Effects of Dibutyryl Cyclic AMP, Ouabain, and Xanthine Derivatives on Crossbridge Kinetics in Rat Cardiac Muscle

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In a previous communication, we showed that β-adrenergic stimulation of cardiac muscle was associated with an increase in the rate of cycling of crossbridges as measured by perturbation analysis in the frequency domain. In this analysis, the frequency at which dynamic stiffness is a minimum (f_{min}) is taken as a measure of the rate of crossbridge cycling. In this paper, we test the hypothesis that the β-adrenergic receptor–induced increase in crossbridge cycling rate is mediated by elevation of the intracellular level of cyclic AMP. The approach taken is to compare the effects on f_{min} in rat papillary muscles during Ba^{2+}-activated contractures of 1) an agonist of cyclic AMP that can easily penetrate the cell, namely, dibutyryl cyclic AMP, 2) agents that block cyclic AMP phosphodiesterase, namely, the xanthine derivatives isobutylmethylxanthine and caffeine, and 3) an inotropic agent that does not affect the intracellular level of cyclic AMP, namely, ouabain. Our results showed that dibutyryl cyclic AMP at a dose of 5 mM has the same actions as β-adrenergic stimulation: it potentiated the isometric twitch force, reduced the time to peak tension and time to half relaxation, and shifted f_{min} by a factor of 1.8±0.1 (n=5). Isobutylmethylxanthine at up to 1.1 mM also acted in the same manner, increasing f_{min} by a factor of 1.8±0.2 (n=6), but ouabain, at a dose (0.03 mM) sufficient to potentiate twitch force by 40±2% (n=4), was without effect on the time course of the twitch nor was f_{min} changed (n=4). Our findings support the hypothesis that a β-adrenergic receptor–mediated increase in crossbridge cycling rate is due to an increase in intracellular cyclic AMP level and illustrate the usefulness of the frequency domain analysis approach in the analysis of the mechanism of action of inotropic agents. (Circulation Research 1991;68:702–713)

The mechanical manifestations of β-adrenergic stimulation of the myocardium in the time domain are well known: increase in the maximal speed of shortening, increase in the rate of rise of isometric tension, increase in peak twitch tension, and decrease in time to peak (TTP) and time to half relaxation (TT½R). These changes have been generally regarded as satisfactorily explained by an increase in the number of activated crossbridges as a result of enhanced numbers of activated troponin C by calcium ions.1,2 Using frequency-domain analysis of dynamic stiffness, we3 have recently shown that β-adrenergic stimulation of rat papillary muscle in Ba^{2+}-activated contracture is associated with a shift, in the direction of higher frequency, of the dynamic stiffness and phase-versus-frequency plots. Such shifts can be quantified by noting the change in the frequency at which dynamic stiffness exhibits a minimum (f_{min}). β-Adrenergic stimulation is associated with an increase in f_{min} in an isomyosin-dependent manner: for heart tissue rich in V_{1} isomyosin (V_{1} hearts), the increase is 50%, whereas for heart tissue rich in V_{3} isomyosin (V_{3} hearts), the increase is 25%.4 A similar observation has been reported by Berman et al4 with rabbit papillary muscle. Such a shift in f_{min} is interpreted as a change in crossbridge dynamics; the rationale for such an interpretation is supported by the work of others5–8 and by our own contributions9–11 in which we demonstrated a correlation between the mechanical and the myothermic and biochemical properties of muscle rich in V_{1} and V_{3} isomyosins. We concluded4 that β-adrenergic stimulation causes an enhancement in the rate of crossbridge cycling and that this effect of β-adrenergic stimulation is independent of the effects of β-adrenergic stimulation on the number of active crossbridges during contraction.

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Many different mechanical parameters have been used as a measure of myocardial contractility, such as the maximal speed of shortening and the rates of rise of tension or pressure. These parameters ultimately depend at the molecular level on two basic parameters, namely, the number of active crossbridges during contraction and the rate at which they cycle. Thus, β-adrenergic receptor–mediated cardiac inotropy is associated with an enhancement of both parameters. The product of the extensive factor (number of active crossbridges) and the intensive factor (cycling rate) defines the hydrolysis rate of ATP, the mechanical power and rate of tension-related heat liberation, and hence the contractility of the myocardium.

β-Adrenergic agents are currently thought to act on target cells in two ways. The first is the well-established pathway whereby β-adrenergic agents activate a stimulatory G protein (Gs), which in turn elevates an intracellular second messenger, cAMP, by the activation of adenylyl cyclase. Recently, it has been shown that β-adrenergic receptor–stimulated Gs can also directly regulate calcium channels in mammalian cardiac cells independent of cAMP.12 It would seem more plausible that β-adrenergic action on crossbridge cycling is mediated via the cAMP system, since Gs, a low abundance membrane protein in the sarcolemma, is qualitatively and quantitatively inept to act directly on crossbridges. If β-adrenergic receptor–mediated enhancement of crossbridge cycling is indeed brought about via cAMP, the following predictions can be made on the effects of positive inotropic agents on crossbridge kinetics: 1) agonists of cAMP as well as agents that elevate intracellular cAMP should also enhance crossbridge cycling rate, and 2) inotropic agents that do not elevate intracellular cAMP should have no effect on crossbridge cycling rate.

