluorescence was transformed to $[Ca^{2+}]_i$ by a pseudo-ratio method: $[Ca^{2+}]_i = K_d \times (F/F_0)/[(Kd/[Ca^{2+}]_{i,rest}) + 1 - (F/F_0)]$;[21] where K_d is a dissociation constant for fluo-3, F is the fluorescence intensity, F_0 is the intensity at rest, and $[Ca^{2+}]_{i,rest}$ is the $[Ca^{2+}]_i$ at rest. The K_d and $[Ca^{2+}]_{i,rest}$ were assumed to be 1.1 μmol/L and 150 nmol/L, respectively.[22]

Ca^{2+} Signal Recording in Permeabilized Myocytes

Image acquisition for the quantitative analyses of caffeine-induced Ca^{2+} transients (CaffCaT) and Ca^{2+} sparks was done in the line-scan mode of LSCM. Fluo-3 fluorescence was transformed to $[Ca^{2+}]_i$ by a pseudo-ratio method in which K_d was assumed to be 400 nmol/L.[23] The SR Ca^{2+} content was evaluated by measuring the amplitude of the CaffCaT obtained after rapid application of 10 mmol/L caf-

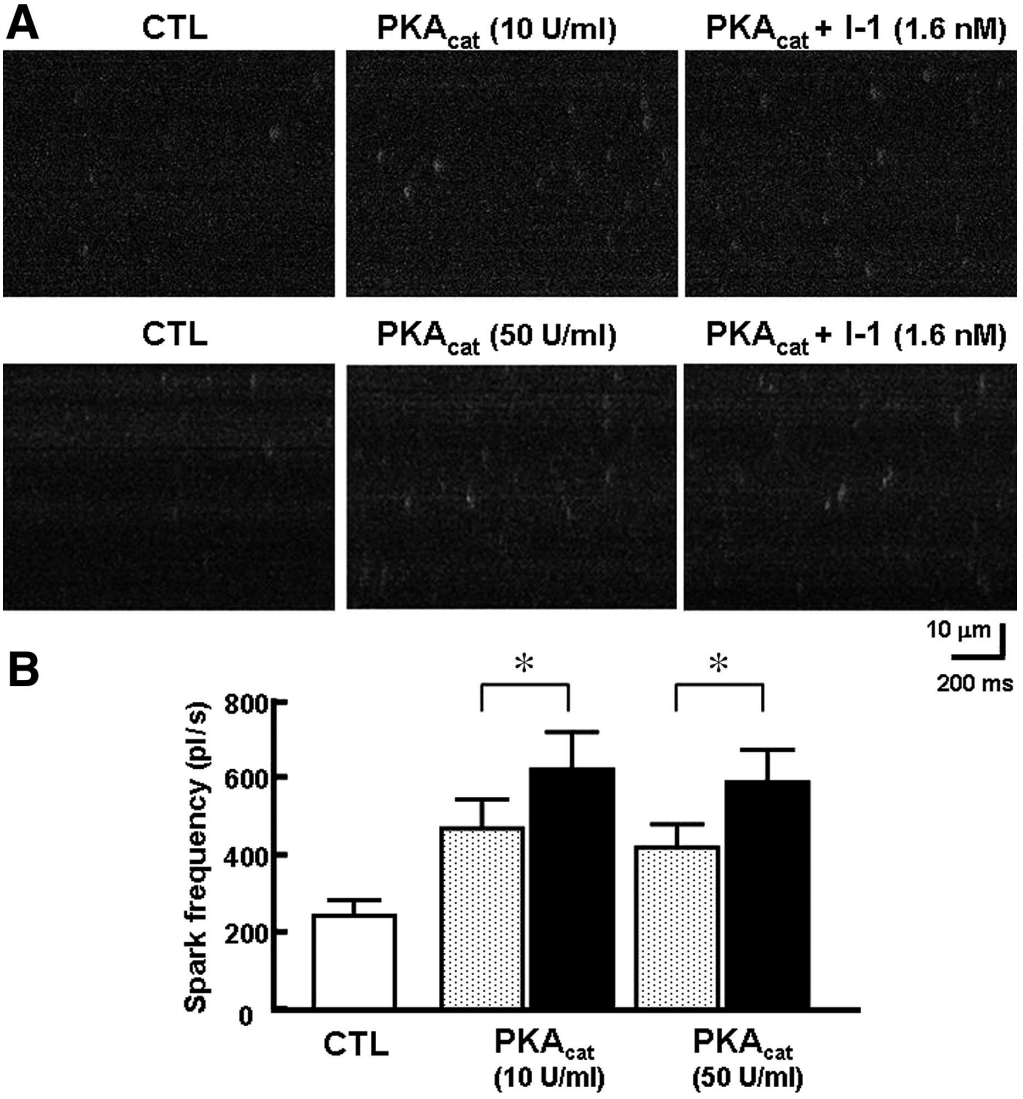

Figure 3. Effects of protein kinase A catalytic subunit (PKA_{cat}) and inhibitor-1 (I-1) on Ca^{2+} sparks in permeabilized cardiac myocytes. (**A**) Representative line scan images of Ca^{2+} sparks at 50 nmol/L $[Ca^{2+}]_c$. With the control perfusion (CTL), Ca^{2+} sparks occurred at a frequency of 235.3±37.7 pl/s. The perfusion of 10 U/ml PKA_{cat} (**Upper**) and 50 U/ml PKA_{cat} (**Lower**) significantly increased the Ca^{2+} spark frequency. The addition of I-1 further increased the Ca^{2+} spark frequency (PKA_{cat}+I-1). It is also evident that the Ca^{2+} sparks in 50 U/ml PKA_{cat} are larger than those in 10 U/ml PKA_{cat}. (**B**) Summary of the Ca^{2+} spark frequency. The perfusion of 10 U/ml PKA_{cat} significantly increased the Ca^{2+} spark frequency, but a higher dose (50 U/ml) did not increase it further (dotted columns). The addition of I-1 further increased the Ca^{2+} spark frequency, both at 10 U/ml and at 50 U/ml PKA_{cat} (solid columns). Data are mean±SEM. *P<0.05 vs PKA_{cat} only, by 2-way repeated ANOVA.

