Multifunctional Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase Mediates Ca\(^{2+}\)-Induced Enhancement of the L-type Ca\(^{2+}\) Current in Rabbit Ventricular Myocytes

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Abstract The intracellular mechanism underlying the Ca\(^{2+}\)-induced enhancement of the L-type Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) was examined in adult rabbit cardiac ventricular myocytes by using patch-clamp methodology. Internal Ca\(^{2+}\) was elevated by flash photolysis of the Ca\(^{2+}\) chelator Nitr 5, and intracellular Ca\(^{2+}\) levels were simultaneously monitored by Fluo 3 fluorescence. Flash photolysis of Nitr 5 produced a rapid (<1-second) elevation of internal Ca\(^{2+}\), which led to enhancement (39% to 51% above control) of the peak inward Ca\(^{2+}\) current after a delay of 20 to 120 seconds. Internal dialysis of myocytes with synthetic inhibitory peptides derived from the pseudosubstrate (peptide 273-302) and calmodulin binding (peptide 291-317) regions within the regulatory domain of multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaM kinase) blocked enhancement of I\(_{\text{Ca}}\) produced by elevation of internal Ca\(^{2+}\) but not that produced by \(\beta\)-adrenergic stimulation. These inhibitory peptides also had no effect on the elevation of internal Ca\(^{2+}\) produced by flash photolysis of Nitr 5. A pseudosubstrate inhibitory peptide derived from protein kinase C had no significant effect on Ca\(^{2+}\)-dependent enhancement of I\(_{\text{Ca}}\). We conclude that CaM kinase mediates the Ca\(^{2+}\)-induced enhancement of I\(_{\text{Ca}}\) in mammalian cardiac myocytes by a mechanism likely involving direct phosphorylation of the L-type Ca\(^{2+}\) channel complex or an associated regulatory protein. (Circ Res. 1994;75:854-861.)

Key Words • Ca\(^{2+}\)/calmodulin kinase • Ca\(^{2+}\) current • cardiac myocytes • Fluo 3 • Nitr 5

Ca\(^{2+}\) entry during the cardiac action potential serves to trigger Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) leading to myofilibrillar contraction and force generation (for review, see Reference 1). The inward Ca\(^{2+}\) current thus plays a critical role in excitation-contraction coupling, and factors that alter this influx would be anticipated to influence myocardial contractility. The cardiac L-type Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) is known to be modulated by a variety of agents, including hormones, neurotransmitters, and drugs (for review, see Reference 2). Studies primarily from the laboratories of Reuter, Trautwein, Tsien, and Sperelakis (for review, see Pelzer et al\(^2\) and Hartzell\(^3\)) have shown that \(\beta\)-adrenergic stimulation of I\(_{\text{Ca}}\) occurs via the cAMP/protein kinase A-signaling cascade, leading to a phosphorylation-dependent increase in current magnitude. Similarly, recent findings have suggested that I\(_{\text{Ca}}\) in a number of cell types, including cardiac myocytes,\(^4\)\(^5\) may also be modulated via the protein kinase C (PKC)-signaling cascade,\(^6\) leading to increases in I\(_{\text{Ca}}\). However, the functional importance of this latter pathway in the extrinsic regulation of cardiac Ca\(^{2+}\) channel activity is less clear.

In the absence of external stimuli, changes in stimulation frequency alone are reported to alter Ca\(^{2+}\) current properties. This frequency-related regulation can involve both a decrease\(^7\)\(^10\) and/or increase\(^11\)\(^-\)\(^15\) of the macroscopic Ca\(^{2+}\) current. In both cases, the cellular mechanisms underlying each of these regulatory events are poorly understood. Enhancement, or facilitation, of I\(_{\text{Ca}}\) appears to occur indirectly, via a Ca\(^{2+}\)-mediated intermediate step, such as an enzymatic event.