In the present study, we attempted to verify these predictions on the effects of various inotropic agents on crossbridge cycling rate, using Ba2+-activated rat papillary muscles and pseudo–random binary noise (PRBN)–modulated length perturbations to carry out dynamic stiffness analysis. The effects on dynamic stiffness and consequently on crossbridge cycling rate of four positive inotropic agents, dibutyryl cAMP (DcAMP), isobutylmethylxanthine (IBMX), caffeine, and ouabain, were analyzed. In addition, the actions of these inotropic agents on isometric twitch characteristics of parallel preparations of papillary muscle were also analyzed using the same concentrations of drugs as those for frequency-domain analyses. These results enabled correlations to be made between results obtained using frequency-domain analyses with those using time-domain methods.

It will be shown that dibutyryl cAMP and the xanthine derivatives that inhibit cAMP phosphodiesterase enhance the crossbridge cycling rate, whereas ouabain, which does not affect the cAMP system, has no such action. These experiments imply that β-adrenergic stimulation enhances crossbridge cycling rate in cardiac muscle by elevating the intracellular level of cAMP.

Materials and Methods

Experimental Apparatus and Data Analysis

Perturbation of muscle length was achieved by means of an electromagnetic vibrator (Ling Altec, Royston, England),13 and muscle force was measured by means of a piezoresistive strain gauge (model 801, Akers, Horten, Norway). The muscle bath was a rectangular prism cut from a solid aluminum block. Temperature control was achieved by means of a Peltier device (model 801-2001-01, Cambion, Comatsu Electronics, Tokyo), and all experiments were carried out at 25°C. A detailed description is given in a previous paper.3

Electrical stimulation of intact muscle was achieved via platinum wire electrodes located 5 mm on either side of the muscle. The rectangular stimulating pulses had a strength of 40 V and width of 2 msec. At 25°C, pulse frequency was 12 pulses/min.

The signal used to perturb the length of the muscle was software-generated and was introduced to the muscle via digital-to-analog conversion and the length driver. The length signal and the resulting force response of the muscle were passed back to the control computer (model 1000E, Hewlett-Packard Co., Palo Alto, Calif.) via analog-to-digital conversion and stored on hard disk. When all the required dynamic length–tension records had been collected, the data files were processed by means of fast Fourier transforms to yield dynamic stiffness and phase values. The data were smoothed, using a three-point convolution procedure, and displayed on a digital plotter (model 7225A, Hewlett-Packard). The signal used to perturb the length of the muscle was PRBN. A detailed description of this signal and the associated data analysis are given in a previous article.14

The shape of the dynamic stiffness and phase plots obtained by means of small-amplitude PRBN length perturbation agree with those obtained when using small-amplitude sinusoidal length perturbation.14 The plots displayed a characteristic minimum in stiffness and accompanying minimum/maximum feature in phase response (Figure 6). They are in agreement with plots obtained by other investigators who used sinusoids for perturbing muscle length.15,16 fmin corresponds to the point of inflection in the rising part of the phase curve.

Solutions

The standard solution used was a modified Krebs-Henseleit solution, with the following composition (mM): NaCl 120, KCl 4.69, CaCl2 1.5, MgCl2 0.54, KH2PO4 1.02, NaHCO3 25, and dextrose 10. This was further modified as follows to obtain the solutions listed: 1) for calcium-free solution, CaCl2 was omitted; 2) for barium contracture solution, CaCl2 was replaced by 0.5 mM BaCl2.
All solutions were kept at pH 7.4 by continuous bubbling with 95% O₂-5% CO₂.

The drugs used were IBMX and caffeine (both supplied by Sigma Chemical Co., St. Louis), propanolol hydrochloride (ICI Australia, Melbourne), and DcAMP and strophanthin-G (ouabain) (Boehringer Mannheim Corp., Indianapolis, Ind.). All drugs were of analytical grade. Stock solutions of all drugs were made up by dissolving in the above "standard solution," except for the stock propanolol, which was a ready preparation of 1 mg/ml in sterile physiological saline. The volume of drug solution added was typically 2% of the bath volume.

Muscle Preparation

The experiments were carried out on papillary muscles taken from the right ventricle of Sprague-Dawley rats. V₁ hearts were obtained from 3–5-week-old rats; V₂ hearts were obtained from mature rats that had been treated with propylthiouracil.17

The rats were killed by decapitation, and each heart was immediately removed via a sternotomy and placed in the standard solution kept at 25°C. As described previously,1 a long, thin papillary muscle was selected from the right ventricle and secured between force gauge and length driver.

Experimental Protocol

After mounting, the muscle was stretched by 30% of its resting length to the operating length (L₀), and the Ca²⁺-free solution was replaced with standard solution. L₀ is about 95% of the length at which the muscle produces maximum twitch force. The muscle was electrically stimulated every 5 seconds, and the isometric twitch was displayed on a digitizing oscilloscope (model 5223, Tektronix, Beaverton, Ore.) and on a chart recorder (model 7402A, Hewlett-Packard).

