Protein kinase A does not alter unloaded velocity of sarcomere shortening in skinned rat cardiac trabeculae

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Jannsen, Paul M. L., and Pieter P. de Tombe. Protein kinase A does not alter unloaded velocity of sarcomere shortening in skinned rat cardiac trabeculae. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2415–H2422, 1997.—Whether β-adrenergic stimulation affects the cross-bridge cycling rate independently of its effect on Ca\(^{2+}\) handling by the cardiac myocyte is still unknown. An increase in cross-bridge cycling rate may result in increased unloaded velocity of sarcomere shortening (V\(_o\)). To test this hypothesis directly, skinned rat cardiac trabeculae were attached between a silicon strain gauge (3.5 kHz resonant frequency) and a fast displacement motor. V\(_o\) was measured by a modified “Edman slack test” during a single maximal activation using seven to eight sarcomere-length step releases (measured by laser diffraction) ranging between 0.12 and 0.20 µm (15.0 ± 0.1°C). β-Adrenergic stimulation was mimicked by exposing the trabeculae to the catalytic subunit of protein kinase A (PKA). Treatment with PKA (3 µg/ml; 45 min) caused a significant (P < 0.01) increase (41 ± 13%) in the Ca\(^{2+}\) concentration required for half-maximal steady-state tension development. Neither maximum tension nor V\(_o\) was affected by treatment with PKA, suggesting that β-adrenergic stimulation does not affect the rate-limiting step of cross-bridge cycling during unloaded shortening in myocardium.

Edman slack test; contractile protein phosphorylation; β-adrenergic stimulation; cross-bridge cycling; calcium sensitivity

STIMULATION of β-adrenergic receptors has a profound impact on myocardial contractile state. This is evidenced by an increase in both the rate and level of force development, as well as an increase in the rate of force relaxation (30). The response to β-adrenergic stimulation is mediated by adenosine 3′,5′-cyclic monophosphate-dependent protein kinase A (PKA). The increased PKA activity results in phosphorylation of several proteins intimately involved in Ca\(^{2+}\) handling, such as phospholamban, the sarcomemmal Ca\(^{2+}\) channel, and troponin I (16, 32). It has been suggested, therefore, that the effects of β-adrenergic stimulation in the heart are mainly due to altered Ca\(^{2+}\) handling (13, 24, 34). Recent studies on both mechanical (2, 20, 31, 33) and biochemical (36) properties of isolated myocardium, however, have suggested that the inotropic effects of β-adrenergic stimulation may be due, in part, to a direct effect of PKA-induced protein phosphorylation on cross-bridge cycling kinetics. However, this hypothesis has been challenged by other investigators (4, 7, 9, 19). Therefore, whether β-adrenergic stimulation affects cross-bridge cycling kinetics is currently still under debate.

An increase in cross-bridge cycling kinetics is expected to result in an increase in the unloaded velocity of sarcomere shortening (V\(_o\)). Measurement of V\(_o\) has recently been used to investigate the effect of β-adrenergic stimulation in single isolated myocytes by two groups of investigators who derived opposing results. Strang et al. (33) observed a significant increase in V\(_o\), whereas Hofmann and Lange (19) found no change in V\(_o\) after PKA treatment. These conflicting results may have resulted from technical limitations that hamper accurate assessment of shortening velocity in single-skinned cardiac myocytes. These include a low signal-to-noise ratio for force measurement, various degrees of uncontrolled damaged-end compliance, and rundown of the preparation. In the present study, therefore, we reexamined the impact of PKA treatment on V\(_o\) in skinned cardiac trabeculae. This multicellular preparation presents certain advantages over the use of single isolated myocytes. First, the preparation is sufficiently stable, at low temperatures, to allow for the measurement of V\(_o\) at maximal activation during a single activation-relaxation cycle. Adoption of this protocol, therefore, reduces the extent of rundown of the preparation because of the substantial reduction in the number of activation-relaxation cycles required to measure V\(_o\). Second, skinned cardiac trabeculae can be attached to the apparatus via aluminum T clips, which limits the extent of uncontrolled sarcomere length (SL) shortening during maximal activation to −50 mm (~2.5%); in addition, it allows for the use of laser diffraction techniques to monitor SL just before the length release steps required for the measurement of V\(_o\). Adoption of this technique ensures that each release is initiated from the same SL and, therefore, from the same level of isometric force development. In addition, it ensures that V\(_o\) is measured within a range of SL at which there is no restoring force to limit V\(_o\). Finally, as a result of the stability of the preparation, it is possible to measure V\(_o\) both before and after exposure to PKA in each individual skinned cardiac trabecula. This allows for the use of paired statistical analyses to allow detection of small differences in V\(_o\) induced by PKA treatment.

