Corrections

PHYSIOLOGY. For the article “α1-Adrenoceptor stimulation potentiates L-type Ca\(^{2+}\) current through Ca\(^{2+}\)/calmodulin-dependent PK II (CaMKII) activation in rat ventricular myocytes,” by Jin O-Uchi, Kimiaki Komukai, Yoichiro Kusakari, Toru Obata, Kenichi Hongo, Hiroyuki Sasaki, and Satoshi Kurihara, which appeared in issue 26, June 28, 2005, of Proc. Natl. Acad. Sci. USA (102, 9400–9405; first published June 17, 2005; 10.1073/pnas.0503569102), the authors note that the panels in Fig. 4 were incorrectly labeled, due to a printer’s error. The corrected figure and its legend appear below.

![Fig. 4](image)

**Table 1. Potency of chemicals and reversibility of their effects**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Inhibition of gravitropism, IC(_{50})*</th>
<th>Inhibition of growth</th>
<th>Inhibition of hypocotyl bending</th>
<th>Vacuolar effects</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Hypocotyl</td>
<td>Root</td>
<td>Hypocotyl length( ^{1#} )</td>
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</tr>
<tr>
<td>2,4D</td>
<td>NA</td>
<td>0.02 ( \mu M )</td>
<td>NA</td>
<td>96.8 (0.02 ( \mu M ))</td>
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<tr>
<td>S403629</td>
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<td>0.2 ( \mu M )</td>
<td>90.4 (0.1 ( \mu M ))</td>
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<tr>
<td>S271050</td>
<td>2 ( \mu M )</td>
<td>NA</td>
<td>88.7 (2 ( \mu M ))</td>
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</tr>
<tr>
<td>S5850247</td>
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<td>0.3 ( \mu M )</td>
<td>93.8 (0.3 ( \mu M ))</td>
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<tr>
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<td>8 ( \mu M )</td>
<td>NA</td>
<td>21.8 (10 ( \mu M ))</td>
</tr>
</tbody>
</table>

*Estimated concentration resulting in 50% inhibition after 24-h gravistimulation.

†Lengths are reported as a percentage of the untreated controls.

‡Length measurements performed at the concentrations indicated.

www.pnas.org/cgi/doi/10.1073/pnas.0503569102

PLANT BIOLOGY. For the article “The power of chemical genomics to study the link between endomembrane system components and the gravitropic response,” by Marci Surpin, Marcela Rojas-Pierce, Clay Carter, Glenn R. Hicks, Jacob Vasquez, and Natasha V. Raikhel, which appeared in issue 13, March 29, 2005, of Proc. Natl. Acad. Sci. USA (102, 4902–4907; first published March 16, 2005; 10.1073/pnas.0500222102), the authors note that in Table 1, the concentrations are incorrectly presented as “M” instead of “\( \mu M \)” due to a printer’s error. The corrected table appears below. In addition, the authors note the last sentence of the Fig. 3 legend, “Chemical structures are shown in Table 1,” should be removed. The structures now appear online in Table 2, which is published as supporting information on the PNAS web site. These errors do not affect the conclusions of the article.

![Chemical structures](image)

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For the article “A quantitative assessment of models for voltage-dependent gating of ion channels,” by Michael Grabe, Harold Lecar, Yuh Nung Jan, and Lily Yeh Jan, which appeared in issue 51, December 21, 2004, of Proc. Natl. Acad. Sci. USA (101, 17640–17645; first published December 10, 2004; 10.1073/pnas.0408116101), the authors note that Fig. 3a was computed with a tilt angle different from that detailed in the Results. The corrected curves, presented below, are ~7% smaller in magnitude than the curves presented in Fig. 3a Left, but their shape and spacing are preserved. The correction will not alter the curve shown in Fig. 3a Right. The corrected activation energy barrier for the paddle model is ~75 k_BT, and the corresponding activation time is 10^{28} ms or 10^{17} years. The conclusions of the article remain unchanged. The corrected figure and its legend appear below.