To ensure that the observed inotropic effects are those of the unadulterated muscle, the experiments on isometric twitches and on fₘᵢₙ were done on separate papillary preparations.

Effect of Inotropic Agents on Isometric Twitch

When the isometric twitch response had stabilized, it was recorded by means of a Polaroid oscilloscope camera (model C59A, Tektronix) and was taken as the control response. The inotropic agent was then added to the standard solution in the muscle bath, and the time course of its effect on the twitch profile was recorded by means of the Polaroid camera.

Effect of Inotropic Agents on fₘᵢₙ

After the twitch had stabilized, the muscle was washed in several changes of Ca²⁺-free solution until the twitch force had become negligibly small.9,18 The Ca²⁺-free solution was then replaced by the Ba²⁺-contracture solution leading to the development of contracture tension. When the contracture tension had stabilized, the control computer recorded the dynamic length and tension signals. This recording formed the control response. Inotropic agents were added to the muscle bath as described above, followed by mechanical perturbation analyses to monitor the time course of their effect on fₘᵢₙ.

Effect of IBMX on Twitch and fₘᵢₙ in the Presence of Propranolol

Procedures were followed as above; however, the muscle was allowed to equilibrate for 10 minutes in the presence of propranolol (3 µg/ml) before the addition of IBMX.

Results

Effect of IBMX, DcAMP, and Ouabain on Isometric Twitch Parameters

Figure 1 shows the effect of inotropic agents, IBMX, DcAMP, and ouabain, on the time course of the twitch force profile of the V₁-type papillary muscles. In all cases, the agent potentiated twitch force. DcAMP and IBMX, but not ouabain, had the additional effect of accelerating the time course of the twitch. IBMX and DcAMP share these properties with adrenaline.3 The potentiating effect of adrenaline on twitch force and speed is blocked by propranolol.3

As shown in Figure 2 and Table 1, unlike adrenaline, the potentiating effect of IBMX on twitch force and speed was not blocked by propranolol (by paired t test, p>0.1) but was delayed. Without propranolol, it took 4.1±0.3 minutes (mean±SEM; n=7) for the effect of IBMX on the twitch profile to reach steady state, whereas in the presence of propranolol the time taken was 6.3±0.9 minutes (mean±SEM; n=6).

To quantify the effect of the inotropic agents on the isometric twitch profile, we measured the following parameters: isometric force, TTP, and TT½R. The ratios (experimental/control) for these parameters are plotted against time in Figures 3 and 4. The increase in speed of the twitch profile when the muscle was challenged by DcAMP and IBMX (Figures 1a and 1b) was reflected in a decrease in both TTP and TT½R. When the muscle was challenged with ouabain, which was seen not to alter the speed of the twitch profile (Figure 1c), TTP and TT½R (Figure 4) remained unchanged over the period of observation (53.4 minutes). The doses of inotropic agents used gave maximal steady-state responses, and these values are displayed in Table 1. The table also gives the mean ratios (experimental/control) of the isometric force, TTP, and TT½R. From this table we can infer that if we quantify a duration of the isometric twitch force by the sum (TTP+TT½R), then, relative to control, the 50% increase in twitch force induced by DcAMP was accompanied by a shortening of the duration of the twitch by 45%, IBMX increased the twitch force by 30% and reduced the twitch duration by 35%. Ouabain increased the twitch force by 40% and left the duration of the twitch unchanged.

Effect of Inotropic Agents on fₘᵢₙ

Intact papillary muscles developed active tension when standard physiological saline solution was re-
placed by activating solution containing Ba²⁺, as described previously.⁹ An example of the time course of the effect of an inotropic agent (IBMX) on the contracture tension, evoked by Ba²⁺, together with applied length perturbations and the resulting dynamic perturbations of muscle force, is shown in Figure 5. In contrast to the potentiating effect of the three inotropic agents on isometric twitch, no well-defined effect was evident on their contractural tension.

Analysis of the PRBN length changes and the resulting force changes of recording 5 in Figure 5 gave rise to the characteristic dynamic stiffness and phase data shown in Figure 6i, which displays a minimum in dynamic stiffness at a characteristic frequency accompanied by the minimum/maximum feature in the phase response.⁹¹⁶ \( f_{\text{min}} \) corresponds to the point of inflection in the rising part of the phase curve. The effect of adding IBMX (0.6 mM) to a V₁-type muscle is shown to shift the dynamic stiffness and phase curves to higher frequencies, indicating that IBMX initiated an increase in crossbridge dynamics.⁶⁸⁹

The effect of DcAMP on dynamic stiffness and phase is similar to that of IBMX (Figure 6ii). Table 1 gives the means of the ratios (experimental/control) of the values of \( f_{\text{min}} \) for each inotropic agent used. It shows that in the mean, the magnitude of the shift in \( f_{\text{min}} \) brought about by DcAMP is comparable with that obtained with IBMX.