In the present study we found a significant reduction in the sensitivity of the contractile system to Ca\(^{2+}\) for force development after PKA treatment. However, we did not observe an impact of PKA treatment on the unloaded velocity of sarcomere shortening. These results suggest that β-adrenergic stimulation does not affect the rate-limiting step of cross-bridge cycling during unloaded shortening in myocardium.

MATERIALS AND METHODS

Muscle preparation. Rats (LBNF-1; 200–250 g) were anesthetized with halothane, and the hearts were rapidly excised.
All procedures used in the present study were in accordance with institutional guidelines regarding the care and use of laboratory animals. After exsion, the heart was placed in a dissection dish and immediately perfused through the aortic stump with a Krebs-Henseleit solution that also contained 20 mmol/l 2,3-butanedione monoxime to prevent beating of the heart during dissection. Thin, uniform, and unbranched trabeculae were carefully dissected from the free wall of the right ventricle. After dissection, the trabeculae were transferred to a cold (4°C) standard relaxing solution to which 1% (vol/vol) Triton X-100 was added. The trabeculae were left in this solution for a minimum of 2 h to allow solubilization of all membranous structures. The average size of the trabeculae was 1.35 ± 0.07 mm in length, 219 ± 23 µm in width, and 80 ± 8 µm in thickness (means ± SE, n = 15; measured at SL = ~2.0 µm in relaxing solution). Next, the skinned trabeculae were attached to aluminum T-dips and mounted in the experimental setup. The T-dips were glued with superglue to the small stainless steel hooks that extended from both the motor arm and the force transducer to ensure rigidity of attachment.

Solutions. Three bathing solutions were used: a relaxing solution, a preactivating solution with low Ca²⁺-buffering capacity, and an activating solution. The ionic compositions of these solutions are shown in Table 1. The solutions were calculated using the methods of Fabiato and Fabiato (14). To achieve a range of free Ca²⁺ concentration (ICa²⁺), activating and relaxing solutions were appropriately mixed with an assumed apparent stability constant of the Ca²⁺-ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetracetic acid complex of 10⁶⁻³⁹. The ionic strength of the solutions was kept at 200 mmol/l by adding the appropriate amount of potassium propionate. The pH was adjusted to 7.0 at 15.0°C with KOH (see also Table 1). The PKA solution was a standard relaxing solution to which 3 µg/ml protein kinase catalytic subunit (prepared from porcine heart) and 5 mmol/l diithiothreitol were added. The PKA-inhibitor solution was prepared from the PKA solution by adding 63 µg/ml protein kinase inhibitor (prepared from porcine heart). All chemicals were of the highest purity available (Sigma Chemical, St. Louis, MO).

Experimental apparatus. The skinned trabeculae were attached on one end to a servomotor (model 308B, Cambridge Technology, Watertown, MA; ~0.2-ms 90% step-response time) and on the other end to a modified silicon strain gauge (model AE801, SenSonor, Horten, Norway). The natural resonance frequency of the force transducer was ~3.5 kHz with the muscle attached. The muscle bath was formed by a 100-µl trough milled in an anodized aluminum block through which water was circulated to allow for temperature control. Throughout the experiment, temperature was monitored and kept constant at 15.0 ± 0.1°C. SL was measured as described previously (6, 8, 35). Briefly, the intensity distribution of the first-order diffraction band was monitored by a 512-element photodiode array that was scanned every 580 µs. Median SL was calculated by an analog circuit after a correction had been made for zero-order light scattering. Potential sources of error associated with the use of laser diffraction techniques to measure SL include Bragg angle reflection artifacts and inhomogeneity of the muscle preparation. De Tombe and ter Keurs (8) have previously shown that the possible error in the measurement of SL due to these artifacts is <4% in our measurements. Tension was calculated as force divided by cross-sectional area. The latter was calculated from the muscle dimensions by assuming an ellipsoid shape. Force and SL signals were recorded on a strip chart recorder; force, SL, and muscle length were also stored on computer disk for off-line analysis (10 kHz/channel sampling frequency).