Calmodulin is a ubiquitous intracellular Ca\(^{2+}\) (Ca\(_{\text{b}}\))-binding protein that mediates the Ca\(^{2+}\)-dependent activation of a number of enzymes and cellular processes (for review, see Reference 16). One such target enzyme is the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaM kinase), a ubiquitous multimeric enzyme complex comprising 8 to 12 catalytic subunits (for review, see Reference 17). The predominant cardiac isoforms of CaM kinase (\(\delta\) and \(\epsilon\)) have recently been cloned and expressed in COS cells\(^18\) and displayed strong similarities to the \(\alpha\) and \(\beta\) isoforms previously identified in the brain.\(^19\) Recent studies have implicated CaM kinase in the Ca\(^{2+}\)-dependent modulation of ion currents in a number of preparations\(^19\)\(^-\)\(^22\) as well as the intracellular Ca\(^{2+}\) release channels (ryanodine receptors) from cardiacc\(^23\) and skeletal\(^24\) muscles. We sought to examine whether CaM kinase plays a role in the Ca\(^{2+}\)-dependent enhancement of I\(_{\text{Ca}}\) observed in various cardiac preparations.\(^11\)\(^-\)\(^15\)\(^25\) The L-type Ca\(^{2+}\) current was examined in acutely isolated adult rabbit ventricular myocytes by using whole-cell voltage-clamp methodology. For our studies, we chose to manipulate Ca\(_{\text{b}}\) by flash photolysis of the calcium chelator Nitr-5 rather than with increased stimulation frequency.\(^12\)\(^25\)\(^26\) In this

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way, voltage-dependent inactivation of ICa,L was minimized, and the kinetics of Ca2+ elevations could be more precisely controlled. Our findings indicate that inhibition of CaM kinase selectively blocks the Ca2+-mediated enhancement of ICa,L in cardiac myocytes without affecting the modulation of ICa,L via the β-adrenergic receptor/cAMP pathway.

Materials and Methods

Preparation of Single Ventricular Myocytes

Single ventricular myocytes were prepared according to the method of Fedida et al.27 Briefly, New Zealand White rabbits (2 to 3 kg body weight) were killed by pentobarbital (50 mg/kg IV) overdose. Hearts were rapidly excised and placed in ice-cold nominally Ca2+-free HEPES-buffered saline solution (see below). The aorta was cannulated, and the heart was perfused in a retrograde fashion with a nominally Ca2+-free perfusate for 20 minutes at 37°C. This was followed by a 15-minute perfusion with a collagenase-containing solution (see below) and finally by a 15-minute perfusion with a low-Ca2+ (0.2 mmol/L) solution. The left ventricle and septum were cut away, coarsely minced, and placed in a beaker containing low-Ca2+ solution with 1% (wt/vol) bovine serum albumin at 37°C. Myocytes were dispersed by gentle agitation, collected in serial aliquots, and then maintained in standard saline solution containing 1.8 mmol/L CaCl2.

Solutions

The standard Ca2+-containing saline solution was composed of (mmol/L) NaCl 135.0, HEPES (free acid) 1.0, NaH2PO4 0.33, glucose 10.0, KCl 5.4, MgCl2 1.0, and CaCl2 1.8. The nominally Ca2+-free solution was identical, except CaCl2 was omitted. The low-Ca2+ solution contained 0.2 mmol/L CaCl2. The collagenase solution was prepared in nominally Ca2+-free saline containing 60 U/mL collagenase (Worthington Biochemicals) and 0.1 U/mL type XIV protease (Sigma Chemical Co.). Solutions were adjusted to pH 7.4 with 10N NaOH.

The experimental bath solution contained (mmol/L) NaCl 120.0, CaCl2 2.5, KCl 4.0, MgCl2 0.5, CsCl 20.0, HEPES 10.0, and glucose 10.0; pH was adjusted to 7.4 with 10N NaOH. Tetrodotoxin (0.03 mmol/L) was also present in the majority of experiments. The standard intracellular pipette solution contained (mmol/L) CsCl2 120.0, tetraethyllumonium chloride (TEA) 10.0, MgATP 1.0, NaGTP 1.0, CaCl2 1.0, phosphocREATine 5.0, and HEPES 10.0; pH was adjusted to 7.2 with 1N CsOH. Some cells were dialyzed with a second set of intracellular solutions containing (mmol/L) Cs+ 130 to 145, Cl- 50 to 140, aspartate 0 to 105, TEA 10, Mg2+ 2 to 3, ATP 2 to 3, GTP 2, Ca2+ 1.25, and HEPES 10; pH was adjusted to 7.2 with 1N CsOH. Data from cells dialyzed with these solutions and the standard solutions were pooled since responses to Nitr 5 photolysis were not significantly different between the two groups. Unless otherwise noted, all chemicals were from Sigma.