An example of the effect of ouabain (0.03 mM) on dynamic stiffness and phase of a V₁-type papillary muscle is shown in Figure 6iii. There is no shift in \( f_{\text{min}} \) between the control response and the ouabain response up to 30 minutes after the addition of the drug. The insensitivity of \( f_{\text{min}} \) to ouabain in contracture solution contrasts with the enhancement of twitch force in standard solution brought about by this agent (Figure 1c) but is consistent with the
TABLE 1. Effects of Inotropic Agents on Isometric Twitch Force, Time to Peak Tension, and Time to Half Relaxation Parameters in Intact V1 and V3 Papillary Muscle and on the Dynamic Stiffness Parameter Obtained During Ba2+-Induced Contracture

<table>
<thead>
<tr>
<th>Inotropic agents</th>
<th>Dose (mM)</th>
<th>Twitch parameters</th>
<th>Dynamic stiffness parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>tₙ (min)</td>
<td>Force</td>
</tr>
<tr>
<td>IBMX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₁</td>
<td>1.1</td>
<td>4</td>
<td>3.6±0.06</td>
</tr>
<tr>
<td>V₃</td>
<td>0.6</td>
<td>. . . .</td>
<td>. . . .</td>
</tr>
<tr>
<td>IBMX (V₁)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Propranolol</td>
<td>0.6</td>
<td>7</td>
<td>4.1±0.34</td>
</tr>
<tr>
<td>+Propranolol</td>
<td>0.6</td>
<td>6</td>
<td>6.25±0.85</td>
</tr>
<tr>
<td>Caffeine (V₁)</td>
<td>4</td>
<td>4</td>
<td>12±1.5</td>
</tr>
<tr>
<td>DcAMP (V₁)</td>
<td>5</td>
<td>5</td>
<td>10±1</td>
</tr>
<tr>
<td>Ouabain (V₁)</td>
<td>0.03</td>
<td>3</td>
<td>23.4±3</td>
</tr>
<tr>
<td>Adrenaline (V₁)</td>
<td>3x10⁻⁴</td>
<td>6</td>
<td>4.5±0.5</td>
</tr>
</tbody>
</table>

Values are mean±SEM. tₙ: Time for parameters to reach steady state; Force, isometric force; TTP, time to peak tension; TT/2R, time to half relaxation; fₘᵢₙ: frequency at which dynamic stiffness is minimum; IBMX, isobutylmethylxanthine; V₁, V3, isomyosin; V₁, V₃; isomyosin; DcAMP, dibutyryl cyclic AMP. The mean fₘᵢₙ for 35 control V₁ muscles was 2.5±0.1 Hz; the mean for five control V₃ muscles was 1.2±0.06 Hz. Mean ratios (experimental/control) are given for isometric force, TTP, TT/2R, and fₘᵢₙ. Data for adrenaline are from experiments reported previously.³

insensitivity of the twitch parameters, TTP and TT/2R, to this drug (Figure 4), whereas the increase in fₘᵢₙ due to the action of IBMX and DcAMP, indicating a speeding up in crossbridge dynamics, is accompanied by a shortening of the duration of the isometric twitch force (Table 1). The lack of effect of ouabain on fₘᵢₙ was confirmed in four V₁-type muscles and one V₃-type muscle.

Figure 7 shows the time course of changes in fₘᵢₙ due to the action of DcAMP (5 mM, panel a) or IBMX (0.6 mM, panel b). It can be seen that the time course of these changes is similar to that of TT/2R brought about by the same agents shown in Figure 3a (DcAMP) and Figure 3b (IBMX). In contrast, ouabain (0.03 mM) leaves both fₘᵢₙ and TT/2R unchanged. Both frequency- and time-domain parame-

![Figure 3](http://circres.ahajournals.org/)
ters have reached their steady-state values at the same
time: 4 minutes after the addition of IBMX and 9
minutes after the addition of DcAMP.
Again, as shown by a paired t test, propranolol does
not significantly block the effect of IBMX on $f_{min}$
($p>0.1$), although it does delay the progress of the $f_{min}$
shift. This is in keeping with the effect of propranolol
on the isometric twitch parameters described above.
It is interesting to note that, contrary to our results
on adrenaline, in which we showed that the $V_3$-type
muscles are less sensitive to adrenaline than are the
$V_1$-type muscles, the effect of IBMX on $V_3$-type
muscles is more dramatic than that on $V_1$-type
muscles (see Table 1). From Table 1, it is seen that
the effect of IBMX on the $f_{min}$ of a $V_3$-type muscle is 1.3
times that on the $f_{min}$ of a $V_1$-type muscle. This
difference is statistically significant ($p<0.05$).

**Correlation of $f_{min}$ and Isometric Twitch Parameters**
The results presented so far suggest that an inverse
relation exists between $f_{min}$ and isometric twitch pa-
rameters, TTP and TT/$2R$; that is, an increase in $f_{min}$
is accompanied by a decrease in TTP and TT/$2R$.

**Figure 4.** Time course of changes in relative twitch force,
time to peak tension (TTP), and time to half relaxation
(TT/$2R$) of rat papillary muscles after the addition of 0.03 mM
ouabain. The values plotted are mean $\pm$ SEM of the ratio
of experimental/control values.

