Apelin decreases the SR Ca$^{2+}$ content but enhances the amplitude of [Ca$^{2+}$]$_i$ transient and contractions during twitches in isolated rat cardiac myocytes

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Apelin decreases the SR Ca$^{2+}$ content but enhances the amplitude of [Ca$^{2+}$]$_i$ transient and contractions during twitches in isolated rat cardiac myocytes. Am J Physiol Heart Circ Physiol 294: H2540–H2546, 2008. First published April 18, 2008; doi:10.1152/ajpheart.00046.2008.—Apelin has been reported to have a positive inotropic action in the isolated rat heart. However, the effect of apelin on sarcoplasmic reticulum (SR) Ca$^{2+}$ content and its influence on intracellular Ca$^{2+}$ transient during excitation-contraction coupling remains poorly understood. In the present study, we determined the effect of apelin on Ca$^{2+}$ transient and contractions in isolated rat cardiomyocytes. When compared with control, treatment with apelin caused a 55.7 ± 13.9% increase in sarcromere fraction shortening and a 43.6 ± 4.56% increase in amplitude of electrical-stimulated intracellular Ca$^{2+}$ concentration ([E[Ca$^{2+}$]$_i$] transient (n = 14, P < 0.05). But SR Ca$^{2+}$ content measured by caffeine-induced [Ca$^{2+}$]$_i$, ([C[Ca$^{2+}$]$_i$]) transient was decreased 8.41 ± 0.92% in response to apelin (n = 14, P < 0.05). Na$^+$/Ca$^{2+}$ exchanger (NCX) function was increased since half-decay time of C[Ca$^{2+}$]$_i$ was decreased 16.22 ± 1.36% in response to apelin. Sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) activity was also increased by apelin. These responses can be partially or completely blocked by chelerythrine chloride, a PKC inhibitor. In addition, to confirm our data, we used indo-1 as another Ca$^{2+}$ indicator and rapid cooling as another way to measure SR Ca$^{2+}$ content, and we observed similar results. So we conclude that apelin has a positive inotropic effect on isolated myocytes, and increased amplitude of E[Ca$^{2+}$]$_i$ is at least partially involved in the mechanism. NCX function and SERCA activity are increased by apelin, and the SR Ca$^{2+}$ content is decreased by apelin during twitches. PKC played an important role in these signaling mechanisms.

excitation-contraction coupling; intracellular calcium concentration; positive inotropic action; protein kinase C; sarcoplasmic reticulum

APELIN, AN ENDOGENOUS LIGAND for apelin-angiotension receptor-like 1, was first isolated from the bovine stomach in 1998 (49). Now we know that apelin and its receptor are highly expressed in many tissues throughout the body, including the nervous system, vascular endothelium, heart, lung, and kidney (13, 14, 23, 26, 28, 31, 34, 41). The peptide has also been shown to be involved in the regulation of the immune system (20), nerve signal (40), hemodynamic homeostasis (15, 42, 47), and human immunodeficiency virus infection (9, 56). The presence of apelin and its receptors in the heart and blood vessels suggests that this peptide may have a role in the cardiovascular system. Intravenous injection of apelin in anesthetized rats decreases blood pressure (28), mediated by a nitric oxide-dependent mechanism (50). Apelin was also reported to have positive inotropic action in the isolated perfused rat heart (46), and the mechanism of apelin’s positive inotropic action may involve the activation of PLC, PKC, sarcolemmal Na$^+$/H$^+$ exchanger (NHE), and Na$^+$/Ca$^{2+}$ exchanger (NCX) (46). However, the effect of apelin on isolated myocytes remains unknown.

Contraction of the cardiac myocyte is initiated by Ca$^{2+}$ influx via the L-type Ca$^{2+}$ current (I$_{Ca,L}$), which subsequently triggers a much larger Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) by Ca$^{2+}$-induced Ca$^{2+}$ release (16). Membrane depolarization during an action potential activates L-type Ca$^{2+}$ channels (LTCCs). These channels open transiently and serve as the major pathway for Ca$^{2+}$ entry into myocytes. The subsequent local elevation in intracellular Ca$^{2+}$ concentration ([E[Ca$^{2+}$]$_i$]) activates Ca$^{2+}$ release channels (ryanodine receptors (RyR)) in the SR, providing the Ca$^{2+}$ required for contraction. This whole sequence of events determines the excitation-contraction (EC) coupling. We hypothesized that apelin had a positive inotropic effect on isolated myocytes and that the mechanism may involve the enhancement of amplitude of [Ca$^{2+}$]$_i$ transient during EC coupling.