feine dissolved in a Na^+- and Ca^{2+}-free solution with 0.5 mmol/L EGTA. This solution was introduced into the chamber via a quick-switching device at a flow rate of 5–7 ml/min, resulting in a CaffCaT that peaked within 200 ms. Ca^{2+} sparks were detected visually and identified as local peak elevations of fluorescent intensity (F) that were 1.5-fold the surrounding background levels (F_0) and having a half amplitude that exceeded 8 ms. The number of Ca^{2+} sparks counted per line scan image was normalized spatially (per picoliter) and temporally (per s) as Ca^{2+} spark frequency (pl/s).[24]

Chemicals and Data Analyses

All chemicals were obtained from Sigma (St Louis, MO, USA) and fluorescent dyes were purchased from Molecular Probes (Eugene, OR, USA). Data are presented as means± SEM, and the number of cells or experiments is shown as n. Statistical analyses were performed using Student's t-test or repeated ANOVA. The differences in each parameter were compared using Scheffé's post-hoc test. The probability was considered significant at P<0.05.

Results

Effects of Isoproterenol (ISO) and Caly A on Twitch CaTs and Cell Shortening in Intact Cardiac Myocytes

In cardiac myocytes, PPs 1 and 2A de-phosphorylate the SR Ca^{2+} handling proteins and the L-type Ca^{2+} channels, thereby negatively controlling excitation–contraction (E-C) coupling. Initially, we examined whether a perfusion of

Caly A, a non-specific PP inhibitor, would modify the ISO-induced changes in twitch CaTs and cell shortening in intact cardiac myocytes. As shown in **Figure 1A**, ISO (10 nmol/L) increased the peak level of CaTs, decreased the diastolic $[Ca^{2+}]_i$, and shortened the decay time of $[Ca^{2+}]_i$. Following the addition of Caly A (100 nmol/L) for 2 min, the peak CaTs further increased. **Figures 1B–D** summarizes the effects of ISO and Caly A on CaTs. The changes in CaTs were associated with an increase in cell shortening (**Figure 1E**). However, Caly A by itself increased the peak of both CaTs and cell shortening, although it did not alter the decay rate of $[Ca^{2+}]_i$ or the resting $[Ca^{2+}]_i$ in the presence or absence of ISO.