Voltage Clamp

Isolated quiescent ventricular myocytes were studied with patch-clamp methodology in the whole-cell recording configuration28 by using an Axopatch 1D amplifier (Axon Instruments). Micropipettes were pulled from glass capillary tubing (outer diameter, 1.5 mm; inner diameter, 1.1 mm; Clark Electromedical Instruments) and heat-polished to a tip resistance of 1.5 to 3.5 MΩ when filled with the intracellular solution (see above).

The cell membrane was depolarized every 10 seconds to 0 or +10 mV from a holding potential of ~40 mV, and transmembrane current was sampled at 5 kHz. A low stimulation rate was chosen to minimize the contribution of basal Ca2+ entry to enhancement of the Ca2+ current. Voltage-clamp protocols and data acquisition were performed using PCLAMP software (version 5.5, Axon Instruments) on a microcomputer (386/25 MHz, Santron Computer Inc) with a Labmaster A/D, D/A converter (Scientific Solutions). Analog data were also digitized (Neuro-corder) and stored on a videotape recorder. All experiments were performed at room temperature (20°C to 23°C) on the stage of a Diaphot inverted epifluorescence microscope (Nikon Corp).

Peptide Kinase Inhibitors

Synthetic peptides, derived from the autoregulatory domain of CaM kinase and PKC, were dialedyzed into myocytes via the recording micropipette. Two peptides, corresponding to the calmodulin-binding region (291-317) and the autoinhibitory region (273-302) of CaM kinase (see Fig 3), were used, along with a pseudosubstrate peptide (19-36) from PKC.29 Such peptides have been shown to selectively and potently (Ki = 2 μmol/L or less) block kinase activities both in vitro30-32 and in situ.19,21,26 A truncated peptide, CaMK (308-317), derived from the calmodulin-binding region, with no inhibitory activity was used as a control. Peptides were added to the intracellular pipette solution at the following final concentrations (μmol/L): CaM (273-302) 10, CaMK (291-317) 20, CaMK (308-317) 20, and PKC 19-36 50. Myocytes were dialyzed for 8 to 10 minutes in the whole-cell configuration before starting the experiment.

CaM kinase–derived peptides were prepared by using a solid-phase peptide synthesizer (Applied Biosystems) and purified by reverse-phase high-performance liquid chromatography, and sequences were confirmed by automated sequencing. The pseudosubstrate PKC inhibitory peptide (PKC 19-36, Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn) was purchased from Gibco BRL.

Intracellular Ca2+

The fluorescent Ca2+ indicator Fluo 3 (penta-ammonium salt, CalBiochem) was added to the pipette solution (0.3 mmol/L) and dialyzed into the cytoplasm. Fluo 3 is compatible in UV photolysis experiments with caged compounds (see below) because its excitation (490 nm) and emission (>510 nm) maxima are unaffected by UV wavelengths. A 100-W mercury vapor lamp was used to deliver excitation light using a 470- 490-nm low-pass filter (all filters and the dichroic mirror were from Nikon). Excitation light was reflected by a 510-nm dichroic mirror to reach the microscope stage. The resultant (>510-nm) epifluorescence passed through the dichroic mirror and barrier filter (520 to 560 nm) and was collected by a photomultiplier tube (Hamamatsu R 268). Photomultiplier output was filtered at 20 Hz with an eight-pole Bessel low-pass filter (Frequency Devices). It is important to note that in the recording chamber, the cell was tightly “windowed” by using an adjustable-field diaphragm placed proximal to the photomultiplier tube. This ensured that the cell was the primary source of the measured fluorescence and that stray fluorescence from other sources (ie, pipette tip) could be effectively excluded.

Resting Ca2+ in the various solutions used was estimated as the calculated concentration of free Ca2+ in the pipette solution (91 to 179 mmol/L). Multiple-equilibria calculations were performed by using commercially available software (CHELATOR, copyright 1992, Theo J.M. Schoenmakers, Department of Animal Physiology, University of Nijmegen, Toernooiveld, Netherlands).