The aims of our study were to test whether apelin has positive inotropic action on individual myocytes and to evaluate the effect of apelin on EC coupling by direct measurement of the cell contraction; the amplitude and the time course of the electrical-stimulated [Ca$^{2+}$]$_i$, ([E[Ca$^{2+}$]$_i$]) transients, sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) activity, and caffeine-induced [Ca$^{2+}$]$_i$, ([C[Ca$^{2+}$]$_i$]), and rapid cooling (RC) of the cell to calculate the SR Ca$^{2+}$ content in isolated myocytes. Since PKC has been shown to alter Ca$^{2+}$ transients, contractility, and SR Ca$^{2+}$ handling (7, 21, 33, 43) and is involved in the apelin effect on myocardium-positive inotropic action (46), we also studied the possible role of PKC in these effects.

MATERIALS AND METHODS

Myocyte isolation. Myocytes were isolated using standard procedures (55). Briefly, hearts were rapidly excised from adult male Sprague-Dawley rats (250–280 g) under pentobarbital sodium anesthesia (140 mg/kg ip), mounted on a Langendorff perfusion apparatus, and perfused with Ca$^{2+}$-free Tyrode solution containing (in mmol/l) 143.0 NaCl, 5.4 KCl, 0.5 MgCl$_2$, 0.3 Na$_2$HPO$_4$, 5.0 HEPES, and 5.0 glucose (pH 7.4, equilibrated with O$_2$) for 5 min at 37°C. The heart was then perfused with the same solution containing 0.4 g/l collagenase II (283 U/mg; Worthington Biochemical; Lakewood, NJ) and 0.7 g/l bovine serum albumin until it became flaccid (10 to 30 min). After perfusion with Ca$^{2+}$-free Tyrode solution for 5 min to remove enzymes, the digested tissues were separated and filtered. The resultant cell suspension was rinsed several times with progressive in-

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creases in [Ca\(^{2+}\)] to 1.8 mmol/l. The experimental protocol was approved by the China Institutional Ethics Review Committee for Animal Experimentation.

Measurement of intracellular Ca\(^{2+}\). We used two different Ca\(^{2+}\) indicators and protocols to measure intracellular Ca\(^{2+}\). First, freshly isolated myocytes were placed on laminin-coated glass coverslips and allowed to attach for 30 min before they were loaded with fura-2 AM (0.5 μmol/l; Alexis Biochemicals, San Diego, CA) for 30 min. All fura-2 experiments were conducted at room temperature. The free [Ca\(^{2+}\)], of loaded cardiac myocytes was measured as the fluorescence ratio (360 to 380 nm) (55). The myocytes were superfused with Ca\(^{2+}\)-containing Tyrode solution and then with 1 mM (17, 46) apelin-16 (Phoenix) or apelin + 5 μM chelerythrine chloride (CHE; PKC inhibitor). The E[Ca\(^{2+}\)], transients were elicited by field stimulation (0.5 Hz). The myocytes were allowed to equilibrate for about 10 min after added apelin or apelin + CHE. The application of CHE alone without apelin had no effect on the myocytes (Table 1). Second, the cells were loaded during 30 min with 5 μmol/l indo-1 AM and 0.01% pluronic before each experiment (1, 2). Myocytes were washed twice with fresh HEPES solution (without albumin) and kept for 15 min to complete deesterification of indo-1 AM. The loaded myocytes were attached to a poly-L-lysine (0.1 g/l)-treated coverslip placed on a microscope stage of an inverted fluorescence microscope (Olympus, Tokyo, Japan). A temperature-controlled perfusion chamber with two needles at opposite sides for perfusion purposes (height, 0.4 mm; diameter, 10 mm; and volume, 30 μl) was tightly positioned over the coverslips. The contents of the chamber could be replaced within 100 ms. The field stimulation (0.5 Hz) was elicited, and indo-1 fluorescence was measured in a dual emission mode, excited at 340 nm with xenon lamp flashes (100 W). Dual wavelength emission was measured at 410 and 516 nm, respectively. Fluorescence signals were recorded at a sample rate of 1 kHz. We calculated free cellular [Ca\(^{2+}\)], according to the ratio equation (11, 18, 19): [Ca\(^{2+}\)] = \frac{K_d \cdot (R - R_{\text{Hmax}})}{R_{\text{Hmax}} - R \cdot R_{\text{Hmax}}} \cdot \text{nmol/l}, where R is the ratio of fluorescence signals at 410 and 516 nm, R_{\text{Hmax}} is the ratio at saturating [Ca\(^{2+}\)], R_{\text{Hmax}}, is the ratio at zero [Ca\(^{2+}\)], K_d was 250 nmol/l, and β is 2.1. In indo-1 AM-loaded myocytes, the final free indo-1 is compartmentalized mainly in the cytosol and mitochondria (45). To calculate “true” cytosolic free [Ca\(^{2+}\)], from measured overall values, both the fraction of mitochondrially localized indo-1 and mitochondrial free [Ca\(^{2+}\)], were measured (32). “True” free cytosolic [Ca\(^{2+}\)], was calculated from measured cellular [Ca\(^{2+}\)],, correcting measured values for compartmentalization and mitochondrial [Ca\(^{2+}\)]. Total cytosolic Ca\(^{2+}\) content (cytosolicically bound plus free cytosolic Ca\(^{2+}\)) was calculated using “true” free [Ca\(^{2+}\)], and data on cytosolic Ca\(^{2+}\) buffer capacity in the literature (24). In the second protocol, the same cell was superfused in turn with Ca\(^{2+}\)-containing Tyrode solution (control solution) and then with 1 mM apelin-16 and then washout with control solution and lastly with apelin + 5 μM CHE. We tested three cells to find out that, repeatedly, the application of electric stimulation and RC would not affect the baseline of the Ca\(^{2+}\) activity (data not shown).