These findings suggested that inhibition of PPs could augment the inotropic effects of ISO in cardiac myocytes without altering the decay rate of $[Ca^{2+}]_i$ or resting $[Ca^{2+}]_i$. Because the type 1 PP mainly de-phosphorylates PLB, PP inhibitors are expected to phosphorylate PLB, thereby activating SR Ca^{2+} uptake via SERCA. Therefore, the inotropic effect of Caly A is likely to be related to an additional increase in the SR Ca^{2+} loading.

Effects of PKA_{cat} and I-1 on SR Ca^{2+} Loading in Permeabilized Myocytes

Inhibition of PPs may also increase the Ca^{2+} influx via L-type Ca^{2+} channels. Furthermore, Caly A blocks both PP1 and PP2A, and may have non-specific effects on cellular Ca^{2+} regulation. To investigate the direct and selective effects of the PKA catalytic subunit (PKA_{cat}) and PP1 on the SR Ca^{2+} handling, we used myocytes permeabilized with saponin in the next series of experiments. **Figure 2A** shows representative recordings of CaffCaTs for the estimation of SR Ca^{2+} loading. The application of caffeine-derived Ca^{2+} from the SR, and a transient increase in $[Ca^{2+}]_i$ was observed. The exposure to I-1 (1.6 nmol/L) for 2 min had no effect on CaffCaT in the absence of PKA_{cat} (**Figure 2B**). After 5 min exposure to PKA_{cat} (10U/ml), the amplitude of CaffCaT increased and additional exposure to I-1 for 2 min further increased it. **Figure 2B** shows the dose-dependent effects of PKA_{cat} on the amplitude of CaffCaTs between 5U/ml and 50U/ml. After the addition of I-1, the amplitude of CaffCaT increased significantly, except at 50U/ml. The higher concentration of I-1 (3.2 nmol/L) exerted no additional augmentation of CaffCaT, when added to 5U/ml PKA_{cat} (**Figure 2C**).

These results suggest that inhibition of PP1 could amplify PKA_{cat}-dependent SR Ca^{2+} loading.

Effects of PKA_{cat} and I-1 on the Ca^{2+} Sparks

SR Ca^{2+} loading is determined by the balance between Ca^{2+} uptake by SERCA and the Ca^{2+} release (leak) from RyRs. To investigate the effects of PKA and I-1 on the properties of SR Ca^{2+} release, we observed the frequency and individual characteristics of Ca^{2+} sparks in permeabilized myocytes. **Figure 3A** shows representative line scan images of Ca^{2+} sparks. Under the control perfusion, the Ca^{2+} sparks were observed at the frequency of 235.3±37.7 pl/s (**Figure 3B**). Perfusion of 10 U/ml PKA_{cat} significantly increased the frequency of Ca^{2+} sparks. The application of I-1 (1.6 nmol/L) further increased the Ca^{2+} spark frequency, whereas a higher dose of PKA_{cat} (50 U/ml) did not. **Table** lists the amplitude and full duration of the half maximum (FDHM) of individual Ca^{2+} sparks. The amplitude of individual Ca^{2+} sparks did not increase at 10 U/ml PKA_{cat} but increased significantly at 50 U/ml PKA_{cat}. The FDHM of individual Ca^{2+} sparks shortened at 10 U/ml PKA_{cat}, but was prolonged at 50 U/ml PKA_{cat}. The addition of I-1 to 10 U/ml PKA_{cat} did not modify either the amplitude or FDHM of individual Ca^{2+} sparks.

Table. Effects of PKA_{cat} and I-1 on the Characteristics of Individual Ca^{2+} Sparks

	n	Amplitude (nmol/L)	FDHM (ms)
Control	70	60.6±7.7	25.8±14.9
10 U/ml PKA_{cat}	56	66.5±11.0	17.5±7.9*
+I-1	48	60.6±11.0	17.6±9.3*
50 U/ml PKA_{cat}	42	93.8±16.7*	32.0±9.0*
+I-1	52	95.5±14.3*	30.3±8.4*

The amplitude of individual Ca^{2+} sparks did not increase at 10 U/ml PKA_{cat} but increased significantly at 50 U/ml PKA_{cat}. The FDHM of individual Ca^{2+} sparks shortened at 10 U/ml PKA_{cat}, but was prolonged at 50 U/ml PKA_{cat}. The addition of I-1 to 10 U/ml PKA_{cat} and 50 U/ml did not modify either the amplitude or FDHM of individual Ca^{2+} sparks. Data are shown as mean± SEM.