Experimental protocol. The protocol consisted of two series of measurements: a first series under control conditions (pre-PKA), and a second series after exposure of the trabecula to the catalytic subunit of PKA (post-PKA). To test whether the effects of PKA treatment were specific for PKA, the protocol was repeated in a separate group of trabeculae in which the muscle preparations were simultaneously exposed to both PKA and a PKA-specific inhibitor.

After being mounted, the trabeculae were stretched to a resting SL of 2.2–2.3 µm (near-maximal active tension) and the bath was flushed two times with the relaxation solution. Before each activation, the muscle was incubated for 3–4 min in the relaxing solution, followed by 3–4 min of incubation in the preactivating solution. All solution exchanges were performed with three to four bath volumes to ensure complete replacement of the solution. Although end compliance was minimized, it could not altogether be eliminated. Hence, in each trabecula a series of test contractions was conducted to examine the extent of SL shortening during maximal activation (which was always <50 nm; see Fig. 1). Resting SL was then adjusted in each trabecula such that active SL was 2.2 µm at maximum activation. It was found after two to three preactivations that maximal activation that lasted 5 s did not require further adjustment throughout the experiment. Next, the sensitivity of force development to Ca²⁺ was determined by activating the muscle during a series of preactivating-activating relaxation cycles using nine levels of free [Ca²⁺] in the activating solution. Furthermore, a contraction at saturating [Ca²⁺] both before and after this series of measurements was used to test for force rundown of the preparation. Subsequently, V₀ was measured during a contraction at the maximum level of activation at saturating [Ca²⁺] (see below). After these measurements were taken (that is, after the pre-PKA series), the trabeculae were incubated with the PKA solution at 20.0 ± 0.1°C for 45 min. It was found in preliminary experiments that incubation at 20°C (rather than at 15°C) was essential to ensure a maximal effect of PKA stimulation on the force-[Ca²⁺] relationship. After incubation, the solution was replaced three times by the standard relaxation solution over a period of ~15 min. Next, the force relationship and V₀ at saturating [Ca²⁺] were again determined (post-PKA series).

V₀ was measured during a contraction at the level of activation at saturating [Ca²⁺] using the “Edman slack test” (12, 21) (see Figs. 1 and 2). Briefly, force was allowed to stabilize after the onset of the contraction for ~20 s. Next, a series of seven to eight quick releases (~1 ms) of increasing magnitude was imposed onto the muscle preparation. The

Table 1. Composition of solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>MgCl₂</th>
<th>Na₂ATP</th>
<th>EGTA</th>
<th>HDTA</th>
<th>Ca²⁺-EGTA</th>
<th>K Prop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxing</td>
<td>6.63</td>
<td>5.98</td>
<td>20</td>
<td></td>
<td></td>
<td>41.2</td>
</tr>
<tr>
<td>Preactivating</td>
<td>6.31</td>
<td>5.98</td>
<td>0.5</td>
<td>19.5</td>
<td></td>
<td>41.8</td>
</tr>
<tr>
<td>Activating</td>
<td>6.20</td>
<td>6.11</td>
<td></td>
<td>20</td>
<td></td>
<td>41.5</td>
</tr>
</tbody>
</table>

Ca²⁺-ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetracetic acid (EGTA) was made by mixing equimolar amounts of CaCl₂ and EGTA. In addition, all solutions contained (in mmol/l) 10 phosphorocreatine, 100 N,N-bis[2-hydroxyethyl]-1,2-aminethanesulfonic Acid (BES), 0.1 leupeptin, 0.1 phenazine methosulfate (PMSF), 1 diithioctetol (DTT), and 4 U/ml creatine phosphokinase. Free Mg²⁺ and Mg-ATP concentrations were calculated at 1 and 5 mmol/l, respectively. Relaxing and activating solutions were appropriately mixed to obtain a range of free Ca²⁺ concentrations ([Ca²⁺]) in the activating solution. An apparent stability constant of Ca²⁺-EGTA complex of 10⁶⁻³⁹ at 15°C was assumed.
step size of these releases (SL_{step}) was calibrated in the relaxed preparation (range: SL = 0.12–0.20 µm). The muscle was returned to its initial length using a slow ramp stretch (500-ms duration) starting 200 ms after the step release. As shown by the typical experiment in Fig. 1, force development increased to levels above the steady-state during the ramp stretch and then returned within a few seconds to the steady-state level after the stretch. Thus each release was initiated from both the same SL (that is, within ~10 nm) and the same level of isometric force development. Adoption of this protocol allows for the collection within 1 min of a complete Edman slack test containing seven to eight releases during a single contraction at maximal activation.