This relation is further elaborated on in Figure 8, in
which these twitch parameters obtained at various
times after application of IBMX and DcAMP are
plotted against $f_{min}$ values obtained at corresponding
times in parallel preparations after the application of
the drugs at the same concentrations. The plots were
obtained from the means of the measured values of the
twitch parameters and $f_{min}$ at set times after the
addition of the inotropic agent. The inverse relation
between TTP and TT/$2R$ against $f_{min}$ is consistent
with the hypothesis that, in the case of IBMX,
DcAMP, and adrenaline, the increase in twitch speed
is partially brought about by an increase in the rate of
crossbridge cycling. The similarity of the relations
suggests a common mechanism of action between the
inotropic agents IBMX, DcAMP, and adrenaline.
The corresponding correlation of twitch force with
$f_{min}$ is shown in Figure 9. The corresponding increase
in twitch force for a particular $f_{min}$ is greater for
IBMX than for DcAMP or adrenaline.

**Effect of Caffeine on Isometric Twitch Parameters
and $f_{min}$**
Caffeine, also a methylxanthine and a phosphodi-
erase inhibitor but well known to have other phar-
macological effects, displays an effect different from
that of IBMX. As shown in Table 1, with IBMX, an
increase in $f_{min}$ was accompanied by a decrease in both
TTP and TT/$2R$, whereas with caffeine, the increase
in $f_{min}$ was not accompanied by any significant change
in twitch speed. In other words, at a dose that gave an
increase in crossbridge cycling rate comparable with
that of IBMX, as indicated by $f_{min}$, the time taken for
the twitch force to develop and to relax remain
unchanged. This indicates that the mode of action of
this inotropic agent is intrinsically different from that
of IBMX. This difference is further emphasized in the
relation between $f_{min}$ and twitch force. In the case of
caffeine, an increase in $f_{min}$ is accompanied by a
decrease in twitch force (Table 1), whereas Figure 9

**Figure 5.** Chart recording of Ba$^{2+}$-induced contracture in
$V_1$-type papillary muscle (upper tracing) and perturbations of
muscle length (lower tracing). Successive pseudo-random
binary noise length perturbations are labeled 1-12; other length
cr-anges represent step perturbations used to evaluate the
delayed tension response of the preparation. The arrow in-
dicates when 0.6 mM isobutylmethylxanthine was added into
the muscle bath. The vertical scale is $25 \times 10^{-3}$ N. The horizontal
scale is 1 minute.
reveals that for IBMX the $f_{\text{min}}$ increase is accompanied by a substantial increase in twitch force.

**Discussion**

**Pharmacology of the Positive Inotropic Agents**

The positive inotropic action of catecholamines is largely due to an increase of intracellular cAMP resulting from the stimulation of β-adrenergic receptors.\(^1\)\(^-\)\(^2\) cAMP exogenously applied to cardiac tissue does not mimic the inotropic action of catecholamines, because of the relative impermeability of the myocardial cells to cAMP.\(^19\) On the other hand, DcAMP, an analogue of cAMP, which is more lipid soluble, has been shown to mimic the action of β-adrenergic receptor activation.\(^20\) The inotropic actions of DcAMP are not blocked by the β-adrenergic receptor blocker propranolol.\(^20\)\(^-\)\(^21\) Our results on rat papillary muscle also showed that DcAMP has the same actions on twitch parameters and $f_{\text{min}}$ as β-adrenergic stimulation, consistent with the assumption that this agent acts simply as an agonist of cAMP.

IBMX and caffeine are methylxanthine derivatives known to inhibit cAMP phosphodiesterase, an action that would be expected to lead to an accumulation of cAMP in the cell.\(^22\)\(^-\)\(^24\) The observed actions of these agents in increasing $f_{\text{min}}$ are consistent with this view. However, methylxanthines are known to have other actions. Theophylline, another methylxanthine, has been shown to release endogenous catecholamines from heart tissue, and a large component of the inotropic action of this agent can be blocked by β-adrenergic antagonist propranolol or by depletion of tissue catecholamines by the prior administration of reserpine.\(^25\)\(^-\)\(^26\) If the methylxanthines used in these experiments exert their influence principally by the release of endogenous catecholamines, the results would have no more significance than those for adrenaline already reported.\(^3\) To assess the role of endogenous catecholamine release by IBMX, we compared the effects of IBMX with and without pretreatment of papillary muscles with propranolol. Our data showed that propranolol pretreatment did not alter the steady-state values of isometric twitch parameters and $f_{\text{min}}$ produced by IBMX; these findings are consistent with the view that the inotropic actions of IBMX are due to its inhibition of cAMP phosphodiesterase rather than the release of endogenous catecholamines. However, we observed that propranolol retarded the time course of action of IBMX on isometric twitch characteristics: the time
taken for these changes to reach steady state was approximately doubled in the presence of propranolol. These observations suggest that the rate of intracellular accumulation of cAMP was slowed down as a result of blocking β-adrenergic receptors, suggesting that IBMX may indeed cause the release of endogenous catecholamines from the heart but that this action is of minor significance in the inotropic action of IBMX.

The stimulatory action of IBMX, DcAMP, and adrenaline on $f_{\text{min}}$ is associated with a reduction of TTP and TT½R. Caffeine is well known to have a complex pharmacology. Caffeine increases TTP and TT½R in skeletal muscle27 and in cardiac muscle28 of several mammalian species by releasing Ca²⁺ from the sarcoplasmic reticulum29 via a direct action on the Ca²⁺-release channel.30 It appears likely that this additional action of caffeine explains why IBMX reduced TTP and TT½R, whereas caffeine apparently has no appreciable action on these parameters.