**Fig. 3.** Total electrostatic solvation energy of the S4 segment and gating charge movement for lipid-exposed (a), translation (b), and rotation (c) models. (Left) Plots of the solvation energy. (Right) Plots of the total gating charge along the reaction pathway. The dashed curve represents the effect of the S3b helix on the lipid-exposed model. The solvation energy profile for the rotation model is rugged because of the complicated interfacial geometry.
α₁-Adrenoceptor stimulation potentiates L-type Ca²⁺ current through Ca²⁺/calmodulin-dependent PK II (CaMKII) activation in rat ventricular myocytes

Jin O-Uchi‡,†, Kimiaki Komukai§, Yoichiro Kusakari*, Toru Obata§, Kenichi Hongo‡, Hiroyuki Sasaki§, and Satoshi Kurihara*

*Department of Physiology (II), ‡Division of Cardiology, and §Division of Molecular Cell Biology, The Jikei University School of Medicine, 3-25-8 Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan

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α₁-Adrenoceptor stimulation (α₁ARS) modulates cardiac muscle contraction under physiological conditions by means of changes in Ca²⁺ current through L-type channels (I_Ca,L) and Ca²⁺ sensitivity of the myofilaments. However, the cellular mechanisms of α₁ARS are not fully clarified. In this study, we investigated the role of Ca²⁺/calmodulin-dependent PK II (CaMKII) in the regulation of I_Ca,L during α₁ARS in isolated rat ventricular myocytes by using the perforated patch–clamp technique. CaMKII inhibition with 0.5 μM KN-93 abolished the potentiation in I_Ca,L observed during α₁ARS by 10 μM phenylephrine. In the presence of PKC inhibitor (10 μM chelerythrine), the potentiation of I_Ca,L by phenylephrine also disappeared. In Western immunoblotting analysis, phenylephrine (≥1 μM) increased the amount of autophosphorylated CaMKII (active CaMKII) significantly, and this increase was abolished by CaMKII inhibition or PKC inhibition. Also, we investigated changes in the subcellular localization of active CaMKII by using immunofluorescence microscopy and immunoelectron microscopy. Before α₁ARS, active CaMKII was exclusively located just beneath the plasmalemma. However, after α₁ARS, active CaMKII was localized close to transverse tubules, where most of L-type Ca²⁺ channels are located. From these results, we propose that CaMKII, which exists near transverse tubules, is activated and phosphorylated by α₁ARS and that CaMKII activation directly potentiates I_Ca,L in rat ventricular myocytes.

Abbreviations: α₁ARS, 1A R Si nr a t 1-ARS stimulation; PKC, protein kinase C; PKII, protein kinase II; CaMKII, Ca²⁺/calmodulin-dependent PK II; I_Ca,L, L-type Ca²⁺ current through L-type channel; SA, standard area; T-tubules, transverse tubules; WGA, wheat germ agglutinin.

†To whom correspondence should be addressed. E-mail: o-uchi@jikei.ac.jp.

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defined as the difference between the peak current and the residual current at the end of the pulse. The application of 10 μM nifedipine in the perfusate almost completely blocked this current (data not shown), indicating that the measured current was ICa,L. All experiments were performed at room temperature (≈25°C).

Western Immunoblotting. Contents of total and phosphorylated CaMKII (active CaMKII) were determined by Western immunoblotting using whole-cell protein extracts (12). Cells were treated with various concentrations of phenylephrine (0–100 μM) for 15 min and then protein extracts were prepared. For testing the effect of chemicals (prazosin, KN-93, and chelerythrine) used in electrophysiological experiments, cells were exposed to Tyrode’s solution containing these chemicals for 15 min before application of phenylephrine and then to the same solutions containing 10 μM phenylephrine for 15 min. Samples (50 μg per well) were electrophoresed in 12% SDS/PAGE gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad) and exposed to primary antibodies against active CaMKII and total CaMKII. Immunoreactive bands were visualized by enhanced chemiluminescence using the ECL-plus detection kit (Amersham Biosciences) and quantified by using densitometry (ATTO, Tokyo). Analysis of the change in total or active CaMKII. Immunoreactive bands were visualized by enhanced chemiluminescence using the ECL-plus detection kit (Amersham Biosciences) and quantified by using densitometry (ATTO, Tokyo). Analysis of the change in total or active CaMKII was performed by using densitometry with the significance level set at P < 0.05.