Data processing and statistical analysis. Sigmoidal force-[Ca^{2+}] relationships were fit by a nonlinear fit procedure (25) to a modified Hill equation

\[ F = F_{\text{max}} \cdot [\text{Ca}^{2+}] \cdot n / ([\text{Ca}^{2+}] + E_{C_{50}}) \]  

where \( F \) is steady-state force, \( F_{\text{max}} \) is the maximum saturated value that \( F \) can attain, \( E_{C_{50}} \) is the [Ca^{2+}] at which \( F \) is 50% of \( F_{\text{max}} \) and represents a compound affinity constant, and \( n \) represents the slope of the force-[Ca^{2+}] relationship (the Hill coefficient). The fit parameters that resulted from the nonlinear fit of the data to Eq. 1 for the pre- and post-PKA were next subjected to a paired Student's t-test, i.e., the fit parameters were treated statistically as if they were obtained by direct measurement (28). In addition, the pooled data from all experiments were analyzed according to Meddings et al. (26) using a simultaneous nonlinear fit to Eq. 1 of the pre- and post-PKA data to directly test for an effect of PKA treatment on the force-pCa relationship. For the purpose of displaying the data obtained in all trabeculae, steady-state force data were normalized to steady-state force measured during the maximal level of activation in the pre-PKA series.

\( V_o \) was calculated as illustrated in Fig. 2 (further analysis of the data shown in Fig. 1). First, for each of the quick releases, the duration of unloaded sarcomere shortening after the step release (T_{slack}) was determined by measuring the duration between the quick release and the time point at which force redeveloped (which is the moment at which the slack introduced by the quick release is just taken up by the muscle preparation). This time point was calculated from a linear regression fit of the force data during the initial phase of force redevelopment and its intersection with the force baseline (cf. dashed lines in Fig. 2A). The force baseline was established by a linear regression fit of the force data during unloaded sarcomere shortening of the largest release step (i.e., SL_{step} = 0.20 µm); this baseline was then applied to all releases. Next, SL_{step} was plotted as a function of T_{slack} (cf. Fig. 2B); the slope of this relationship, which was calculated by linear regression, corresponds to \( V_o \).

To assess the impact of PKA treatment on \( V_o \), the data set obtained in all muscle preparations was analyzed by multiple linear regression (17) using the following model

\[ \text{SL}_{\text{step}} = \alpha + (\beta \cdot \text{T}_{\text{slack}}) + (\gamma \cdot \text{pka}) + (\delta \cdot \text{pka} \cdot \text{T}_{\text{slack}}) \]  

where pka is a dummy variable coding for the pre-PKA (pka = 0) and post-PKA (pka = 1) series data, respectively; and \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \) are regression coefficients. In addition, Eq. 2 was extended with additional dummy variables to allow for inter-experiment variation for both the \( \alpha \) and \( \beta \) parameters (17). Note that a significant coefficient \( \beta \) in Eq. 2 would indicate a significant correlation between SL_{step} and T_{slack}, irrespective of PKA treatment, whereas a significant coefficient \( \delta \) would...

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Fig. 1. Original recordings of force (F), muscle length (ML), and sarcomere length (SL) during determination of unloaded velocity of sarcomere shortening (\( V_o \)) by modified slack test. A: SL (top) and F (bottom) recorded by chart recorder. Wash-in of activating solution is indicated by upward arrow, and wash-in of relaxing solution is indicated by downward arrow. B: SL (top), ML (middle), and F (bottom) sampled by computer at a faster rate (10 kHz), superimposed for each release. Calibrations as indicated; trabecula was 1.5 mm in length, 220 µm in width, and 60 µm in thickness; temperature was 15°C; free [Ca^{2+}] was calculated at 39.8 µmol/l (pCa 4.4). Note that 1st quick release was not sampled at fast rate in this example due to a computer trigger error; magnitude of 2nd release step, therefore, was identical to that of 1st release step.