Measurement of cell shortening. Shortening of myocytes was simultaneously measured with E[Ca\(^{2+}\)]. Myocytes were perfused with Tyrode solution at 22°C and 1 ml/min flow rate. Myocytes were equilibrated at 22°C for about 20 min before use. All experimental protocols were carried out at room temperature. The contractile shortening of ventricular myocyte was measured by a video-based motion edge-detection system (IonOptix, Milton, MA) (52) and an inverted microscope (Olympus).

Measurement of caffeine contracture. C[Ca\(^{2+}\)], transient amplitude was used as a measurement of SR Ca\(^{2+}\) content (22, 51, 54). In the presence of caffeine, the elimination of Ca\(^{2+}\) is mainly attributed to NCX. The time course of the decay of C[Ca\(^{2+}\)], transient is used as an index of NCX function (38, 39, 51). A rapid application of caffeine to the myocyte was performed just 2 s after the stimulation stopped to measure SR Ca\(^{2+}\) content during electrical stimulation switches.

RC used to estimate SR Ca\(^{2+}\) content. SR Ca\(^{2+}\) content was measured by another method since this was the most important observation in the current study. RC causes complete depletion of Ca\(^{2+}\) from SR- and Ca\(^{2+}\)-released remains confined to the cytoplasm (5, 6). RC was carried out by a rapid superfusion with ice-cold Tyrode solution of the same composition; a low temperature (0–1°C) was reached within 200 ms. SR Ca\(^{2+}\) content was calculated from the increase of total cytosolic Ca\(^{2+}\) following RC and a fractional SR volume of 10%. RC was applied 2 s after the cessation of stimulation.

Measurement of Ca\(^{2+}\)-ATPase activity. SR was prepared according to the methods of Jones (25) as modified by Kodavanti et al. (27) and Pande et al. (36). The myocytes were put in homogenizing medium containing (in mmol/l) 50.0 NaHPO\(_4\), 10.0 Na\(_2\)EDTA, and 25.0 NaF (pH 7.4). The minced ventricular tissue was placed in 10 ml of ice-cold homogenizing medium and homogenated three times. An additional 5 ml of homogenizing medium was added, and the homogenate was sedimented twice for 20 min at 14,000 g at 4°C. The supernatant was recentrifuged at 45,000 g for 30 min. The pellet obtained after this centrifugation, consisting of crude membrane vesicles (SR), was suspended in storage buffer containing 30.0 mmol/l histidine, 0.25 mmol/l sucrose, 0.10 mmol/l EDTA, and 10.0 mmol/l NaF (pH 7.4) to a final concentration of 30–40 mg/ml protein and stored at −80°C until used. The activity of Ca\(^{2+}\)-ATPase was determined with a kit (Jiancheng, Nanjing, China) by measuring the inorganic phosphate (P) liberated from ATP hydrolysis (27). Ca\(^{2+}\)-ATPase activity was assayed in a medium containing (in mmol/l) 50.0 histidine, 3.0 MgCl\(_2\), 100.0 KCl, 5.0 sodium azide, and 3.0 ATP and 50.0 μmol/l CaCl\(_2\) (pH 7.0) (36). Cardiac SR membranes were added to the reaction mixture at a final concentration of 20–25 μg of protein per milliliter, preincubated for 10 min at 37°C, and the reaction was