*P<0.01 vs CTL by ANOVA followed by Scheffé's post-hoc test.

PKA_{cat}, protein kinase A catalytic subunit; I-1, inhibitor-1; FDHM, full duration of the half maximum.

This result suggests that the addition of I-1 to 10 U/ml PKA_{cat} might only increase SR Ca^{2+} loading, whereas the higher dose of PKA_{cat} (50 U/ml) might also modify Ca^{2+} gating of RyRs, thereby altering the characteristics of individual Ca^{2+} sparks.

Effect of I-1 on the Ca^{2+} Spark Frequency After Abrupt Inhibition of SR Ca^{2+} Uptake

Finally, to verify that I-1 did not modify the Ca^{2+} gating of RyRs, we observed Ca^{2+} spark frequency and CaffCaT after abrupt inhibition of SR Ca^{2+} uptake, which was achieved by exposing permeabilized cells to cyclopiazonic acid (CPA: 10 μmol/L), an irreversible inhibitor of SERCA. **Figure 4A** shows that the perfusion of PKA_{cat} increased the Ca^{2+} spark frequency, and after exposure to CPA, the Ca^{2+} spark frequency decreased gradually in accordance with the decline in CaffCaT (**Figure 4B**); Ca^{2+} sparks disappeared at approximately 140s. If I-1 directly enhanced SR Ca^{2+} release, then abrupt inhibition of SR Ca^{2+} uptake might result in rapid decay of both Ca^{2+} spark frequency and CaffCaT because of SR Ca^{2+} depletion. Actually, the perfusion of ryanodine (500 nmol/L, 2 min), which increases the open probability of RyRs;[25] transiently increased the Ca^{2+} spark frequency, and the Ca^{2+} spark frequency rapidly decreased and disappeared up til 60 s after the exposure to CPA (time constant: 40.2±7.6 s in PKA_{cat} and 27.3±3.0 s in PKA_{cat}+ryanodine, P<0.05). However, there was no difference in the rate of decay of the Ca^{2+} spark frequency (time constant: 31.4±8.7 s in PKA_{cat}+I-1, P=NS vs PKA_{cat}) or CaffCaT (**Figure 4B**). Furthermore, the relationship between Ca^{2+} spark frequency and CaffCaT did not differ in the presence or absence of I-1 (**Figure 4C**). Because the direct activation of Ca^{2+} gating of RyRs is expected to increase the Ca^{2+} spark frequency at a comparable SR Ca^{2+} loading, this finding also suggests that I-1 did not have a direct effect on SR Ca^{2+} release.

Discussion

Caly A augmented the inotropic effect of ISO without altering the decay rate of $[Ca^{2+}]_i$ or resting $[Ca^{2+}]_i$ in intact cardiac myocytes, although by itself it modified CaTs and cell shortening. I-1 had an amplifying effect on the PKA_{cat}-