Fig. 2. Calculation of \( V_o \) by Edman slack test (further analysis of Fig. 1 data). A: superimposed recordings of force data obtained during first 30 ms after 8 quick releases of increasing magnitude. After a period of unloaded shortening (T_{slack}), force abruptly redeveloped. T_{slack} was calculated from a linear regression fit to force data during initial phase of force redevelopment and its intersection with force baseline (cf. dashed lines). B: SL release step size (SL_{step}) plotted as a function of T_{slack}. Linear regression (solid line) revealed a \( V_o \) of 5.6 µm/s and an intercept of 0.106 µm.
indicate an effect of PKA treatment on the coefficient $\beta$ (i.e., $V_o$).

Commercially available software (SYSTAT, INSTAT) was used for all statistical analyses. Data are presented as means ± SE; a value of $P < 0.05$ was considered significant.

RESULTS

Figure 1 shows original recordings obtained during a typical maximal contraction-relaxation cycle of a skinned trabecula in which $V_o$ was measured. Figure 1A shows SL (top) and force (bottom) as recorded by chart recorder. After the activation solution is washed in (indicated by the upward arrow), force rapidly increased to attain a steady state within ~20 s. During this time, SL followed a complex pattern of shortening that settled to reach a new steady state at 2.2 µm. Uncontrolled SL shortening during activation is a consequence of damaged-end compliance and is always observed in isolated myocardium (8, 23, 35). Therefore, resting SL was adjusted in each trabecula such that active SL was 2.2 µm at maximum activation (see METHODS). During the steady state, a series of quick releases was imposed so as to measure $V_o$ by the Edman slack test. This method is illustrated in greater detail in Fig. 1B showing superimposed SL (top), muscle length (middle), and force (bottom) during the releases as sampled by computer at a faster rate (10 kHz). During the period of unloaded shortening, SL could not accurately be assessed by laser diffraction due to buckling of the muscle preparation. After the period of unloaded shortening, that is, at the time of force redevelopment and when the muscle preparation was just taut again (21), SL had decreased by ~60–140 nm. The amount of sarcomere shortening is due to the intrinsic elasticity of the sarcomeres (1, 15) and the actual extent of unloaded shortening of the sarcomeres. Next, force redeveloped in an exponential fashion while the sarcomeres continued to shorten, presumably due to stretching of the damaged-end series elasticity. The time at which force redeveloped was used to calculate the duration of unloaded sarcomere shortening. This is shown in greater detail in Fig. 2 (further analysis of data in Fig. 1). Figure 2A shows, superimposed, the initial 30 ms of force data after the quick releases, and Fig. 2B shows the relationship between $T_{slack}$ and $SL_{step}$. Several observations are apparent from these data. First, the high signal-to-noise ratio of the force record obtained from the isolated trabecular preparation allows for accurate determination of the time of force redevelopment (Fig. 2A, dashed lines; see METHODS). Second, these data illustrate the relatively small compliance of the attachment between the muscle preparation and the measurement apparatus, as evidenced by the abrupt transition between baseline force and force redevelopment after each of the quick releases. Finally, the duration of unloaded shortening was linearly proportional to the extent of the quick release, as illustrated in Fig. 2B. One potential confounding factor in the use of the Edman slack test protocol is that $V_o$ may change over time during the contraction as a result of ADP accumulation within the muscle preparation (5). To prevent ADP buildup, the solutions contained an abundance of phosphocreatine kinase and phosphocreatine so as to rapidly regenerate ATP from ADP and phosphocreatine. Furthermore, in a series of preliminary studies, $V_o$ was calculated from two consecutive slack test protocols performed during a single maximal contraction of twice the normal duration. On average, $V_o$ calculated from the second slack test was 99.5 ± 2.8% of $V_o$ calculated from the first slack test. Finally, two additional trabeculae, the order of the applied step releases was reversed during a second maximal contraction; $V_o$ calculated from this second slack test was 100 and 97% of $V_o$ calculated from the first slack test, respectively. Hence, $V_o$ appeared to be stable for the duration of the maximal contraction in our experiments.