The mechanism of action of cardiac glycosides does not involve the cAMP system.31,32 Cardiac glycosides block sarcolemmal Na⁺,K⁺-ATPase, thereby increasing the intracellular Na⁺ concentration. This decreases the amount of Ca²⁺ transported out of the myocyte via the Na⁺-Ca²⁺ exchange mechanism, thereby increasing the intracellular Ca²⁺ pool. As a result, more Ca²⁺ enters the cell, and a Ca²⁺ transient of greater magnitude can be recorded intracellularly.33,34 Our results on the action of ouabain on the isometric twitch contractions also confirmed previous reports that cardiac glycosides have little effect on the time course of the isometric twitch.35 In particular, ouabain does not accelerate the rate of isometric twitch relaxation, which is so prominent a feature in the action of catecholamines36 and of IBMX and DcAMP reported here. Cardiac glycosides mobilize Ca²⁺ to produce an increase in the number of crossbridges activated but leave crossbridge kinetics unaffected. The absence of an effect on $f_{\text{min}}$ is consistent with this view.

**Effects of the Inotropic Agents on the Mechanics of Cardiac Muscle**

Before our recent study, the mechanical effects of inotropic agents have only been studied in the time domain.37 The force-velocity relation is generally regarded as a fundamental property of skeletal muscles. Quick-release experiments showed that DcAMP,30 the methylxanthines,26,28 the cardiac glycosides,35,38 and indeed most inotropic interventions37 shifted the force-velocity curve of cardiac muscle upward and to the right, increasing maximal velocity of shortening at zero external load as well as increasing the maximal force of isometric contraction. For cardiac muscle, an increase in maximal velocity of shortening may result from an increase in either the level of activation39,40 or in the crossbridge cycling rate.3,9,41 Therefore, cardiac force-velocity data fail to distinguish between the two possible fundamental mechanisms for enhancing contractility: the number of active crossbridges and the rate at which they cycle (see the introductory section).

Although the mechanical effects on cardiac muscle of catecholamine, cAMP analogues, phosphodiesterase inhibitors, and cardiac glycosides are indistinguishable by force-velocity analysis, our data showed that the profiles of the isometric twitch, which are much easier to obtain than force-velocity data, paradoxically do reveal a difference in actions of various inotropic agents. Adrenaline, DcAMP, and IBMX showed a reduced TTP and TT½R, whereas ouabain did not affect these parameters. These time-domain results are strongly correlated with results of frequency-domain analysis.

The importance of frequency-domain analysis in cardiac muscle mechanics lies in its ability to resolve changes in $f_{\text{min}}$, a parameter indicative of crossbridge
cycling rate, independent of changes in Ca^{2+}-activated force.9 Our present experiments clearly showed that DcAMP, IBMX, and caffeine mimicked the action of β-adrenergic stimulation in increasing f_{\text{min}} of V_{1}-type muscle. These results support the hypothesis that a β-adrenergic receptor–mediated increase in crossbridge cycling rate is due to an increase in intracellular cAMP level rather than due to the direct action of G_{\text{β}} on crossbridges. The lack of effect of ouabain on f_{\text{min}} at a dose shown to elicit an increase of isometric force by 40% is consistent with the view that the inotropic action of cardiac glycosides is due solely to its influence on the number of active crossbridges and not to any influence on the rate of crossbridge cycling. Frequency-domain analysis emerges as a useful method of classifying inotropic agents according to their action on crossbridge kinetics.

**Correlation Between f_{\text{min}} and the Isometric Twitch Parameters**

The results presented in Figure 8 suggest that crossbridge cycling rate in cardiac muscle is inversely related to TTP and TT\(\frac{1}{2}\)R. A similar relation has been found in skeletal muscle: over a very wide range of values, TTP and TT\(\frac{1}{2}\)R are inversely related to the maximal speed of shortening,42 which in turn is proportional to the rate at which myosin hydrolyzes ATP in solution.43 The generality of the relation between twitch kinetics and crossbridge kinetics raises the question whether under certain circumstances there is a causal relation between them.

It has previously been suggested that, during an isometric twitch, most activated crossbridges would only have time to undergo a single cycle.9 In both cardiac33,44 and skeletal muscle fibers,45,46 the Ca^{2+} transient reaches a peak value very early in the twitch and virtually regains resting value at the peak of twitch tension, independent of the value of TTP. The inverse relation between crossbridge dynamics and dynamic parameters of the twitch may be explained by assuming that the time course of the Ca^{2+} transient in striated muscles normally allows only a small fixed number of cycles of the crossbridge. DcAMP and IBMX may not alter the number of cycles, but caffeine, by mobilizing Ca^{2+}, may increase this number.