### Table 1. Myocytes shortening and [Ca\(^{2+}\)], transient parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Apelin</th>
<th>Apelin + CHE</th>
<th>CHE</th>
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<tr>
<td>n</td>
<td>33</td>
<td>14</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>E[Ca(^{2+})], amplitude (F360/F380)</td>
<td>0.942±0.029</td>
<td>1.343±0.021*†</td>
<td>1.479±0.051*</td>
<td>0.973±0.018</td>
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<tr>
<td>Diastolic [Ca(^{2+})], (F360/F380)</td>
<td>0.654±0.05</td>
<td>0.692±0.033</td>
<td>0.709±0.047</td>
<td>0.673±0.038</td>
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<tr>
<td>Twitch FS, %</td>
<td>12.746±0.624</td>
<td>19.49±1.531†</td>
<td>14.109±0.945*</td>
<td>12.559±0.571</td>
</tr>
<tr>
<td>+dL/dt, mm/s</td>
<td>281.495±12</td>
<td>324.332±10*</td>
<td>330.396±15*</td>
<td>294.361±14</td>
</tr>
<tr>
<td>–dL/dt, mm/s</td>
<td>248.792±10</td>
<td>269.669±12*†</td>
<td>251.276±14</td>
<td>245.672±11</td>
</tr>
<tr>
<td>[Ca(^{2+})], TTP, ms</td>
<td>68.627±2.885</td>
<td>59.263±2.224*</td>
<td>57.894±2.572*</td>
<td>69.365±2.113</td>
</tr>
<tr>
<td>[Ca(^{2+})], TTP, s</td>
<td>303.345±2.331</td>
<td>209.909±4.133†</td>
<td>305.962±4.579</td>
<td>305.772±2.99</td>
</tr>
<tr>
<td>C[Ca(^{2+})], amplitude (F360/F380)</td>
<td>4.452±0.031</td>
<td>4.076±0.031†</td>
<td>4.503±0.021</td>
<td>4.472±0.019</td>
</tr>
<tr>
<td>C[Ca(^{2+})], TTP, s</td>
<td>3.028±0.025</td>
<td>2.535±0.036*</td>
<td>3.107±0.054</td>
<td>3.098±0.044</td>
</tr>
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</table>

Values are means ± SE; n, number of cardiomyocytes from 14 rat hearts. ±dL/dt, maximal velocities of shortening and relengthening. E[Ca\(^{2+}\)], and C[Ca\(^{2+}\)], electrical-stimulated and caffeine-induced intracellular Ca\(^{2+}\) concentrations, respectively; F360/F380, fluorescence ratio (360 to 380 nm); FS, fractional shortening; TTP, time to peak; Tso, half-decay time. The control is the combination of the baseline values of the 2 groups: apelin and apelin + chelerythrine chloride (CHE). *P < 0.05 vs. control; †P < 0.05 vs. apelin + CHE. CHE group has no significant difference compared with control.
initiated by the addition of ATP. The ATP hydrolysis that occurred in the absence of Ca\(^{2+}\) (1 mmol/l EGTA) was subtracted to determine the activity of Ca\(^{2+}\)-stimulated ATPase. Ouabain was added fresh to a final concentration of 1 mmol/l in the media, which remained unchanged throughout the incubation. Mitochondrial contamination was assessed by determining the activity of azide-sensitive ATPase, that is, that activity inhibited by 5 mmol/l sodium azide (29).

Statistical analysis. All values are presented as means ± SE. Differences were compared by one-way ANOVA followed by Student-Newman-Keuls test as appropriate. \(P < 0.05\) was considered to be statistically significant; \(n\) represents the number of cardiomyocytes or hearts. All of the statistical tests were carried out with SPSS (v. 11.0).

RESULTS

Single-myocyte sarcomere fractional shortening (FS, normalized by resting cell sarcomere length) and E[Ca\(^{2+}\)], transients were measured simultaneously when myocyte twitches were under steady state at 0.5 Hz stimulation. Cell [Ca\(^{2+}\)], transient and shortening data (Table 1) showed in Figs. 2 and 3 are normalized by data under the control condition (myocytes in Tyrode solution). Apelin condition means the addition of 1 nM apelin-16 in Tyrode solution, and apelin + CHE condition means the addition of 1 nM apelin-16 and 5 \(\mu\)M CHE in Tyrode solution. In the indo-1 protocol, the diastolic “true” free [Ca\(^{2+}\)], was not different in four solutions (Fig. 4B).