Figure 3 shows the relationship between steady-state force and free [Ca$^{2+}$] in the bathing solution obtained in the PKA series (Fig. 3, A and C) and in the PKA + inhibitor series (Fig. 3, B and D). The data obtained under control conditions are indicated by open symbols; the data obtained after treatment with PKA (or PKA + inhibitor) are indicated by filled symbols. Figure 3, A and B, shows data from a representative muscle preparation, whereas Fig. 3, C and D, shows the average normalized data obtained in all trabeculae. The solid lines indicate the best fit of the force-[Ca$^{2+}$] coordinates to a modified Hill equation; the average fit parameters obtained in all trabeculae in the two groups are shown in Table 2. Consistent with previous reports (18, 19, 32, 33), treatment with PKA in skinned myocardium induced a rightward shift in the force-[Ca$^{2+}$] relationship. Thus PKA treatment resulted in a 41 ± 13% increase in the EC$_{50}$ parameter, indicating that PKA treatment caused a significant reduction in the sensitivity of the muscle preparation to Ca$^{2+}$. However, neither maximum steady-state force at saturating [Ca$^{2+}$] nor the slope of the force-[Ca$^{2+}$] relationship (as indexed by the Hill coefficient) was significantly affected by PKA treatment. Analysis of the pooled force-[Ca$^{2+}$] relationship according to Meddings et al. (26) resulted in similar results, that is, a significant increase in the EC$_{50}$ parameter ($P < 0.1$) without significant changes in either maximum force or the Hill coefficient. The rightward shift of the force-[Ca$^{2+}$] relationship was a specific effect of PKA treatment, because it could be blocked entirely by simultaneous incubation with the protein kinase-specific inhibitor (19). This is evidenced by the data shown in Fig. 3, B and D, and Table 2. Finally, PKA treatment did not affect the passive properties of the muscle preparation: passive force development in the relaxing solution was 3.6 ± 0.8 and 3.5 ± 0.8 mN/mm$^2$ in the control condition and post-PKA treatment, respectively ($P = 0.6$).

Figure 4 shows the relation between $T_{slack}$ of the muscle preparation and $SL_{step}$ in the PKA series (Fig. 4, A and C) and in the PKA + inhibitor series (Fig. 4, B and D). The data obtained under control conditions are indicated by open symbols; the data obtained after treatment with PKA (or PKA + inhibitor) are indicated by filled symbols. Figure 4, A and B, shows data from
the representative muscle preparation (cf. Fig. 3). $T_{\text{slack}}$ was linearly proportional to $SL_{\text{step}}$ in all muscle preparations and in both groups. However, the relationship was not affected by PKA treatment alone or PKA treatment in the presence of the specific inhibitor. That this was also observed in the pooled averaged data is shown in Fig. 4, where it is seen that the data from the pre-PKA and post-PKA series cluster close to a common regression line. Multiple linear regression analysis using the data from all muscle preparations confirmed this conclusion. The regression coefficients obtained by this analysis are presented in Table 3.

Multiple linear regression analysis using the data from all muscle preparations confirmed this conclusion. The regression coefficients obtained by this analysis are presented in Table 3. $T_{\text{slack}}$ was significantly correlated to $SL_{\text{step}}$ in both groups, regardless of PKA treatment status (coefficient $\beta$), whereas treatment with PKA resulted in only a small and nonsignificant increase in the slope of the relation (coefficient $\delta$; 0.3%) and a small but significant increase in the elevation of the relationship (coefficient $\gamma$; 3.1%). A similar result was obtained in the PKA inhibitor series. Thus, although PKA treatment in skinned myocardium significantly affected the force-[Ca$^{2+}$] relationship, it did not affect $V_0$ at maximal activation. Finally, the standard error of the relationship between $SL_{\text{step}}$ and $T_{\text{slack}}$ was 3.6% in the pre-PKA series and 3.4% in the post-PKA series.

