8-Bromo-cGMP Reduces the Myofilament Response to \( \text{Ca}^{2+} \) in Intact Cardiac Myocytes

Ajay M. Shah, Harold A. Spurgeon, Steven J. Sollott, Ante Talo, Edward G. Lakatta

Abstract  The role of cGMP in myocardial contraction is not established. Recent reports suggest that nitric oxide, released by endothelial cells or within myocytes, modifies myocardial contraction by raising cGMP. We studied the effects of 8-bromo-cGMP (8bcGMP, 50 \( \mu \text{mol/L} \)) on contraction (cell shortening) and simultaneous intracellular \( \text{Ca}^{2+} \) transients (indo 1 fluorescence ratio) in intact adult rat ventricular myocytes (0.5 Hz and 25°C). 8bcGMP reduced myocyte twitch amplitude and time to peak shortening (−19.6±4.2% and −17.6±1.3%, respectively) and increased steady-state diastolic cell length (+0.6±0.1 \( \mu \text{m} \), mean±SEM, \( n=8 \); all \( P<.05 \)) but had no effect on shortening velocity, systolic or diastolic fluorescence ratio, or time to peak fluorescence rate (all \( P>\text{NS} \)). In 7 of 13 myocytes, this negative inotropic effect was preceded by a transient positive inotropic effect, with small increases in twitch amplitude, shortening velocity, and cytosolic \( \text{Ca}^{2+} \) transient. Analysis of 8bcGMP effects on both the dynamic and steady-state relationship between cell shortening and intracellular \( \text{Ca}^{2+} \) (during twitch contraction and tetanic contraction, respectively) indicated reduction in the myofilament response to \( \text{Ca}^{2+} \) in all cases. These 8bcGMP effects were inhibited by KT5823 (1 \( \mu \text{mol/L} \)), an inhibitor of cGMP-dependent protein kinase, or by the presence of isoproterenol (3 \( \text{nmol/L} \)). 8bcGMP had no effect on cytosolic pH in cells (\( n=4 \)) loaded with the fluorescent probe carboxysemaphthoradfluor-1. These data indicate that cGMP may modulate myocardial relaxation and diastolic tone by reducing the relative myofilament response to \( \text{Ca}^{2+} \), probably via cGMP-dependent protein kinase. (Circ Res. 1994; 74:970-978.)

Key Words  cGMP • cardiac contraction • diastolic tone • myofilaments • relaxation

The role of cGMP in regulating myocardial contraction has been controversial.\(^1\) Myocardial cGMP is known to be elevated by cholinergic stimulation,\(^2\) but this effect has not been clearly correlated with inotropic activity. This is perhaps not surprising, since cholinergic agonists have several cellular actions that may be independent of cGMP, eg, phosphatidilinositol hydrolysis and modulation of K\(^+\) channels.\(^3\) Other studies have either reported depression or no change in myocardial peak isometric force after the addition of exogenous cGMP or lipid-soluble analogues.\(^4\)\(^7\) In some cases, negative inotropic effects have been observed only when preparations have been pre-stimulated with cAMP-elevating interventions,\(^8\) suggesting interaction between cGMP- and cAMP-mediated mechanisms. Recent studies by two groups suggest that elevation of cGMP results in relatively subtle contractile effects influencing predominantly myocardial relaxation: the onset of relaxation occurs earlier with a consequent slight reduction in peak force but with relatively little or no effect on the early systolic phase of contraction, eg, no change in the rate of force development or in maximal unloaded shortening velocity.\(^9\)\(^11\) These effects, observed both in ferret and cat isolated papillary muscle preparations, may easily be overlooked if peak isometric force alone is measured.

A different approach has been to study the effect of cGMP on myocardial subcellular mechanisms and on isolated cardiac proteins.\(^1\) cGMP has been reported to have several actions, including (1) reduction in sarcoplasmic \( \text{Ca}^{2+} \) influx through L-type channels in amphibian and avian myocytes, by activation of a cGMP-stimulated phosphodiesterase that reduces cAMP levels,\(^12\)\(^13\) (2) an increase in sarcoplasmic \( \text{Ca}^{2+} \) influx as a result of cGMP-mediated inhibition of a different phosphodiesterase, with a consequent increase in cAMP levels,\(^14\) (3) reduction in sarcoplasmic \( \text{Ca}^{2+} \) influx in avian and in mammalian cells, by a direct effect of cGMP-dependent protein kinase (PKG),\(^15\)\(^16\) (4) reduction or prolongation of action potential duration,\(^17\)\(^18\) (5) reduction in cell volume by inhibition of Na\(^+\)/K\(^+\)/2Cl\(^−\) cotransport,\(^19\) (6) phosphorylation of troponin I by PKG,\(^20\)\(^21\) and (7) phosphorylation of phospholamban by PKG.\(^22\) These different and sometimes contradictory data may be explained, in part, by the use of nonphysiological high concentrations of cGMP in some studies and by the use of different experimental conditions and preparations (eg, different tissues and species). Furthermore, most of these studies of cGMP have failed to correlate biochemical and electrophysiological effects with changes in contractile behavior in intact cells.