Effect of apelin on amplitude of E[Ca\(^{2+}\)], transients and contractions in isolated myocytes. The traces in Fig. 1A are representative E[Ca\(^{2+}\)],-transient images recorded from myocytes under different conditions. It is clear that the amplitude of the E[Ca\(^{2+}\)], transients recorded in the apelin myocyte is larger than the one in the control myocyte during twitches. Figure 1B shows original recordings of cell contractions. Figure 2A shows that the amplitude of E[Ca\(^{2+}\)], transients was increased by 43.6 ± 4.56% in the apelin condition versus the control condition (\(n = 14\), \(P < 0.05\)). We observed that apelin increased the sarcomere FS and that the effect lasted for at least 30 min. Figure 2B shows that FS was increased by 55.7 ± 13.9% after apelin administration. When we used the indo-1 as the Ca\(^{2+}\), indicator and calculated the total cytosolic Ca\(^{2+}\), we got similar results as shown in Fig. 4. Apelin increased the amplitude of electric stimulation-induced total cytosolic Ca\(^{2+}\) change versus control (\(n = 5\), \(P < 0.05\), Fig. 4, A and C).

Effect of apelin on time course of E[Ca\(^{2+}\)], transients in isolated myocytes. The cause of the increased Ca\(^{2+}\)-transient amplitude in the presence of apelin was further investigated by examining the time course of the E[Ca\(^{2+}\)], transients. The time to peak (TTP) of E[Ca\(^{2+}\)], indicates the speed of Ca\(^{2+}\) release via RyR from SR (37). Apelin significantly decreased (by 12.53 ± 3.8%) the TTP of E[Ca\(^{2+}\)], during steady-state twitches compared with that of the control group (\(n = 14\), \(P < 0.05\), Fig. 2C). Half-decay time (T50) of E[Ca\(^{2+}\)], transient reflects the Ca\(^{2+}\) clearance speed from cytoplasm (3, 30). In the rat cardiomyocytes, immediately after contraction, most (>90%) of the Ca\(^{2+}\) is taken back to the SR via SERCA (4, 44). Figure 2D shows that T50 of E[Ca\(^{2+}\)], transients was reduced by 30.82 ± 1.17% by apelin (vs. control, \(n = 14\), \(P < 0.05\)). The decreased T50 of E[Ca\(^{2+}\)], mainly reflects an increase in SERCA activity.

Effect of apelin on amplitude and time course of C[Ca\(^{2+}\)], transients. During a normal twitch, the amplitude of the E[Ca\(^{2+}\)], transient highly depends on the SR Ca\(^{2+}\) content. To assess the SR Ca\(^{2+}\) content, we used rapid caffeine application.
Caffeine keeps the RyR open, and all Ca\(^{2+}\) stored in the SR is released. Application of caffeine caused a rapid increase of [\(\text{Ca}^{2+}\)]\(_i\), (C[\(\text{Ca}^{2+}\)], transient) as a result of SR Ca\(^{2+}\)-release (Fig. 3A). The amplitude of C[\(\text{Ca}^{2+}\)], transient is an index of Ca\(^{2+}\) content in SR (22). It was surprising to find that SR Ca\(^{2+}\) content estimated by amplitude of C[\(\text{Ca}^{2+}\)], transient, showed in Fig. 3, A and B, was slightly but significantly (\(P < 0.05\)) reduced (8.41 ± 0.92%) by apelin compared with control (\(n = 14\)). Thus we subsequently analyzed the relationship between the amplitude of E[\(\text{Ca}^{2+}\)], and the SR Ca\(^{2+}\) content (C[\(\text{Ca}^{2+}\)]). The ratio of E[\(\text{Ca}^{2+}\)], to C[\(\text{Ca}^{2+}\)], (E[\(\text{Ca}^{2+}\)]/C[\(\text{Ca}^{2+}\)]), in the presence of apelin was largely enhanced versus control (0.325 ± 0.003 vs. 0.204 ± 0.002, \(n = 14, P < 0.05\)). In our study, apelin reduced the SR Ca\(^{2+}\) content but increased the amplitude of E[\(\text{Ca}^{2+}\)], transient during twitches. During a relaxation course after a twitch, close to 7% of [\(\text{Ca}^{2+}\)] is pumped out of the cell via NCX (4, 44), but in the presence of caffeine, the RyR of SR remains open continuously and the extrusion of [\(\text{Ca}^{2+}\)] from the cytoplasm across the sarcolemma is mainly through the NCX. To assess NCX function, we measured the \(T_{50}\) of C[\(\text{Ca}^{2+}\)], transient. Figure 3C showed that apelin reduced \(T_{50}\) of C[\(\text{Ca}^{2+}\)] transients by 16.22 ± 1.36% versus control condition (\(n = 14, P < 0.05\)). The decrease of C[\(\text{Ca}^{2+}\)], \(T_{50}\) reflects the increase of NCX function.