**Figure 8.** Plots of twitch parameters, time to peak tension (TTP) (panel a) and time to half relaxation (TT\(\frac{1}{2}\)R) (panel b), against the frequency at which dynamic stiffness is minimum (F_{\text{min}}) for isobutylmethylxanthine (IBMX, ●), dibutyryl cyclic AMP (DcAMP, ▲), and adrenaline (■). The points used in these plots are the mean values obtained from Figures 3 and 7, and the lines represent least-square fits. The data for adrenaline are from experiments reported previously.3

**Figure 9.** Plots of twitch force against the frequency at which dynamic stiffness is minimum (F_{\text{min}}) for isobutylmethylxanthine (IBMX, ●), dibutyryl cyclic AMP (DcAMP, ▲), and adrenaline (■). The points used in this plot are the mean values obtained from Figures 3 and 7, and the lines represent least-square fits. The data for adrenaline are from experiments reported previously.3
ber, prolonging the twitch, so that inverse changes in \( f_{\text{min}} \) and TTP and TV\( \% \)R are not seen with this agent.

The possibility that the time course of the cardiac isometric twitch may reflect the time course of the crossbridge cycle gives a new insight to the studies of Sonnenblick in the 1960s on the effects of noradrenaline and strophanthidin on the active-state curves of cat papillary muscles. Sonnenblick found that strophanthidin shifted the active-state curve vertically, whereas noradrenaline shifted the active-state curve upward and to the left, reducing the time taken to reach peak active state and accelerating the rate of its decay. These results correlate with our findings that ouabain did not increase \( f_{\text{min}} \) but adrenaline did.\(^3\) This correlation suggests that the time course of the active state as measured by Sonnenblick reflects crossbridge kinetics.

It should be noted, however, that adrenaline also phosphorylates phospholamban,\(^2\) resulting in reaccumulation of Ca\(^{2+}\) ions by the sarcoplasmic reticulum. This action, which is not shared by ouabain, may also have an influence on the active state.

Our data on adrenaline, DCAMP, and IBMX also showed that twitch force is directly correlated with \( f_{\text{min}} \). This simply reflects the fact that cAMP has the dual effect of increasing the number of active crossbridges by mobilizing Ca\(^{2+}\) as well as enhancing crossbridge cycling rate. It is of interest, however, that the slope of the regression line relating force and \( f_{\text{min}} \) for IBMX is considerably higher than that for DCAMP and adrenaline. The reason for this difference is obscure. A possible explanation is that there exist different pools of intracellular cAMP and that different inotropic agents affect these pools differently.

**Difference in Response Between \( V_1 \) and \( V_3 \) Hearts**

Our earlier work showed that the \( f_{\text{min}} \) response to adrenaline of \( V_1 \) and \( V_3 \) hearts differed quantitatively. The \( f_{\text{min}} \) response of \( V_1 \) hearts increased by 50%, but the response of \( V_3 \) hearts was only 25%, suggesting that the magnitude of the response may be myosin-isofrom dependent. This differential response to \( \beta \)-adrenergic stimulation was interesting in the light of the work of Winegrad and coworkers\(^{47,48} \) on isozyme-specific modification of myosin-ATPase by cAMP. Using a quantitative method to measure myosin ATPase activity in a cryostat section of rat cardiac tissue, these workers showed that \( \beta \)-adrenergic stimulation or incubation of a tissue section in the presence of cAMP and phosphodiesterase inhibitor enhances \( V_1 \) myosin ATPase activity while inhibiting \( V_3 \) ATPase activity. This increase in ATPase could be due to enhanced crossbridge cycling or to a recruitment of crossbridges. These authors appear to favor the latter view, since they have shown earlier that treatment of a hyperpermeable cardiac muscle preparation by a mixture of cAMP, phosphodiesterase inhibitor, and detergent causes an increase in the maximal force that calcium ions can elicit.\(^49\)

We are surprised to find, in the present work, that IBMX induced a significantly greater response in \( V_1 \) hearts than in \( V_3 \) hearts. The reason for this difference in relative sensitivity to adrenaline and IBMX between \( V_1 \) and \( V_3 \) hearts is not clear. These observations suggest that the difference is extrinsic to the myosin isoforms and that the \( f_{\text{min}} \) shift is unrelated to the isofrom-specific enhancement of myosin ATPase observed by Winegrad and colleagues.\(^47,48\)

**Possible Mechanisms of Action of cAMP on Crossbridge Kinetics**

No information is currently available on the molecular events linking cAMP and crossbridge cycling rate. The enhancement of the Ca\(^{2+}\) transient after \( \beta \)-adrenergic stimulation is due to cAMP-dependent phosphorylation of several proteins.\(^1,2\) The direct action of \( \beta \)-adrenergic receptor–stimulated G\(_s\) on the Ca\(^{2+}\) channel may also play a role.\(^12\) These actions on Ca\(^{2+}\) are unlikely to have any effect on crossbridge cycling kinetics, since \( f_{\text{min}} \) is insensitive to the level of activating Ca\(^{2+}\) concentration.\(^9\)