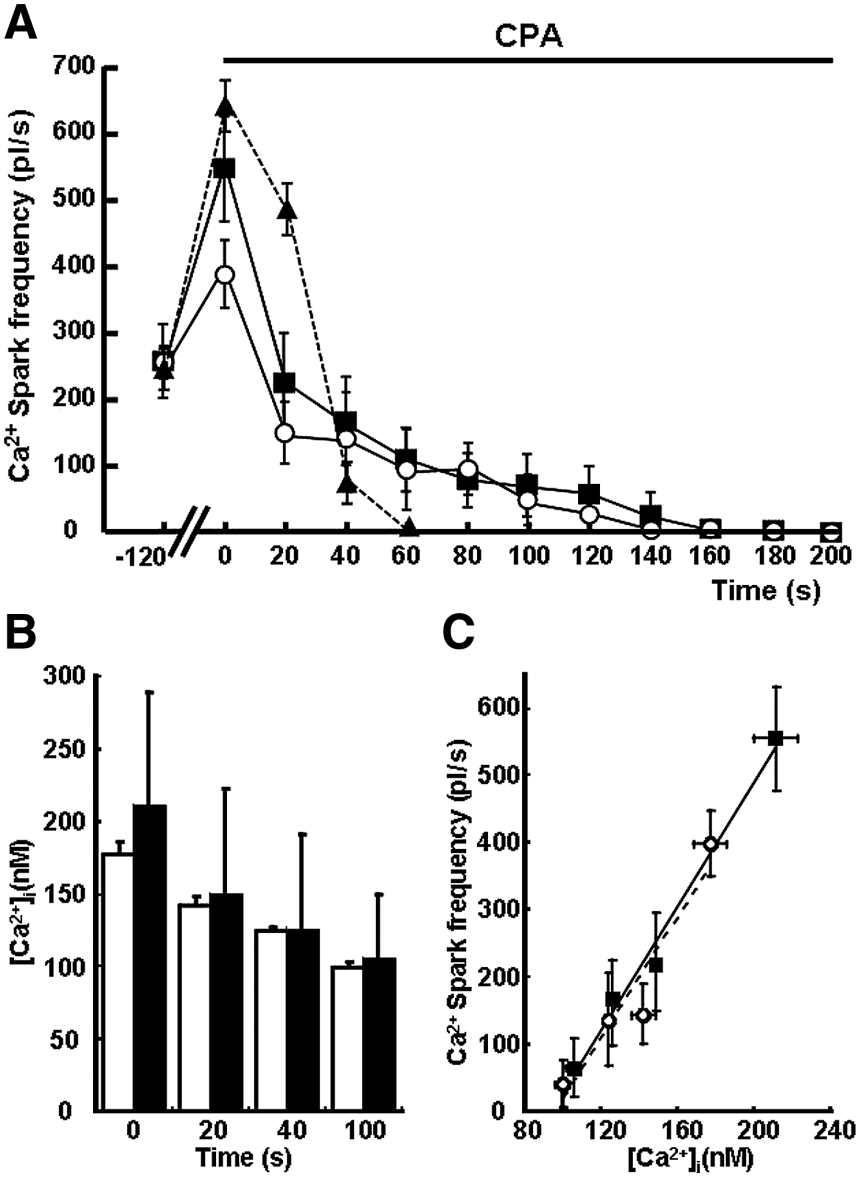

Figure 4. Effect of inhibitor-1 (I-1) on Ca^{2+} spark frequency and caffeine-induced Ca^{2+} transient (CaffCaT) after abrupt inhibition of the sarcoplasmic reticulum Ca^{2+} uptake. (**A**) After the initial increase in the Ca^{2+} spark frequency by protein kinase A catalytic subunit (PKA_{cat}) (10 U/ml), exposure to cyclopiazonic acid (CPA) (bar) caused a gradual decrease in the Ca^{2+} spark frequency, in both the absence (open circles) and presence (solid squares) of I-1. There was no difference in the decay rate of Ca^{2+} spark frequency. These time courses were very different from that in the presence of ryanodine (500 nmol/L), where the Ca^{2+} spark frequency transiently increased and then rapidly decreased and disappeared up to 60 s (filled triangles and broken line). (**B**) Change in CaffCaTs before (0 s) and 20, 40, and 100 s after exposure to CPA. The time-dependent decline in CaffCaTs was similar in the absence (open columns) or in the presence (solid columns) of I-1. (**C**) Relationship between Ca^{2+} spark frequency and CaffCaT before (0 s), and at 20, 40, and 100 s after exposure to CPA. The regression line was similar in the absence (broken line) or presence (straight line) of I-1. Data are mean± SEM.

induced increases in SR Ca^{2+} loading and in the Ca^{2+} spark frequency without direct modification of SR Ca^{2+} release in permeabilized myocytes. We have clarified, for the first time, the direct effects of PKA_{cat} and I-1 on SR Ca^{2+} handling and our findings suggest the possibility of specific inhibition of PP1 to improve cellular Ca^{2+} handling in heart failure.