**DISCUSSION**

On average, maximal steady-state force development in the control condition was 64.4 mN/mm$^2$. This amount of steady-state force is comparable to that found in previous studies using skinned cardiac preparations (6, 7, 11, 22). The extent of force rundown was virtually eliminated in the present study, that is, each determination of the force-[Ca$^{2+}$] relationship was associated with a 0.4 ± 2.2% increase in maximum force development at saturating [Ca$^{2+}$]. Likewise, maximum force development in the final test contraction in the PKA inhibitor series was 103 ± 4.3% of the first test contraction in that group of muscle preparations. The lack of force rundown in the present study is likely due to both the use of a low temperature (15°C) and the limiting of the measurement of $V_0$ by the Edman slack
test at saturating levels of \([\text{Ca}^{2+}]\) to a single contraction. On average, \(V_o\) in the control series was 6.14 µm/s; this value is comparable to previous studies on intact electrically stimulated rat cardiac trabeculae (8, 9). At a sarcomere length of 2.2 µm, the calculated \(V_o\) was 2.8 muscle lengths/s, which is comparable to previous studies on skinned single isolated rat cardiac myocytes (19, 33).

Incubation of the trabeculae with PKA resulted in a significant rightward shift in the force-[Ca\(^{2+}\)] relationship that could be blocked entirely by simultaneous incubation with a PKA-specific inhibitor. On average, the EC\(_{50}\) parameter of the fit to the modified Hill equation increased from 1.95 ± 0.12 to 2.70 ± 0.22 µmol/l after 45 min of exposure to PKA at 20°C. These values translate into a rightward shift of 0.14 pCa units (\(-\log[\text{Ca}^{2+}]\)), which is comparable to the shift found previously by de Tombe and Stienen (7) in skinned rat cardiac trabeculae and by other investigators in skinned single isolated rat myocytes (19, 33). Strang et al. (33) and Hofmann and Lange (19) demonstrated that PKA treatment results in a substantial phosphorylation of the troponin I and C protein subunits. Likewise, it has been shown that \(\beta\)-adrenergic stimulation in unskinned myocardium leads to phosphorylation of these same contractile protein subunits (32). Thus it is likely that treatment with PKA in skinned myocardium mimics the effects of \(\beta\)-adrenergic stimulation on contractile function. The present study therefore supports the hypothesis that phosphorylation of the troponin I and C protein reduces the \(\text{Ca}^{2+}\) responsiveness of the contractile apparatus for force development (19, 32, 33). It should be noted, however, that the extent of contractile protein phosphorylation induced by PKA treatment in the present study may differ from that in intact myocardium after \(\beta\)-adrenergic stimulation.

Although PKA treatment affected the \(\text{Ca}^{2+}\) responsiveness of the myofilaments, it did not alter \(V_o\). Our results are in contrast to the results of Strang et al. (33), who observed a 41% increase in \(V_o\) in PKA-treated skinned cardiac myocytes compared with a control.
group. Likewise, previous mechanical measurements have suggested a direct effect of β-adrenergic stimulation in intact myocardium to increase cross-bridge cycling rate by ~50% (2, 20). Our results, however, are consistent with the findings of Hofmann and Lange (19), who observed no change in \( V_o \) in skinned cardiac myocytes after PKA stimulation. Our results are also consistent with a previous study in intact rat cardiac trabeculae in which de Tombe and ter Keurs (9) found that β-adrenergic receptor stimulation with isoproterenol was without an effect on the maximum velocity of sarcomere shortening during a twitch. Finally, de Tombe and Stienen (7) recently observed that PKA stimulation is also without an effect on the economy of force maintenance in skinned rat cardiac trabeculae. The standard error of the slope of the relationship between \( SL_{\text{step}} \) and \( T_{\text{slack}} \) was 3.6% in the pre-PKA series and 3.4% in the post-PKA series. Therefore, it is unlikely that we would not have been able to detect an effect of PKA treatment on \( V_o \), if the effect were as large as some of these studies suggest. It is not possible, using the current data, to determine whether a possible effect on cross-bridge kinetics would result if troponin I or C protein were to be phosphorylated in isolation. Furthermore, it is not possible at present to determine the relative degrees to which the various residues of either troponin I or C protein are phosphorylated by PKA treatment. Hence, some of the variable results regarding the effect of PKA treatment on cross-bridge cycling may be due to variable degrees of contractile protein phosphorylation induced by PKA between the studies. Future experiments employing transgenic models in which specific phosphorylation sites of contractile proteins are modified are required to address this important issue in detail (29). In addition, determination of cross-bridge kinetic parameters with other techniques, such as dynamic transfer function analysis of sarcomere length to force generation (3), may help resolve some of the variable results that have been obtained thus far.