Two myofibrillar proteins have been reported to be consistently phosphorylated by \( \beta \)-adrenergic stimulation: troponin I\(^{50,51} \) and C protein.\(^52\) Phosphorylation of troponin I is associated with a reduction in Ca\(^{2+}\) sensitivity of the myofilibr as measured by myofibrillar ATPase activity\(^ {53,54} \) or the force:\( \text{PCa}^{2+} \) relation in skinned papillary muscle preparation.\(^55\) Troponin phosphorylation is associated with an increase in the rate of dissociation of Ca\(^{2+}\) from the Ca\(^{2+}\)–troponin C complex,\(^56\) resulting in a reduced affinity of troponin for Ca\(^{2+}\). The effect per se would have led to a negative rather than a positive inotropic effect, but apparently the concurrent \( \beta \)-adrenergic effects on Ca\(^{2+}\) movements are more than adequate to compensate for this. The functional significance of troponin I phosphorylation is to ensure adequate ventricular filling in the face of adrenergically mediated tachycardia by causing a more rapid relaxation of the heart. This function does not exclude the possibility that it may play an additional role in modulating crossbridge kinetics. The availability of a preparation in which troponin I is apparently selectively phosphorylated\(^57\) should help to resolve this issue.

Phosphorylation of C protein in response to \( \beta \)-adrenergic stimulation is interesting in that it is correlated with the change in the time course of the relaxation of the isometric twitch after \( \beta \)-adrenergic stimulation.\(^58\) Furthermore, it does not take place during inotropic interventions that increase intracellular calcium ions without increasing cAMP.\(^59\) However, the function of C protein is unknown, and phosphorylation of C protein in vitro reduced actin-activated myosin-ATPase activity,\(^60\) which is contrary to enhanced crossbridge cycling rate.

The phosphorylation of myosin light chain 2 is unlikely to account for enhanced crossbridge kinetics. First, the level of phosphorylation of myosin light chain 2 is not changed by \( \beta \)-adrenergic stimulation.\(^59\) Second, the specific enzyme that phosphorylates myosin light chain 2, myosin light chain kinase, is
activated by calcium, not cAMP.\textsuperscript{61} It is unlikely that the activity of this enzyme would be elevated under the condition of our experiment, since Ba\textsuperscript{2+} was used for activating the muscle to a constant level during contracture. After the addition of DC\textsubscript{4}AMP or IBMX, or of adrenaline,\textsuperscript{3} there was no consistent change in contracture tension to suggest an elevation of Ca\textsuperscript{2+} or Ba\textsuperscript{2+} that might enhance myosin light chain kinase activity. Third, phosphorylation decreases myosin ATPase rather than enhances it.\textsuperscript{62}

It is conceivable that cAMP may act on crossbridge kinetics through a mechanism independent of protein phosphorylation. In this connection it is important to point out that neither catecholamines\textsuperscript{63–67} nor cAMP\textsuperscript{68} alters the ATPase activities of cardiac myosin and actomyosin. However, Winegrad\textsuperscript{69} has reported that \(\beta\)-adrenergic stimulation increases cardiac contractility via a second cAMP-dependent system that does not involve myofibrillar protein phosphorylation. He postulated that cAMP causes the activation and release of a membrane-bound protein factor that acts on myosin to increase mechanical response to calcium ions. This system acts selectively on V\textsubscript{i}, isomyosin, and the biochemical correlate of this phenomenon, an increase in actomyosin-ATPase, has also been demonstrated in cryostat section of muscle tissues containing V\textsubscript{i}, isomyosins.\textsuperscript{47} A similar mechanism may enhance crossbridge kinetics in addition to increasing the number of active crossbridges. If so, under Winegrad's condition\textsuperscript{69} of enhanced force, \(f_{\text{min}}\) would be expected to be shifted upward. Preliminary attempts to repeat Winegrad's observations on force enhancement in our laboratory on skinned fibers have been unsuccessful.

References

1. Tsien RW: Cyclic AMP and contractile activity in heart. \textit{Adv Cyclic Nucleotide Protein Phosphorylation Res} 1977;8:363–420
2. Katz AM: Role of the contractile proteins and sarcoplasmic reticulum in the response of the heart to catecholamines: An historical review. \textit{Adv Cyclic Nucleotide Protein Phosphorylation Res} 1979;1:303–343
6. Thorson J, White DCS: Role of cross-bridge distortion in the small-signal mechanical dynamics of insect and rabbit striated muscle. \textit{J Physiol (Lond)} 1983;343:59–84
15. Steiger GJ: Tension transients in extracted rabbit heart muscle preparations. \textit{J Mol Cell Cardiol} 1979;7:671–685
30. Rubtsov AM, Murphy AJ: Caffeine interaction with the Ca-release channels of heavy sarcoplasmic reticulum: Evidence that 170 KD Ca-binding protein is a caffeine receptor of the Ca-channels. \textit{Biochem Biophys Res Commun} 1988;154:462–468
34. Wier WG, Hess P: Excitation-contraction coupling in cardiac Purkinje fibers: Effects of cardiovascular steroids on the intracel-
lular Ca\textsuperscript{2+} transient, membrane potential, and contraction. *J Gen Physiol* 1984;83:395–415
42. Close RI: The relation between intrinsic speed of shortening and duration of the active state of muscle. *J Physiol* (Lond) 1965;180:542–559
65. Luchi RJ, Kritcher EM, Conn HL: Modification of cardiac myosin adenosine triphosphatase activity by ions and cardiac drugs. *Circulation* 1964;30:118

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