Inhibition of PPs and E-C Coupling in Intact Myocytes

Inhibition of PPs has various effects on cardiac myocytes, including modification of the response to β-adrenergic receptor stimulation and adenosine receptor stimulation[26] Regarding E-C coupling cascade, the inhibition of PPs augments phosphorylation of PLB, RyRs and L-type Ca^{2+} channels, resulting in increases in SR Ca^{2+} uptake, SR Ca^{2+} release, and trans-sarcolemmal Ca^{2+} influx![4,15,27] In this study, ISO increased the peak of CaTs and twitch cell shortening, decreased the diastolic $[Ca^{2+}]_i$ and hastened $[Ca^{2+}]_i$ decay. Additional perfusion of Caly A at 100 nmol/L further increased the peak of CaTs and twitch cell shortening without altering the decay rate of $[Ca^{2+}]_i$ or resting $[Ca^{2+}]_i$. Caly A at this concentration has been shown to inhibit PP1 and PP2A by 50% and 85%, respectively![5] Taken together, the results suggest that inhibition of PPs could augment ISO-induced inotropic effects without altering the diastolic $[Ca^{2+}]_i$. However, in the present study, Caly A by itself increased the peak of CaTs and cell shortening. Direct modification of Ca^{2+} regulation may be a non-specific effect of Caly A on Ca^{2+} handling proteins, but in intact myocytes that were electrically stimulated, the proteins involved in E-C coupling were expected to be certainly phosphorylated by other protein kinases (eg, CaMKII).

Measurement of CaffCaT and Ca^{2+} Sparks in Permeabilized Cardiac Myocytes

To investigate the selective effect of PP1 inhibition on SR Ca^{2+} handling, we observed the effects of PKA_{cat} and I-1 on SR Ca^{2+} loading and SR Ca^{2+} sparks in saponin-permeabilized cardiac myocytes. Because the permeabilization of the cell membrane causes loss of cytosolic signaling messengers and proteins, it enabled us to precisely control the internal solution and to examine direct modification of SR Ca^{2+} handling proteins. It is well known that PPs are

anchored to RyRs and PLB by anchoring-proteins, and are not lost from the SR by permeabilization.[28]

The concentrations of PKA_{cat} in this study were within the ranges reported previously,[28,29] and we used I-1 at a concentration of 1.6 nmol/L, which was equivalent to the IC_{50} for PPs.[30] A higher concentration of I-1 (3.2 nmol/L) did not increase CaffCaT further (**Figure 2C**).

Effects of PKA_{cat} and I-1 on SR Ca^{2+} Loading

In permeabilized myocytes, in which endogenous phosphorylation of PLB might be lost, perfusion of PKA_{cat} increased SR Ca^{2+} loading dose-dependently, indicating phosphorylation of PLB and activation of SERCA. Following the addition of I-1 to 5 or 10 U/ml PKA_{cat} increased the SR Ca^{2+} loading further, but did not affect SR Ca^{2+} loading when added alone or to 50 U/ml PKA_{cat}. Thus, the effects of I-1 on SR Ca^{2+} loading may differ according to the level of PKA-induced phosphorylation of the SR proteins. One reason why I-1 alone did not affect SR Ca^{2+} loading may be that it has to be phosphorylated by PKA at the phosphorylation site, Thr-35, in order to be in its active form.[16] Another reason may be that the endogenous phosphorylation of PLB might be lost when cells are permeabilized. On the other hand, at the higher (50 U/ml) concentration of PKA_{cat}, SR Ca^{2+} loading may have been near maximum, and the additional effect of I-1 might have been obscured.

Effects of PKA_{cat} and I-1 on SR Ca^{2+} Release

The RyR that controls the SR Ca^{2+} release is regulated by stimuli including $[Ca^{2+}]_i$, SR Ca^{2+} loading, pH_i, PKA, protein kinase C, and CaMKII.[3] In lipid bilayer studies, direct phosphorylation at Ser-2809 on the RyRs by PKA increased the open probability of the RyRs or changed the sensitivity to Ca^{2+} in the cleft.[9] To investigate the effects of PKA_{cat} and I-1 on the properties of SR Ca^{2+} release, we studied the frequency and characteristics of Ca^{2+} sparks, which reflect synchronous activation of a cluster of 6–20 RyRs, producing both diastolic SR Ca^{2+} leak and temporally synchronized SR Ca^{2+} release during E-C coupling.[21,24] In this study, 10 and 50 U/ml PKA_{cat} significantly increased the Ca^{2+} spark frequency and the addition of I-1 further increased it. As mentioned earlier, because the activity of RyR is largely influenced by SR Ca^{2+} loading, it is uncertain whether PKA_{cat} and I-1 directly modified the RyR gating and so we analyzed the characteristics of individual Ca^{2+} sparks. The amplitude of the Ca^{2+} sparks did not increase at 10 U/ml PKA_{cat}, but increased significantly at 50 U/ml. The FDHM of the Ca^{2+} sparks shortened significantly at 10 U/ml PKA_{cat}, whereas it was prolonged at 50 U/ml PKA_{cat}. Although we did not compare the level of phosphorylation, the larger and longer Ca^{2+} sparks at 50 U/ml PKA_{cat} may indicate altered RyR gating because of the excessive phosphorylation of RyRs.[31] In fact, Shorofsky et al demonstrated 'big sparks' in spontaneously hypertensive rat myocytes, which may be caused by an alteration in the micro-domain between L-type Ca^{2+} channels and RyRs.[32] The shortening of the FDHM of the Ca^{2+} sparks at 10 U/ml PKA_{cat} may also imply acceleration of the SR Ca^{2+} uptake by SERCA.[33]