Immunofluorescence Microscopy. After treatment with 100 μM phenylephrine for 15 min, myocytes were fixed in 100% acetone at −20°C for 10 min, incubated with the primary antibody against active CaMKII and WGA-FITC (overnight), followed by Alexa-546-conjugated anti-rabbit secondary antibody for 1 h (17). Immunostaining was visualized with an LSM-510 laser scanning confocal microscope (Zeiss). Control experiments performed by using secondary antibody without primary antibody showed no noticeable labeling.

Immunoelectron Microscopy. For cryoimmunoelectron microscopy, isolated myocytes were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) infused with 2.3 M sucrose containing 20% polyvinylpyrrolidone at 4°C and frozen in liquid nitrogen. Ultrathin cryosections were cut and processed for immunolabeling (18, 19). Rabbit anti-active CaMKII IgG as a secondary antibody and 15 nm of gold-conjugated goat anti-rabbit IgG as a secondary antibody were used. Samples were examined with an H-7500 transmission electron microscope (Hitachi, Tokyo) at an accelerating voltage of 100 KV. Gold-particle density was determined at the level of the Z lines (representing T-tubule location) and at the plasmalemma, as described in ref. 20, with some modifications. Briefly, the density of gold particles within 500 × 500-nm² areas of the section was determined for areas located at the level of the Z lines between the myofibrils (233 areas, presumably indicating T-tubule location) and in proximity of the plasmalemma (83 areas) by using sections from four representative single cells before or after α1-AR agonists, respectively. The counts were normalized by the particles density on areas of the section within the myofibrils (293 areas, taken as background).

Statistics. All data are presented as mean ± SD. Bars indicate SD. Statistical comparisons were carried out by using one-way or one-way repeated measured ANOVA followed by Bonferroni post hoc test with the significance level set at P < 0.05.

Results

Effect of 10 μM Phenylephrine on ICa,L Measured by Using the Perforated Patch–Clamp Technique. Fig. 1 shows a representative result of the effect of 10 μM phenylephrine on ICa,L. The effect of phenylephrine on the Ca2⁺ responsiveness of the contractile element in rat ventricular myocytes is almost saturated at this concentration (5). As reported (8, 9), we observed a transient decrease followed by a sustained increase of ICa,L amplitude. As shown in Fig. 1A, ICa,L transiently decreased for up to 2 min after the application of phenylephrine, and then it gradually increased and reached another steady-state level at 15 min after this application. The amplitudes of ICa,L at 15 and 20 min were not statistically different (114.8 ± 14.5% of control and 117.9 ± 13.5% of control, n = 12, P = 1.00), and thus, the effect of 10 μM phenylephrine on ICa,L potentiation reached a steady state at 15 min. The amplitude of ICa,L returned to the control level at 10 min after removal of phenylephrine (101.4 ± 6.2% of control, n = 5, P = 0.678). The initial transient decrease in ICa,L at 2 min (91.4 ± 6.0% of control, n = 12, P < 0.05) and the later increase at 15 min (114.8 ± 14.5% of control, n = 12, P > 0.001) (Fig. 1C) were small but significant. The increase in ICa,L occurred without changes in the shape of the current-voltage relationship (Fig. 1B). These negative and positive effects of phenylephrine on ICa,L were both completely blocked by the α1-adrenoceptor antagonist prazosin (1 μM) (n = 12, data not shown). In the
relationship (Fig. 2A). Time-dependent changes of I_{Ca,L} after the application of 10 μM phenylephrine in the presence of 0.5 μM KN-93 (n = 12) or 0.5 μM KN-92 (n = 7). After establishing a new steady state for I_{Ca,L} by 10-min application of KN-93 or KN-92, the effect of 10 μM phenylephrine on I_{Ca,L} was observed in the continuous presence of KN-93 or KN-92. The amplitude of the current at each period was normalized to the current before the application of phenylephrine. (Bars indicate SD.) ∗, P < 0.05; ∗∗, P < 0.01; ∗∗∗, P < 0.001, compared with the normalized current in the presence of KN-92 at each time. Phe, phenylephrine.