Photolytic Ca2+ Release

Nitr 5 is a photosensitive BAPTA-based Ca2+ chelator with a high selectivity for Ca2+ (Kf for Ca2+, ~1.5×1011) over Mg2+ (Kf for Mg2+, ~8.5×107). Photolysis with UV light produces a 40-fold decrease in Ca2+ (Kf for Nitr 5, ~6.3×107) but leaves Mg2+ affinity unchanged (Kf for Mg2+, ~8.0×105). Photorelease of Ca2+ is rapid (3000/s) and produces an increase in free Ca2+ independent of other major electro-
lytes. The observed change in Fluo 3 fluorescence after Nitr 5 photolysis in dialyzed cells verified in situ Ca$^{2+}$ release. Nitr 5 (2.5 to 4.0 mmol/L) was included in all pipette solutions except for control experiments, in which 3 mmol/L BAPTA was substituted.

A commercially available xenon flash lamp (H. Rapp, Optoelektronik) was used to deliver one or two 250-kW flashes between successive voltage-clamp steps. Xenon arc lamp bulbs (Advanced Radiation Corp) produce a broad emission spectrum with a maximum near 440 nm. Flash lamp emission was filtered with a WG305 filter (Newport Corp) because wavelengths <300 nm were found to alter holding potentials in voltage-clamped cells. The flash lamp output was manually focused on the bath chamber before each experiment. Proper focusing was confirmed by using photosensitive paper. Flash lamp output did not result in significant photobleaching of the Fluo 3 fluorescence signal (see "Results").

To determine the efficiency of experimental Nitr 5 photolysis, successive flashes were performed on a large inexitable cell (Jurkat T lymphocyte) under the same whole-cell recording conditions as used for myocytes. Maximal Fluo 3 fluorescence was achieved after three or four flashes, indicating no further Ca$^{2+}$ release with Nitr 5 photolysis. Subsequent exposure of the cell to the Ca$^{2+}$ ionophore ionomycin produced a large increase in fluorescence, confirming that the Fluo 3 itself was not saturated after the series of flashes. Similar experiments in myocytes proved uninterpretable, since ionomycin exposure led to hypercontracture and increases in fluorescence associated with cell-shape changes. On the basis of the maximal release of free Ca$^{2+}$ from Nitr 5 observed after three or four flashes, we estimate that a single flash produces photolysis of ~30% of the total Ca$^{2+}$-bound Nitr 5 pool. The paired flash protocol used under whole-cell recording conditions is thus expected to cause photolysis of 50% to 60% of the Ca$^{2+}$-bound Nitr 5, similar to earlier observations by Gurney et al.

**Cell Viability and Data Analysis**

Augmentation of IC$_{a}$ by isoproterenol was used to establish overall cell viability/responsiveness. Such a test was implemented because it was found that only a portion (~30%) of cells exhibited augmentation of IC$_{a}$ after photolytic release of internal Ca$^{2+}$. All cells studied were challenged with L-isoproterenol (final concentration, 10 µmol/L; Sigma) after flash photolysis. Cells were included in the data analysis if they demonstrated augmentation responses to either photolytic Ca$^{2+}$ release or isoproterenol. The threshold for augmentation response was defined as an increase of 5% above the prestimulus peak inward Ca$^{2+}$ current. Peak Ca$^{2+}$ current was measured by using PCLAMP software as the difference between peak inward current and the steady-state current at the holding potential.

Data are presented as mean±SEM. Statistical significance was assessed by one-way ANOVA.

**Results**

After establishment of the whole-cell recording configuration, dialysis of the cell interior with the fluorescent Ca$^{2+}$ indicator dye Fluo 3 and the photolabile Ca$^{2+}$ chelator Nitr 5 was performed for 8 to 10 minutes, over which time IC$_{a}$ magnitude reached a stable level. Transient increases in Fluo 3 fluorescence were observed with each voltage-clamp step from ~40 to 0 mV delivered at 10-second intervals (Fig 1), likely as a result of both inward Ca$^{2+}$ current and Ca$^{2+}$-induced Ca$^{2+}$ release from the SR. Voltage-clamp steps producing Ca$_{in}$ transients did not lead to visually detectable contractions, likely because of the significant buffering of cytosolic Ca$^{2+}$ by Nitr 5 and Fluo 3. After a brief flash discharge from a xenon lamp focused on the cell, an immediate step increase in the level of free Ca$_{i}$ was observed, as judged by Fluo 3 fluorescence (Fig 1, bottom). The fact that this jump in Ca$_{i}$ did not obscure the depolarization-induced Ca$^{2+}$ transients demonstrates that the flash did not lead to Ca$^{2+}$ saturation of the Fluo 3 fluorescence. Importantly, the clear increase in the magnitude of Ca$^{2+}$ transients after the flash further suggests that the observed rise in Fluo 3 fluorescence primarily reflects an elevation of Ca$_{i}$. Furthermore, myocytes still retained their normal rod-shaped appearance after flash-induced elevations of Ca$_{i}$ and continued to do so until the end of the experiment. During voltage-clamp steps, significant cell shortening was not routinely observed. However, this is the sum of spot observations made on a small number of cells and was not made immediately after the flash.