**Effect of apelin on RC-induced total cytosolic Ca\(^{2+}\) change.**

The same cell in different solutions got different total cytosolic Ca\(^{2+}\) change induced by RC 2 s after the stimulation stopped (shown in Fig. 4A). The total cytosolic Ca\(^{2+}\) was calculated from measured free [\(\text{Ca}^{2+}\)], correcting the mitochondrially localized indo-1 and mitochondrial free [\(\text{Ca}^{2+}\)]; and then, in addition, the reported cytosolic Ca\(^{2+}\) buffer capacity. The SR volume was about 10% of the cell, so 10 times of the RC-induced total cytosolic Ca\(^{2+}\) content was the SR Ca\(^{2+}\) content (Fig. 4C). Apelin decreased the SR Ca\(^{2+}\) content versus control (\(n = 5, P < 0.05\), Fig. 4C), similarly to the result gotten from the caffeine experiment.

**Effect of apelin on SERCA.** \(T_{50}\) of E[\(\text{Ca}^{2+}\)], transient reduced by apelin means that apelin probably increased the SERCA activity. To study whether SERCA is involved in the apelin-induced improvement of contractile function, the present study examined Ca\(^{2+}\)-ATPase activity by an optical assay in crude SR extracted from myocytes. As shown in Fig. 5, apelin increased the SR Ca\(^{2+}\)-ATPase activity in myocytes versus control.
control \((n = 7, P < 0.05)\), but the effect was abolished by CHE completely \((n = 7, P < 0.05)\).

**Effects of blockade of PKC on cardiomyocytes contraction, \(E[Ca^{2+}]_i\), \(C[Ca^{2+}]_i\), transients, RC, and SERCA activity.** Figures 1 and 2A show that there is a moderate but significant increase in apelin + CHE condition \((151.2 \pm 1.075\% , n = 14)\) versus apelin condition \((143.6 \pm 4.56\% , n = 14, P < 0.05)\) on the amplitude of \(E[Ca^{2+}]_i\). Figure 4 also shows the similar result using indo-1 and total cytosolic \(Ca^{2+}\) as values \((n = 5, P < 0.05)\). But the further increase in apelin + CHE did not bring the more relevant increase than apelin; on the contrary, we found that apelin + CHE partially decreased the apelin enhancement of contraction \((n = 14, P < 0.05, \text{Figs. 1 and 2})\).

In addition, we found that 5 \(\mu M\) CHE could completely abolish the negative effect of apelin on caffeine-induced SR releasable \(Ca^{2+}\) content \((\text{Fig. 3, A and B})\), but there was no significant difference in amplitude of \(E[Ca^{2+}]_i/C[Ca^{2+}]_i\) between apelin and apelin + CHE condition \((0.325 \pm 0.003\% \text{ vs. } 0.317 \pm 0.003\% , n = 14, P > 0.3)\). In the RC experiment, CHE also erased the effect of apelin reduction in the SR \(Ca^{2+}\) content \((n = 5, P < 0.05, \text{Fig. 4})\). Although 5 \(\mu M\) CHE slightly increased \(Ca^{2+}\) transient amplitude, they largely, but not completely, attenuated the increasing effect of apelin on single myocyte sarcomere FS \((\text{Fig. 2B; apelin, 155.7 \pm 13.9\% , n = 14 vs. apelin + CHE, 119 \pm 9.7\% , n = 14, P < 0.05})\).

Furthermore, we found that there was no significant difference in the TTP of the E[Ca\(^{2+}\)] \(_i\) transient between the apelin and the apelin + CHE groups \((\text{Fig. 2C})\). Figures 2D and 3C show that CHE abolished the effect of apelin on the reduction of \(T_{50}\) of \(E[Ca^{2+}]_i\) or \(C[Ca^{2+}]_i\) transients, which means that PKC may be involved in the effect of apelin on SERCA activity and NCX function. In the direct measurement of SERCA activity, we found that SERCA activity was increased by apelin versus control \((n = 7, P < 0.05)\) but that CHE perished this effect completely \((n = 7, P < 0.05, \text{Fig. 5})\).

**DISCUSSION**

Consistent with previous study in whole isolated rat heart \((46)\), our results showed that apelin had positive inotropic action on individual rat myocytes. With the positive inotropic action, apelin increased the amplitude of \(Ca^{2+}\) transients and decreased the SR \(Ca^{2+}\) content during electrical stimulations. Furthermore, we found that PKC played an important role in the mechanism of the action of apelin on isolated rat myocytes.