In the absence of phenylephrine, the amplitude of I_{Ca,L} was stable for up to 15 min (Fig. 1C).

We explored the effect of intracellular Ca^{2+} buffering on I_{Ca,L} in response to 10 μM phenylephrine (Fig. 1D). After establishing perforated patch, a cell was incubated with 20 μM BAPTA-acetoxyethyl ester, the cell permeable form of BAPTA, for 10 min and we confirmed that the cell did not contract during the pulse to evoke I_{Ca,L} (16). In this condition, phenylephrine did not show either a significant transient decrease in I_{Ca,L} at 2 min (97.2 ± 4.4% of control, n = 10, P = 1.00) or a sustained increase at 15 min (101.3 ± 17.1% of control, n = 10, P = 1.00). Thus, intracellular Ca^{2+} is a key factor for the potentiation in I_{Ca,L} induced by phenylephrine. The different responses of I_{Ca,L} to phenylephrine recorded by conventional patch and perforated patch can be explained by the intracellular Ca^{2+} concentration.

The Role of CaMKII in the Regulation of I_{Ca,L} by α1ARS. Recent studies have reported that CaMKII is involved in various Ca^{2+}-dependent effects both under physiological and pathophysiological conditions in mammalian ventricular myocytes (10). Thus, we investigated the role of CaMKII in the regulation of I_{Ca,L} by α1ARS by using KN-93, a synthetic CaMKII inhibitor. At a concentration of 0.5 μM, KN-93 selectively inhibits CaMKII without affecting other PKs (21). A 10-min exposure to 0.5 μM KN-93/94 significantly decreased I_{Ca,L} from 9.12 ± 3.27 to 5.50 ± 3.39 pA/pF, corresponding to 39.1 ± 25.6% decrease (n = 12, P < 0.001) without changing the shape of the current–voltage relationship (n = 10) (see also ref. 22). In the presence of KN-93, 10 μM phenylephrine produced only a sustained decrease of I_{Ca,L} (Fig. 2A). At 15 min after application of 10 μM phenylephrine, the amplitude of I_{Ca,L} significantly decreased to 66.7 ± 22.0% of the value before application of phenylephrine in the presence of KN-93 (n = 12, P < 0.001) without changing the shape of the current–voltage relationship (n = 10). In contrast, 0.5 μM KN-92, an inactive KN-93 analogue, did not show significant effects on the biphasic change of I_{Ca,L} caused by α1ARS (Fig. 2A). We also investigated the effect of 10 μM phenylephrine in the presence of another CaMKII inhibitor, autacotide-2 inhibitory peptide, a membrane-permeable and a highly specific peptide type inhibitor of CaMKII (23). When we used 10 μM autacotide-2 inhibitory peptide, 10 μM phenylephrine produced only a sustained decrease of I_{Ca,L} without potentiation as in the presence of KN-93 (n = 5, data not shown). Thus, CaMKII inhibition abolished the potentiation of I_{Ca,L} during α1ARS.

The Role of PKC in the Regulation of I_{Ca,L} During α1ARS. PKC may be involved in the α1-adrenoceptor-mediated modulation of cardiac K^{+} channels, intracellular alkalinization and myofibrillar responsiveness to Ca^{2+} (2). Therefore, we investigated the role of PKC in the regulation of I_{Ca,L} by α1ARS by using chelerythrine as a PKC inhibitor. We found that 10 μM chelerythrine selectively inhibits PKC without affecting other PKs (24) and effectively blocks the effect of phenylephrine on the Ca^{2+} responsiveness of the contractile element (5). Exposure to 10 μM chelerythrine for 10 min significantly decreased I_{Ca,L} to a new steady state from 7.42 ± 1.61 to 3.78 ± 1.23 pA/pF, corresponding to a 49.7 ± 13.2% decrease (n = 9, P < 0.001) without changing the shape of the current–voltage relationship. In the presence of chelerythrine, 10 μM phenylephrine produced only a sustained decrease of I_{Ca,L} as observed in the presence of CaMKII inhibitors (Fig. 2B). At 15 min after application of 10 μM phenylephrine, the amplitude of I_{Ca,L} decreased to 42.3 ± 10.9% of the value before application of phenylephrine in the presence of chelerythrine (n = 9, P < 0.001) (Fig. 2B) without changing the shape of the current–voltage relationship (n = 9).