In the example shown, the steady-state Fluo 3 signal remains elevated; however, in a number of cells demonstrating enhancement of IC$_{a}$ after a flash, the Fluo 3 signal decayed over time as IC$_{a}$ was increasing. Such differences may reflect a balance between the rates of Ca$^{2+}$ removal by the cell and dialysis of photo-released free Ca$^{2+}$ into the cytosol from the pipette tip. Flashing cells in which the recording pipette was left in the cell-attached configuration (ie, Nitr 5 and Fluo 3 present only in the tip and not in the cytosol) produced <10% of the change in steady-state fluorescence typically measured from cells in the whole-cell recording mode (ie, Nitr 5 and Fluo 3 present in the cytosol). Therefore, such an experiment rules out the possibility that the step increase in steady-state fluorescence shown in the lower panel of Fig 1 is the result of stray fluorescence arising from Fluo 3 in the pipette tip, which was omitted using an adjustable diaphragm (see "Materials and Methods"). The somewhat slow decay of the individual Fluo 3 transients likely reflects the significant degree of Ca$^{2+}$ buffering by intracellular Fluo 3 and Nitr 5.
After a short but variable delay (20 to 120 seconds), an increase in the magnitude of peak inward current was also observed (Fig 1, top). In five cells, the average increase in peak $I_{Ca}$ was 51.4±20.9% above preflash levels. This increase was completely dependent on the flash-released Ca$^{2+}$, since flash discharges had no effect in myocytes in which Nitr 5 was replaced by equimolar BAPTA (see Fig 6).

Fig 2 shows the current-voltage relation of the peak inward current before and after enhancement; an overall increase in peak $I_{Ca}$ was observed with no apparent shift in the voltage dependence of activation. This effect was observed in four of four cells in which current-voltage relations were measured. That the observed increases in peak inward current primarily reflect changes in $I_{Ca}$ is based on (1) voltage-clamp and ionic conditions that were chosen to isolate the inward Ca$^{2+}$ current (see “Materials and Methods”) and (2) the close similarity of the observed current-voltage relation to that for the rabbit $I_{Ca}$ previously reported under similar recording conditions. However, we considered the possibility that changes in other inward currents (ie, Na$^+$ current [$I_{Na}$]) may still be contributing to the observed increase in total inward current after flash-dependent elevation of $I_{Ca}$. However, the following points argue against such an involvement of $I_{Na}$: (1) Myocytes were voltage-clamped at a holding potential of −40 mV to provide steady-state inactivation of $I_{Na}$. (2) There was not a significant leftward shift observed in the peak of the current-voltage relation after flash-induced enhancement of inward current. (3) The presence of 30 μmol/L external tetrodotoxin had no effect on the enhancement of inward current after flash-dependent elevation of $I_{Ca}$ (data not shown). Furthermore, low concentrations of the Ca$^{2+}$ channel blockers cadmium (100 μmol/L) (Fig 2) or D600 (10 μmol/L) (data not shown) in the bath completely abolished all inward current after enhancement by elevated $C_{Ca}$. Taken together, these findings strongly suggest that the observed enhancement of inward current by elevated $C_{Ca}$ is due primarily to an increase in $I_{Ca}$ alone. As seen in Fig 1, the increases in $I_{Ca}$ and free Ca$^{2+}$ responses showed dissimilar time courses after a flash discharge. Free Ca$^{2+}$ was observed to peak immediately after a flash discharge, whereas $I_{Ca}$ typically began to increase only after a noticeable lag. This apparent lack of coordination between these two responses suggested that Ca$^{2+}$ does not act directly to enhance $I_{Ca}$ but rather that Ca$^{2+}$ may activate an intermediate step that leads to the observed enhancement. To examine whether CaM kinase may be involved in this Ca$^{2+}$-induced enhancement of $I_{Ca}$, myocytes were dialyzed via the recording micropipette with synthetic inhibitory peptides modeled after functionally defined regions within the regulatory domain of CaM kinase (Fig 3). CaMK (291-317) is derived from the calmodulin-binding site and inhibits by preventing the interaction of activated calmodulin with any of its endogenous substrates, including CaM kinase. CaMK (273-302) acts as a pseudosubstrate inhibitor and binds to the catalytic site of CaM kinase, thereby preventing any interaction with native substrate proteins. Thus, both peptides have distinct inhibitory mechanisms in blocking the activity of endogenous CaM kinase.