**Contractions and amplitude of \(Ca^{2+}\) transients.** In perfused rat hearts, apelin has a positive inotropic effect \((46)\). In our study, we demonstrated that apelin also has a positive inotropic effect in isolated single rat cardiomyocytes at concentrations of 1 \(nM\) \((\text{Fig. 1B})\). The positive inotropic effect of apelin could in principle be due to \(Ca^{2+}\) availability and/or \(Ca^{2+}\) responsiveness of the myofilaments. The amplitude of \([Ca^{2+}]_i\) transient has been shown to be increased in heart muscles with apelin, and it was concluded that the increase in force development was due to increased \(Ca^{2+}\) availability rather than changes in myofilament \(Ca^{2+}\) responsiveness \((12)\). In our study, we used two different methods to find that the increased shortening induced by apelin was accompanied by the increased amplitude of \(Ca^{2+}\) transients. Furthermore, we found that the effect of apelin on the amplitude of \([Ca^{2+}]_i\) transients was not decreased but that the sarcomere FS was reduced in the presence of CHE, which provided evidence that apelin increases the \(Ca^{2+}\) transient through a PKC-independent way and also gave the feasibility that there are other PKC-dependent mechanisms, reported as NHE activity and intracellular PH value \((17)\), involved in the positive inotropic effect of apelin. These findings could explain the result that the suppression of PKC with staurosporine and GF-109203X markedly attenuates the apelin inotropic effect \((46)\). Therefore, we suggest that apelin acts on myocyte contraction through at least two different ways: 1) a PKC-independent way with a \(Ca^{2+}\) transient increase and 2) a PKC-dependent way without a \(Ca^{2+}\) transient increase.

**Analysis of the time course of \(E[Ca^{2+}]_i\), transients.** The \(Ca^{2+}\) release by the SR is graded \((8)\): the rate and the amount of \(Ca^{2+}\) released from the SR is variable and depends on the \(Ca^{2+}\) current flowing through the \(I_{Ca,L \ LTCC}\), the amount of \(Ca^{2+}\) stored in the SR, and the availability of RyR for activation. The previous result has demonstrated that \(I_{Ca,L \ LTCC}\) via LTCC is not changed by apelin \((46)\). In the present study, we showed that SR \(Ca^{2+}\) content was decreased but that the TTP of \(E[Ca^{2+}]_i\) transient was reduced. Thus the reasonable explanation is that apelin increased the availability of RyR for activation. The speed of \(Ca^{2+}\) release via RyR of SR was significantly faster in the apelin group, which presented the possibility of increasing the amplitude of \(E[Ca^{2+}]_i\) transients even when the SR \(Ca^{2+}\) content was reduced. The mechanism of this effect is not clear, but our data show that this effect is PKC independent \((\text{Fig. 2C})\). \(T_{50}\) of \(E[Ca^{2+}]_i\) transient was decreased by apelin, indicating that SERCA activity was most likely enhanced. We know that the function of SERCA is regulated by phospholamban (PLB) and that phosphorylation of PLB causes a dissociation of PLB from SERCA, allowing for faster rates of SR \(Ca^{2+}\) uptake and relaxation and enhanced contractility \((53)\). In our study, we do not know whether the PKC phosphorylation of the PLB mech-

![Fig. 6. The possible pathway of the action of apelin on cell contractions. “1”, PKC dependent; “2”, PKC independent; “3”, reported (Ref. 17) but not confirmed directly in our study whether through the pH, Na+/H+ exchanger (NHE), and myofilament response. During “2”, there is no direct evidence to show whether and how apelin acts on ryanodine receptor (RyR). If CHE stopped the “1” pathway, the effect of apelin of decreasing SR \(Ca^{2+}\) content will disappear and the apelin increase of \(Ca^{2+}\) transient will be greater than without CHE, but “3” will be stopped at the same time, so the cell contraction is downregulated lastly. The “1” pathway that decreases SR \(Ca^{2+}\) content will indirectly decrease the \(E[Ca^{2+}]_i\), and counteract the increased \(E[Ca^{2+}]_i\), effect of the “2” pathway. The counteraction effect will disappear when blockade “1” with CHE, so CHE could further increase the apelin effect on \(E[Ca^{2+}]_i\). NCX, Na+/Ca2+ exchanger.](http://ajpheart.physiology.org/DownloadedFrom/10.22033.4)
anism was involved, but we tested SERCA activity by an optical assay, and we found that apelin does increase the SERCA activity through a PKC-dependent pathway.