Interest in myocardial cGMP has been reawakened by recent reports that nitric oxide (NO), either released by endothelial cells within the heart or by cardiac myocytes themselves, can influence myocardial contraction, probably through elevation of cGMP.\(^11\)\(^22\)\(^29\) NO is recognized to be an important signaling molecule in several physiological processes, including endothelial regulation of vascular smooth muscle tone and neuro-

Received July 1, 1993; accepted December 22, 1993.

From the Laboratory of Cardiovascular Science (A.M.S., H.A.S., S.J.S., A.T., E.G.L.), Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, Md; and the Department of Cardiology (A.M.S.), University of Wales College of Medicine, Heath Park, Cardiff, UK.

Correspondence to Dr A.M. Shah, Department of Cardiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, UK, or Laboratory of Cardiovascular Science, GRC/NIA, National Institutes of Health, 4940 Eastern Ave, Baltimore, MD 21224.
transmission. In isolated ferret papillary muscle preparations, NO released from endocardial endothelium elevated myocardial cGMP and induced earlier myocardial relaxation, effects identical to those described with lipid-soluble cGMP analogues, with atrial natriuretic peptide, which activates a plasma membrane guanylyl cyclase receptor (GC-A), and with the NO donor sodium nitroprusside, which stimulates a soluble guanylyl cyclase. Similar effects of NO on left ventricular relaxation have been observed in isolated ejecting guinea pig heart preparations. In cardiac myocytes, NO-induced elevation of myocardial cGMP and associated changes in contractile behavior are reported to occur both with constitutive NO production and after cytokine induction of a Ca2+-independent myocardial NO synthase.

Thus, it seems likely that myocardial cGMP may indeed play a role in regulating myocardial contraction. Some of the more recent data suggest a selective influence of cGMP on relaxation. Because of the similarity of these effects with those observed after changes in resting muscle length, it has been suggested that they might be similarly mediated by changes in myofilament responsiveness to Ca2+. Known to underlie length-induced changes. In the present study, we have investigated the effects of cGMP on contraction and simultaneous intracellular Ca2+ transients, pH, and the membrane action potential in isolated, intact, adult rat cardiac myocytes by use of the lipid-soluble analogue 8-bromo-cGMP (8bcGMP).

Materials and Methods

Cardiac Myocyte Isolation

Single cardiac myocytes were isolated with minor modification of a previously described technique. Briefly, 2- to 4-month-old adult Wistar rats were anesthetized with intraperitoneal sodium pentobarbital, and the heart was rapidly excised and perfused retrogradely with 25 mL nominally Ca2+-free bicarbonate buffer (36°C, pH 7.2). Perfusion was continued with similar bicarbonate buffer containing 0.1% collagenase type B, 0.04 mg/mL protease XVI, and 0.1% bovine serum albumin type V for ~20 minutes. The left ventricle was then mechanically dissociated, and myocytes were resuspended in a series of HEPES buffers with gradually increasing Ca2+ concentration. Cells were finally suspended in HEPES buffer containing (mM/L) NaCl 137, KCl 4.9, MgSO4 1.2, NaH2PO4 1.2, glucose 15, HEPES 20, and CaCl2 1 (pH 7.4) and stored at room temperature until use.

Myocyte Loading With Fluorescent Probes

For assessment of intracellular Ca2+ , myocytes were loaded with the membrane-permeant acetoxyxymethyl ester of indo 1 (indo 1-AM), using a 10-minute exposure to a 25 mM/L solution in HEPES buffer at room temperature. In some experiments, myocytes were loaded with indo 1 free acid, which is selectively localized to the cytosol, by use of a recently described procedure for loading immediately after myocyte isolation. For assessment of changes in cytosolic pH, myocytes were loaded with carboxyxyaninphthordiafluor-1 AM (SNARF-1 AM) by use of a 10-minute exposure to 4 mM/L SNARF-1 AM in HEPES buffer at room temperature as previously described.