Peptides derived from this regulatory domain have been shown previously to block CaM kinase activity in vitro, as well as the modulation of ion currents by CaM kinase in single-cell or multicellular preparations. To verify the specificity of these inhibitory peptides in situ, stimulation of $I_{Ca}$ by the β-adrenergic receptor agonist i-isoproterenol was examined at the end of each protocol. As shown in Fig 4, dialysis of myocytes with either the CaM kinase inhibitory peptide (291-317) (Fig 4A) or (273-302) (Fig 4B) completely prevented enhancement of peak inward $I_{Ca}$ after flash photolysis of intracellular Nitr 5. Two important aspects arise from these observations. First, these results demonstrate a critical role for endogenous CaM kinase in mediating the enhance-
ment of I_Ca by elevated C_a. Second, the role of calmodulin in the modulation of I_Ca may be primarily via CaM kinase, since blocking the effects of activated calmodulin by CaMK (291-317) is equivalent to the more specific blockade of CaM kinase by CaMK (273-302). Calmodulin is the only known endogenous activator of CaM kinase. Importantly, dialysis of myocytes with a truncated control peptide, CaMK (308-317), also derived from the autoregulatory domain of CaM kinase (see Fig 3), did not interfere with the enhancement of I_Ca after flash-dependent elevation of C_a (Fig 4C). This truncated peptide does not inhibit CaM kinase activity in vitro. Thus, only synthetic peptides capable of blocking CaM kinase activity produced functional effects in intact myocytes. In addition, we have examined the effects of the nonpeptide calmodulin inhibitor calmidazolium⁴⁹ on C_a²⁺-induced enhancement of I_Ca. Bath exposure of the cells to the calmodulin inhibitor calmidazolium (5 μmol/L) itself was found to produce an irreversible decrease in I_Ca magnitude (data not shown). Klockner and Isenberg⁴⁰ have previously reported similar effects of calmidazolium on I_Ca in guinea pig ventricular myocytes, with an IC₅₀ value of ≈1 μmol/L. More recently, Nakazawa et al⁴¹ have shown similar potent inhibitory effects of calmidazolium on both I_Ca and radiolabeled dihydropyridine (ie, [³H]nimodipine) binding in rat vascular myocytes.

The data in Fig 4 also demonstrate the specificity of the CaM kinase inhibitory peptides. After failure of elevated C_a to produce enhancement of I_Ca in myocytes dialyzed with either CaMK (291-317) or CaMK (273-302), exposure to L-isoproterenol still produced clear increases in peak I_Ca. The β-adrenergic stimulation of I_Ca was 22.0±5.8% (n=11) above control levels in cells dialyzed with CaMK (291-317) compared with only 8.0±2.7% (n=11) in cells dialyzed with the truncated control peptide CaMK (308-317). In agreement with Gurney et al,⁴⁵ isoproterenol and flash-induced increases in I_Ca were observed to be nonadditive (Fig 4C).