Apelin on the SR Ca\textsuperscript{2+} content. A rapid application of caffeine induces a [Ca\textsuperscript{2+}], transient in myocytes, the amplitude of C[Ca\textsuperscript{2+}], represents the SR Ca\textsuperscript{2+} content, and the T\textsubscript{50} of C[Ca\textsuperscript{2+}], is an index of NCX function. We showed that apelin caused a decrease in SR Ca\textsuperscript{2+} content (Fig. 3, A and B) PKC dependently, and we confirmed this with the RC of myocytes (Fig. 4). The decrease of SR Ca\textsuperscript{2+} content seems incompatible with the increase of SERCA, but we find that apelin increases the NCX activity, too (Fig. 3C). A plausible explanation is that the increase of NCX activity is greater than that of SERCA activity, so the enhanced activity of NCX extrudes more Ca\textsuperscript{2+} and a smaller fraction of the Ca\textsuperscript{2+} will be uptaken by the SERCA during the relaxation result in the decrease of SR Ca\textsuperscript{2+} content in presence of apelin. In our study, apelin caused a 30% and 16% reduction in E[Ca\textsuperscript{2+}], T\textsubscript{50} and C[Ca\textsuperscript{2+}], T\textsubscript{50}, respectively. The T\textsubscript{50} of E[Ca\textsuperscript{2+}] mainly reflects the SERCA activity because the efficiency of SERCA is much higher than that of NCX (4, 44), and in the rat cardiomyocytes, immediately after contraction, most (>90%) of the Ca\textsuperscript{2+} is taken back to the SR via SERCA. However, when caffeine is present, SR cannot hold Ca\textsuperscript{2+} anymore: the extrusion of cytosolic Ca\textsuperscript{2+} is mainly trough NCX, so the speed of decline in C[Ca\textsuperscript{2+}], is slower than in E[Ca\textsuperscript{2+}]. The 30% reduction in E[Ca\textsuperscript{2+}], T\textsubscript{50} suggested that the SERCA activity was enhanced, which was confirmed by the measurement of SERCA activity directly, but the 16% reduction in C[Ca\textsuperscript{2+}], suggested that NCX function increased. In the present study, the measurement of the NCX activity is not direct. For this reason, future studies are needed to test the NCX activity more directly and try to give solid evidence to confirm the relationship between apelin and NCX.

Role of PKC in the effect of apelin. The activation of PKC has many cardiomyocyte effects (10, 35, 48, 57), but the mechanisms by which PKC mediates its effects are not fully understood. In our study, apelin decreased the SR Ca\textsuperscript{2+} content in a PKC-dependent fashion, but the positive effect of apelin on the amplitude of E[Ca\textsuperscript{2+}], is likely PKC independent. In addition, the increasing effect of CHE on E[Ca\textsuperscript{2+}], transient showed in Fig. 2A may be secondary to the larger SR Ca\textsuperscript{2+} content showed in Fig. 3B, since the amplitude of E[Ca\textsuperscript{2+}]/C[Ca\textsuperscript{2+}], was not different between apelin group and apelin + CHE group. The possible explanation, shown in Fig. 6, is that apelin not only has an increase effect on E[Ca\textsuperscript{2+}], but also has a decrease effect on SR Ca\textsuperscript{2+} content, and the decrease effect on SR Ca\textsuperscript{2+} content could decrease the E[Ca\textsuperscript{2+}], indirectly. When we used apelin + CHE solution, the PKC-independent decrease effect on E[Ca\textsuperscript{2+}], was still working, but the decrease effect on SR Ca\textsuperscript{2+} was stopped, so the SR Ca\textsuperscript{2+} content was increased. And consequently, CHE further enhances the E[Ca\textsuperscript{2+}]. The PKC-involved enhancement effect on Ca\textsuperscript{2+} extrusion via NCX, shown in Fig. 3C, could explain the SR Ca\textsuperscript{2+} content decrease, but the decreased T\textsubscript{50} of E[Ca\textsuperscript{2+}], shown in Fig. 2D, by apelin is mainly due to the enhancement of SERCA activity. PKC inhibitor CHE partially blocked the positive inotropic action of apelin (shown in Fig. 2B) without decreasing the amplitude of E[Ca\textsuperscript{2+}], which indicates that a PKC-dependent mechanism was partially involved in the positive inotropic action of apelin. The possible pathways of these effects are shown in Fig. 6.

In conclusion, the present data demonstrate that apelin has a positive inotropic effect on individual isolated rat cardiac myocytes. The increased amplitude of [Ca\textsuperscript{2+}], transient during EC coupling is one of the reasons of this positive inotropic effect; the other mechanism of this effect is PKC mediated. During electrical stimulation twitches, apelin decreases the myocyte SR Ca\textsuperscript{2+} content via a PKC-dependent pathway. NCX function and SERCA activity are also most likely enhanced by apelin PKC dependently.

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