On the other hand, the addition of I-1 to 10 U/ml and 50 U/ml PKA_{cat} did not alter either the amplitude or FDHM of individual Ca^{2+} sparks. Previous studies suggested that both an increase in SR Ca^{2+} loading and modification of RyRs could increase the amplitude and FDHM of individual Ca^{2+} sparks.[3,34,35] The reason for the unchanged characteristics of individual Ca^{2+} sparks in the present study is uncertain, but may be related to the low $[Ca^{2+}]_c$ and/or smaller increases in SR Ca^{2+} loading as compared with the other studies. Thus, I-1 could augment PKA-induced activation of SR Ca^{2+} uptake, with a less direct effect on SR Ca^{2+} release.

To confirm the lack of direct effects of I-1 on SR Ca^{2+} release, we investigated the time course of Ca^{2+} spark frequency and CaffCaT after abrupt inhibition of SR Ca^{2+} uptake. If I-1 directly accelerated RyR gating, the Ca^{2+} spark frequency would initially increase, even with unchanged SR Ca^{2+} loading, and then rapidly reach baseline because of depletion of the SR Ca^{2+} content.[21,25] These changes were observed when a low concentration of ryanodine was perfused (**Figure 4A**), but there was no difference in the decay rate of the Ca^{2+} spark frequency or CaffCaT in the presence or absence of I-1. Furthermore, the relationship between Ca^{2+} spark frequency and CaffCaT did not differ in the presence or absence of I-1. These findings support the supposition that I-1 does not directly modify SR Ca^{2+} release. The mechanism for the selective augmentation of I-1 on PKA_{cat}-activated SR Ca^{2+} loading is unknown, but could be explained by differences in the expression of PP1 and in the extent of inhibition of PP1 by PLB and RyRs.[36]

Physiological and Pathophysiological Implications

In heart failure, although hyper-activation of the β-adrenergic system enhances cardiac output in the short term, it may be cardiotoxic over the long term.[37–39] The chronic stimulation of β-adrenergic receptors would result in downregulation of the receptors themselves, and cause uncoupling from G-protein.[40] In addition, sustained increase in $[Ca^{2+}]_i$ as a result of increased Ca^{2+} influx via the L-type Ca^{2+} channels would be detrimental to cardiomyocytes because of increased energy consumption, fatal arrhythmias and diastolic dysfunction.[3] Therefore, an alternative strategy is needed to improve both systolic and diastolic function with less cardiac toxicity. Agents that enhance SR Ca^{2+} uptake in a cAMP-independent manner may be ideal for improving the SR Ca^{2+} handling in heart failure.[41] Accordingly, the expression of constitutively active I-1 or other specific inhibitors of PP1 may be an approach to altering SR Ca^{2+} cycling to improve depressed cardiac function and prevent the mal-adaptation of the β-adrenergic system in heart failure.

Conclusion

Inhibition of PPs augments the ISO-induced inotropic effect in intact myocytes, and selective inhibition of I-1 amplifies SR Ca^{2+} loading without directly altering SR Ca^{2+} release in permeabilized cells. Selective PP1 inhibition could enhance PLB phosphorylation, resulting in accelerated SR Ca^{2+} uptake without altering RyR gating. We described 1 of the mechanisms for the beneficial effects of PP1 inhibition reported in the treatment of failing hearts.[7] Although the long-term benefit of PP1 inhibition remains undetermined,[36] this strategy holds promise as a therapeutic agent in heart failure.

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