As a result of the simultaneous monitoring of C_a with Fluo 3, we were able to confirm that the CaM kinase inhibitory peptides did not interfere with the elevation of C_a via flash photolysis of Nitr 5. As shown in Fig 5, there were no significant differences in the steady-state levels of C_a after flash release of the caged C_a²⁺ in myocytes dialyzed with no peptides, the truncated control peptide, or the true inhibitory peptides. These observations thus demonstrate that the inhibitory peptides CaMK (291-317) and CaMK (273-302) block CaM kinase activation by elevating C_a and not by preventing the necessary rise in C_a to cause activation of CaM kinase. In a related study, dialysis of smooth muscle myocytes with similar CaM kinase inhibitory peptides had no effect on increases in C_a monitored by fura 2.²⁶

In Fig 4B, the steady-state elevation of C_a produced a modest decrease in peak I_Ca, possibly reflecting inactivation of I_Ca by CaMK.⁴⁰ Overall, the magnitude of postflash I_Ca inactivation was 7.1±1.8% of the preflash peak I_Ca and was not significantly different between experimental groups. Lack of notable postflash inactivation of peak I_Ca may be because elevations of C_a were small compared with those found in other studies.⁸

It has also been reported⁴⁻⁵ that PKC may also modulate L-type C_a²⁺ channel activity in rat neonatal cardiac myocytes, on the basis of observed effects of
Fig 5. Effects of Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaM kinase)–derived peptides on steady-state Fluo 3 fluorescence. Histogram shows the percent increases in Fluo 3 fluorescence after flash photolysis of internal Nitr 5 in myocytes dialyzed without CaM kinase–derived peptides (control), with 20 μmol/L CaMK (308-317), with 20 μmol/L CaMK (291-317), with 10 μmol/L CaMK (273-302), and with 3 mmol/L Nitr 5 replaced by equimolar BAPTA (sham). The difference in the steady-state (ie, between voltage-clamp steps) Fluo 3 signal (output in millivolts) measured before and after flash photolysis of Nitr 5 was used to calculate the percent increases in fluorescence shown. The calculated resting internal Ca\(^{2+}\) ranged from 91 to 179 mmol/L, and maximal levels after flash photolysis of Nitr 5 were estimated to range from 290 to 500 mmol/L. The increases in intracellular Ca\(^{2+}\) after Nitr 5 photolysis were not different (P = .526) between groups dialyzed with Nitr 5. However, these increases were statistically different between cells dialyzed with Nitr 5 and the sham group of cells (P = .009).

Discussion

The findings described in the present study provide the first direct evidence that Ca\(^{2+}\)-induced enhancement of the cardiac L-type Ca\(^{2+}\) channel is mediated by an endogenous Ca\(^{2+}\)/calmodulin-dependent protein kinase, as originally proposed more than 10 years ago by Marban and Tsien.\(^1\) Our conclusion is supported by the observations that (1) Ca\(^{2+}\)-induced enhancement typically developed after a delay of tens of seconds after the elevation of internal Ca\(^{2+}\) and (2) inhibition of the Ca\(^{2+}\)/calmodulin-activated protein kinase, CaM kinase, by synthetic peptides, which block the catalytic site [CaMK (273-302)] and prevent calmodulin binding [CaMK (291-317)], blocked the enhancement of I\(_{\text{Ca}}\) by flash-induced elevation of Ca. These findings are in agreement with a recent study by McCarron et al\(^2\) in which CaM kinase inhibitory peptides were used to demonstrate that CaM kinase also mediates the Ca\(^{2+}\)-induced enhancement of I\(_{\text{Ca}}\) in toad smooth muscle cells. The delay in the onset of enhancement observed in the present study and others\(^15,26,42\) is thus consistent with a mechanism in which Ca\(^{2+}\) acts via an intermediate phosphorylation step. At the single-channel level, Armstrong et al\(^43\) have reported that direct application of CaM kinase, as well as the calytic subunit of protein kinase A, can shift the channel to a mode of long open-time durations. Taken together, such observations in both amphibians and mammals suggest that CaM kinase may be the common mediator of Ca\(^{2+}\)-induced enhancement of I\(_{\text{Ca}}\), in agreement with its ubiquitous distribution among tissues and species. Yet recently, Bates and Gurney\(^44\) have concluded that Ca\(^{2+}\)-induced enhancement in guinea pig myocytes may not involve a phosphorylation event, on the basis of the ineffectiveness of internal dialysis with the ATP analogue AMP-
PMP and the kinase inhibitor H-7 to block Ca\(^{2+}\)-induced enhancement of \(I_{Ca}\). However, in their experiments, such manipulations did not cause complete inhibition of the \(\beta\)-adrenergic stimulation of \(I_{Ca}\), which presumably occurs primarily via the cAMP-dependent protein kinase. Given this circumstance, we believe that the data of Bates and Gurney should be interpreted cautiously when attempting to extrapolate these observations to the Ca\(^{2+}\)-dependent modulation of \(I_{Ca}\).