Activation of CaMKII During α1ARS. Our electrophysiological experiments suggest that potentiation of I_{Ca,L} in response to α1ARS is mediated by both CaMKII and PKC. Although PKC has been suggested to be involved in the α1-adrenoceptor signal-transduction pathway (2), there are no reports that CaMKII is activated by α1ARS in rat ventricular myocytes. The amount of CaMKII is low compared with other PKs; consequently, it is difficult to determine CaMKII activity directly in cardiac muscle (25). To overcome this difficulty, we used an antibody against the autophosphorylation site of CaMKII, Thr-286, and an enhanced chemiluminescence system to measure CaMKII activity in whole-cell lysates in response to α1ARS (12, 13). Active CaMKII increased significantly at concentrations of ≥1 μM phenylephrine (Fig. 3A Bottom), although total CaMKII was not altered (Fig. 3A Top; data not shown). The increase of active CaMKII level induced by 10 μM phenylephrine was completely blocked by the α1-adrenoceptor antagonist prazosin (1 μM), showing that this effect was mediated by α1-adrenoceptor (Fig. 3B). The selective CaMKII inhibitor 0.5 μM KN-93 that we used in the perforated patch experiments partially blocked the basal CaMKII activity in the absence of phenylephrine and also completely blocked the increase in the active CaMKII level by 10 μM phenylephrine (Fig. 3C).

To determine whether PKC could be a regulator of CaMKII activation during α1ARS, the effect of phenylephrine in the presence of chelerythrine on CaMKII activity was determined. Chelerythrine (10 μM) did not produce significant changes in the CaMKII activation before phenylephrine (86.6 ± 25.1% of control, n = 7, P = 0.39). However, the increase in active CaMKII by 10 μM phenylephrine was abolished by chelerythrine (102.2 ± 24.3% of control, n = 7, P = 1.00), as in the case of KN-93, indicating that PKC is involved in the activation of CaMKII during α1ARS (Fig. 3D).

Immunocytochemical Localization of Active CaMKII by α1ARS. It has been reported that CaMKII may directly phosphorylate the L-type Ca^{2+} channel and regulate the positive feedback system that facilitates I_{Ca,L} under physiological conditions (17, 26). Our biochemical experiments showed that CaMKII is significantly activated by α1ARS in adult rat isolated ventricular myocytes.
investigate whether CaMKII could directly regulate the L-type Ca\textsuperscript{2+} channel during \(\alpha_1\)ARS, we determined the localization of active CaMKII in isolated ventricular myocytes by using immunofluorescence microscopy, as shown in Fig. 4. We used WGA-FITC, a marker of sarcolemma including T-tubules (27), and anti-active CaMKII antibody to establish the intracellular localization of active CaMKII (Fig. 4C and D). Before \(\alpha_1\)ARS, active CaMKII was detectable at the plasmalemma (Fig. 4A and E) as reported (17). After \(\alpha_1\)ARS (100 \(\mu\)M phenylephrine) active CaMKII was still present at the plasmalemma (Fig. 4B), but it was also clearly visible along transverse bands that coincide with the location of WGA-FITC. This result suggests that a higher level of active CaMKII was localized at or near the T-tubules after \(\alpha_1\)ARS than in the resting state (Fig. 4F).