It is possible that PKA treatment affects \( V_o \) at reduced levels of contractile activation but not at maximum levels of contractile activation at saturating \([Ca^{2+}]\). It should be pointed out, however, that such an effect would be difficult to determine, because contractile activation itself has been shown to be a determinant of \( V_o \) (10, 27). Furthermore, the accuracy of the Edman slack test is considerably reduced at low levels of contractile activation due to reduced signal-to-noise ratio of the force recording and reduced rate of force redevelopment at reduced levels of \([Ca^{2+}]\) activation (37) after the period of maximum shortening of the muscle to take up the slack.

PKA treatment took place at 20°C, whereas the mechanical measurements were made at 15°C. However, it is unlikely that adoption of this protocol affected the results. In an earlier stage of the study, we performed the experiment at a constant temperature throughout the experiment (20°C). We observed a similar rightward shift in the force-pCa relationship after PKA incubation (0.15 pCa units at 20°C vs. 0.14 pCa units at 15°C). \( V_o \) was 11.74 ± 3.13 \( \mu \)m/s (control) compared with 11.64 ± 2.72 \( \mu \)m/s (PKA; \( P = 0.86 \)). However, the period over which the muscle was slackened was considerably reduced due to the higher shortening velocity at that temperature. This resulted in a higher standard deviation of the slope of the slack tests (9.5 vs. 3.6% in the control series and 8.6 vs. 3.4% in the PKA series at 15°C and 20°C, respectively). Hence, to detect a potentially small effect of PKA treatment on \( V_o \), we required the higher level of resolving power and opted to perform the experiments at 15°C.

In the present study, \( V_o \) was measured with the use of a modified Edman slack test (12, 21). There are several factors that determine whether the method provides an accurate estimate of sarcomere \( V_o \) (cf. Figs. 1 and 2). It is assumed that the sarcomeres are shortening under conditions at which there are no opposing forces to limit the shortening velocity. It is likely that this condition was met in our experiments. Force redevelopment occurred at a time at which \( SL \), as measured by laser diffraction techniques, was well above slack SL (which is ~1.90 \( \mu \)m in rat cardiac trabeculae) (8, 35). In addition, it is essential that the time at which force redevelops can be assessed accurately. This was the case in our study, because force redeveloped abruptly after the period of unloaded shortening. It is also assumed that the properties of the series elastic element are purely elastic without significant aspects of creep or viscous behavior. Although it is difficult to determine whether this condition was met in our study, it is likely that this factor did not play a significant role; all slack test plots were highly linear (\( r^2 = 0.994 ± 0.001 \)). Another reason for the high degree of linearity of the slack plot is likely related to the fact that \( V_o \) was measured during a single contraction in which resting SL was continuously monitored. Adoption of this technique assured that the amount of force development and, therefore, the amount of stretch of the series elastic element were constant for each of the quick releases. Finally, although there was a significant increase in the intercept of the slack test plot after PKA treatment (cf. Fig. 4 and Table 3), it was also small (3.1%). Hence, it is unlikely that a change in the properties of the series elastic element would underlie our inability to find a significant impact of PKA treatment on \( V_o \). Our finding that PKA treatment does not affect the passive property of skinned myocardium is consistent with previous studies (7, 19) but is in contrast with the study of Strang et al. (33), who found a 40% decrease in passive tension after PKA treatment. It is of note that these authors also found an effect of PKA treatment on \( V_o \). Whether these phenomena are related, however, cannot be determined from the present study.

In summary, our results support the hypothesis that PKA treatment results in a reduction of \([Ca^{2+}]\) responsiveness of the contractile apparatus. However, PKA treatment does not alter the unloaded velocity of sarcomere shortening in rat myocardium. This finding suggests that β-adrenergic stimulation does not affect the rate-
limiting step in the cross-bridge cycle during rapid shortening.

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