The effect of CaM kinase inhibitory peptides on Ca\(^{2+}\) current enhancement was found to be selective for CaM kinase, since these peptide inhibitors had no effect on the \(\beta\)-adrenergic (1-isoproterenol)-induced increase in \(I_{Ca}\). This observation is in agreement with biochemical data\(^{30}\) demonstrating no significant inhibition of the cAMP-dependent protein kinase by a CaM kinase inhibitory peptide similar to those used in our study. Biochemical evidence that CaM kinase can phosphorylate the \(\alpha\) and \(\beta\) subunits of the skeletal muscle dihydropyridine receptor/Ca\(^{2+}\) channel in vitro\(^{45,46}\) ties in with a proposed functional role for CaM kinase in the modulation of \(I_{Ca}\) based on electrophysiological findings. Such biochemical data, taken together with observations by us (see Fig 4C) and others\(^{15,47}\) that the Ca\(^{2+}\)-dependent and \(\beta\)-adrenergic–dependent enhancement of \(I_{Ca}\) are nonadditive, suggest that the Ca\(^{2+}\) channel complex may be the final common target for these two intracellular signaling pathways. Interestingly, we observed that a selective and potent peptide inhibitor against PKC had no significant effect on the Ca\(^{2+}\)-induced enhancement of \(I_{Ca}\), even though phorbol ester activation of PKC is reported to modulate Ca\(^{2+}\) channels in neonatal rat cardiac myocytes\(^{4,5}\) and other cell types.\(^{6,47}\) Likely, elevations in Ca\(_0\) alone may not be capable of activating the endogenous isoform of PKC that may mediate this effect, thus accounting for the observed ineffectiveness of the PKC (19-36) inhibitory peptide.

In our experiments, flash-induced elevations of Ca\(_0\) did not produce consistent inactivation or decreases in the magnitude of peak Ca\(^{2+}\) current, even in myocytes not responding to elevated Ca\(_0\) or in cells in which Ca\(^{2+}\)-induced enhancement was blocked by dialysis with CaM kinase inhibitory peptides. This observation is in agreement with an earlier suggestion\(^{11}\) and observations\(^{8,42}\) that Ca\(^{2+}\) current inactivation results from larger increases in Ca\(_0\) than required to produce Ca\(^{2+}\)-dependent enhancement of the current. The amount of internal Ca\(^{2+}\) buffering provided by Nitr 5 may have somewhat limited the magnitude of Ca\(^{2+}\) elevations, thus preventing elevations necessary to produce an increased rate of \(I_{Ca}\) inactivation.

Physiologically, Ca\(^{2+}\)-induced enhancement of \(I_{Ca}\) may participate in the regulation of cardiac excitation-contraction coupling, e.g., by augmenting trigger calcium for Ca\(^{2+}\)-induced Ca\(^{2+}\) release and contributing to the refilling of SR Ca\(^{2+}\) stores. Specifically, the positive force–stimulation frequency relation that is observed in a number of mammalian cardiac preparations, including rabbit,\(^{48}\) is one cellular process to which \(I_{Ca}\) enhancement may contribute. Of clinical importance, Marban and Tsien\(^{11}\) have suggested that Ca\(^{2+}\)-induced enhancement of \(I_{Ca}\) may in part underlie the positive inotropic effect of cardiotoxic steroids (ie, digitalis and strophanthidin) observed in mammalian preparations.

Whether Ca\(^{2+}\)-induced enhancement of \(I_{Ca}\) may also contribute to the pathophysiology underlying Ca\(^{2+}\) overload in the heart is speculative. Ca\(_0\) has been implicated in the modulation of a number of ion transport pathways\(^{49}\) (eg, K\(^{+}\) channels, Na\(^{+}\)-Ca\(^{2+}\) exchange, and SR uptake/release) that influence myocardial excitability and contractility. By allowing greater Ca\(^{2+}\) influx, enhancement of \(I_{Ca}\) may contribute in rendering a damaged or failing myocardium more susceptible to the phenomenon of delayed afterdepolarizations.\(^{50}\)

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