To confirm the subcellular localization of active CaMKII before and after \(\alpha_1\)ARS, immunoelectron microscopy was used. The number of gold particles over a standard area (SA) at the plasmalemma and at the level of the Z lines where T-tubules are located was counted in sections from four representative cells before and after \(\alpha_1\)ARS. Before \(\alpha_1\)ARS, the frequency of gold particles was very low both beneath the plasmalemma (0.10 \(\pm\) 0.31 particles per SA, 0.39 \(\pm\) 1.20 in normalized density, \(n = 30\) areas) and at T-tubules (0.09 \(\pm\) 0.29 particles per SA, 0.36 \(\pm\) 1.15 in normalized density, \(n = 108\) areas) (Fig. 5D). After applica-

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**Fig. 3.** Activation of CaMKII in response to \(\alpha_1\)ARS. (A) (Top and Middle) Western immunoblot analyses showing the activation levels of CaMKII in response to various concentration of phenylephrine (\(n = 8\)). Although the level of total CaMKII protein was not changed, the level of active CaMKII significantly increased at concentrations of phenylephrine \(\geq 1\) \(\mu\)M. (Bottom) Bar graphs show the intensity of the active CaMKII band, normalized to the control, indicating the change of CaMKII activation level. (B–D) (Top and Middle) Western immunoblot analyses showing the activation level of CaMKII during \(\alpha_1\)ARS in the presence of prazosin, KN-93, and chelerythrine, respectively. (Bottom) Increase of the level of active CaMKII during \(\alpha_1\)ARS was completely blocked by prazosin (\(n = 6\)), KN-93 (\(n = 9\)), and chelerythrine (\(n = 7\)), showing the percentage of increase in CaMKII activation. (Bars indicate SD.) \(*, P < 0.05\), compared with the control. N.S., no significant difference between the two experimental results; CTL, control; Phe, phenylephrine; Pra, prazosin; Che, chelerythrine.

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**Fig. 4.** Localization of activated CaMKII in response to \(\alpha_1\)ARS. Immunofluorescence images of ventricular myocytes labeled with active CaMKII antibody (red: A and B) and the plasma membrane marker WGA-FITC (green: C and D) before and after \(\alpha_1\)ARS. (E and F) Overlay images demonstrate that active CaMKII localized near the T-tubules and the sarcolemma after \(\alpha_1\)ARS (F). However, immunoreactivity of active CaMKII was found only at the plasmalemma before \(\alpha_1\)ARS (E). (Scale bars in F, 10 \(\mu\)m.)
tion of phenylephrine (100 μM), as shown in Fig. 5 A and B, the frequency of gold particles (indicating active CaMKII) near T-tubules was increased (1.21 ± 0.92 particles per SA, 3.95 ± 3.00 in normalized density, n = 125 areas), showing ~10-fold enrichment, consistent with the result of immunofluorescence image analysis (compare Figs. 4F and 5E). In contrast, the particle density beneath the plasmalemma remained low (0.49 ± 0.80 particles per SA, 1.60 ± 2.61 in normalized density, n = 53, P = 0.27, compared with that before α1ARS) (Fig. 5C). Thus, immunogold labeling showed ~2.5-fold enrichment of active CaMKII in T-tubules compared with the plasmalemma after α1ARS (Fig. 5E). The particle density in the myofilibrils areas was not significantly different before (0.25 ± 0.48 particles per SA, n = 110) and after α1ARS (0.30 ± 0.52 particles per SA, n = 183).

There seems to be a discrepancy between the results obtained by using light and electron microscopy regarding the location of active CaMKII in the resting cells. The light-microscope images did not show active CaMKII at T-tubules, whereas electron-microscopic images detected a very low level at those sites. The confocal sections are relatively thick compared with the thickness of the T-tubules so that a small portion of each section is occupied by T-tubule membrane and a weak signal in this membrane is not detected. In contrast, the plasmalemma runs from one end of the section to the other, so that the signal from this membrane is detectable even if weak. In the thin sections for electron microscopy, plasmalemma and T-tubule membrane are more equally represented.

Discussion

In this study, we explored the involvement of CaMKII in the signal transduction pathway between α1ARS and the potentiation of I_{Ca,L} in rat ventricular myocytes, and we show direct evidence indicating that CaMKII is activated by α1ARS and has an important effect on I_{Ca,L}.

Our electrophysiological experiments using the perforated patch technique confirm the reported (8, 9) potentiation of I_{Ca,L} during α1ARS in rat ventricular myocytes. When I_{Ca,L} is measured by using the whole-cell patch-clamp technique, phenylephrine does not affect I_{Ca,L} at any concentrations (4, 7), and we can mimic this effect by buffering the intracellular Ca^{2+} with BAPTA–acetoxymethyl ester. Thus, intracellular Ca^{2+} concentration is essential for the potentiation of I_{Ca,L} during α1ARS. This result is consistent with the report that basal intracellular Ca^{2+} is sufficient for the initial CaMKII activation under physiological condition (28).

We chose 10 μM phenylephrine in our electrophysiological experiments because the effect of phenylephrine on I_{Ca,L} potentiation is saturated at this concentration (see Fig. 6, which is published as supporting information on the PNAS web site). α1ARS has two opposite effects on I_{Ca,L}. (i) Higher concentration of phenylephrine (≥10 μM) causes a biphasic response: an initial brief (~2 min) depression, or negative phase, followed by a potentiation or positive phase; and (ii) lower concentration of phenylephrine (≥1 μM) causes a monophasic positive effect (Fig. 6). The positive effect (potentiation) depends on CaMKII activation and PKC activation because CaMKII inhibition or PKC inhibition abolished the potentiation of I_{Ca,L} (Fig. 2 and Fig. 7, which is published as supporting information on the PNAS web site). There is less information about the negative effect of α1ARS on I_{Ca,L}. In these experiments, we focused on the potentiation of I_{Ca,L} during α1ARS (positive effect).

Our electrophysiological experiments using CaMKII inhibitors demonstrated the important role of CaMKII in the potentiation of I_{Ca,L} (positive phase) during α1ARS in cardiac myocytes. Western immunoblotting analysis confirmed an increase in active CaMKII (see also ref. 13) in parallel with the potentiation of I_{Ca,L}. Also, we showed that PKC, which is activated by the Gq-phospholipase C-diacylglycerol pathway (2), is involved in the activation of CaMKII during α1ARS, based on the similar effects of CaMKII and PKC inhibition on I_{Ca,L} in the presence of phenylephrine. It has been reported that, in resting cardiac myocytes, there is significant activation of CaMKII but active CaMKII can be lost when the intracellular Ca^{2+} level is lowered to very low levels by removal of extracellular Ca^{2+} (17). Basal activity of CaMKII is determined mainly by the resting intracellular Ca^{2+} level (and not by the PKC activity). However, after α1ARS, PKC activity has an important role in the additional and sustained
activation of CaMKII. PKC can directly phosphorylate the autophosphorylation site of CaMKII in vitro, thus directly increasing CaMKII activity (29). This report strongly supports our hypothesis that there is a physiological linkage between PKC and CaMKII during α1ARS.

Immunolabeling at the ultrastructural level demonstrates that the level of active CaMKII is very low along the plasmalemma and T-tubules in the resting cells but increases significantly along the T-tubules, where L-type Ca\(^{2+}\) channels are mostly present (11), after α1ARS. The correspondence between the distributions of active CaMKII and L-type Ca\(^{2+}\) channels strongly supports our view that CaMKII directly phosphorylates the L-type Ca\(^{2+}\) channels and potentiates \(I_{\text{Ca,L}}\) in response to α1ARS. Immunofluorescence microscopy confirms the increase in active CaMKII at the T-tubules from an undetectable to a detectable level with stimulation.

It has been reported (10) that CaMKII modulates \(I_{\text{Ca,L}}\) under physiological conditions. Several groups have demonstrated that Ca\(^{2+}\)-dependent \(I_{\text{Ca,L}}\) facilitation is mediated by CaMKII-dependent phosphorylation of L-type Ca\(^{2+}\) channel, and this mechanism is considered to be related to the positive staircase of \(I_{\text{Ca,L}}\) induced by repeated depolarization from physiological holding potential (17, 26, 30). However, the molecular mechanisms of how \(I_{\text{Ca,L}}\) is potentiated by the activation of CaMKII have not been elucidated.

In summary, these experiments show that (i) α1ARS potentiates \(I_{\text{Ca,L}}\) by PKC and CaMKII activation and (ii) activated CaMKII is highly localized close to the T-tubules after α1ARS.

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