Measurement of Contraction and Fluorescence

Single myocytes were studied in a Lucite chamber either on the stage of an ordinary microscope or a modified Zeiss IM-35 fluorescence microscope as described previously. The cham-
myocyte length versus simultaneously measured indo 1 fluorescence during single-twitch contractions, as fully described recently.9 In mechanically unloaded rat cardiac myocytes, which were electrically stimulated to contract from slack length, a quasi equilibrium was achieved between cell re-lengthening and the decline in cytosolic Ca\(^{2+}\) during twitch relaxation. This was manifested as a common trajectory during relaxation in the cell length versus intracellular Ca\(^{2+}\) (or indo 1 fluorescence) phase-plane diagram for twitch contractions of varying amplitude and varying peak intracellular Ca\(^{2+}\) (Fig 1). The common trajectory persisted even when sarcoplasmic reticulum function and, therefore, the kinetics of cytosolic Ca\(^{2+}\) cycling were selectively altered in these myocytes.38 Consequently, we have suggested that the existence of this common relaxation trajectory indicates a dynamic quasi-equilibrium between cytosolic Ca\(^{2+}\) and the response of the myofilaments to Ca\(^{2+}\) (which determines cell length). The position of the trajectory reflects the relative myofilament response to Ca\(^{2+}\). Perturbations that decrease the relative myofilament response to Ca\(^{2+}\) (eg, acidosis or the presence of the nonenzymatic phosphatase butanedione monoxime39,40) shift the relaxation trajectory downward and rightward and result in earlier myocyte relaxation, a briefer twitch, and a longer diastolic myocyte length. Perturbations that increase the relative myofilament response (eg, alkalosis,39,40 α- adrenergic stimulation,41 and Ca\(^{2+}\)-sensitizing drugs42) have the opposite effect. This form of analysis does not elucidate the precise nature of change in myofilament properties (eg, Ca\(^{2+}\)- troponin interaction or myosin ATPase activity) but has the advantage of allowing assessment of myofilament response to Ca\(^{2+}\) in an intact single myocyte during "normal" twitch contraction.

We also studied the steady-state relation between cell length and intracellular Ca\(^{2+}\) in intact single myocytes tetanized by high-frequency (10-Hz) stimulation after exposure to thapsigargin (0.2 μmol/L for 15 minutes), as described recently.43 The latter agent disables the sarcoplasmic reticulum44 and thus enables tetanization of intact myocytes.

Materials

Collagenase type B and 8bcGMP were purchased from Boehringer Mannheim, Indianapolis, Ind; isoproterenol, from Winthrop Pharmaceuticals, New York; protease XVI, from Sigma Chemical Co, St Louis, Mo; and indo 1-AM, indo 1 free acid, and SNARF-1 AM, from Molecular Probes Inc, Eugene, Ore. Bovine serum albumin type V, KT5823 (a specific inhibitor of PKG),46 and thapsigargin were obtained from Calbiochem Corp, La Jolla, Calif. All other chemicals were of the purest reagent grade available. KT5823 was prepared as a 1 mmol/L stock solution in dimethylsulfoxide (DMSO), stored at 4°C, and diluted in HEPES buffer just before use. Thapsigargin was made up as a 5 mmol/L stock solution in DMSO, stored at -20°C, and also diluted in HEPES buffer before use. DMSO at the concentrations used had no effect on myocytes. All other drugs were prepared fresh by dissolution in distilled water and then dilution in HEPES buffer.

Statistics

All data are presented as mean±SEM. Comparisons within groups were made by paired Student's t test using absolute values (even where data are presented as percent change). Between-group comparisons were made by one-way ANOVA. A value of P<.05 was considered significant.

Results

Effect of 8bcGMP on Contraction and Intracellular Ca\(^{2+}\) at 0.5 Hz and 25°C

To assess the effects of physiologically relevant concentrations of intracellular cGMP, we studied the lowest concentration of the lipid-soluble analogue 8bcGMP that produced consistent changes in contraction of intact myocytes. Pilot studies demonstrated that this dose was 50 μmol/L. 8bcGMP at 1 μmol/L had no effect on contraction (n=3), whereas at 10 μmol/L, a small negative inotropic effect (<10% reduction in twitch amplitude) was observed in three of five myocytes. 8bcGMP at 100 μmol/L induced effects similar to those observed at 50 μmol/L (n=3; data not shown).

Fig 2 shows a representative example of the effect of 8bcGMP (50 μmol/L) on isotonic twitch contraction and the associated intracellular Ca\(^{2+}\) transient in a myocyte stimulated at 0.5 Hz and 25°C. 8bcGMP induced a slowly progressive decrease in twitch amplitude, which was maximal 10 to 15 minutes after administration of the drug. The lower tracings illustrate, on an expanded time scale, that the major effect of 8bcGMP was to abbreviate the duration of twitch contraction by inducing an earlier onset of twitch relaxation (decreased time to peak shortening) and to increase the resting (diastolic) cell length but that it had little effect on the velocity of shortening. This "negative inotropic" effect was not accompanied by any significant change in diastolic Ca\(^{2+}\), in the amplitude of the Ca\(^{2+}\) transient, or in the time to peak Ca\(^{2+}\), thus suggesting that 8bcGMP reduced the response of the myofilaments to Ca\(^{2+}\). These effects of 8bcGMP were only reversed after prolonged superfusion with pure HEPES buffer for >20 minutes. Fig 3 shows the mean data for the maximal percent change (compare with baseline values) in contractile parameters 12.5±1.0 minutes after the addition of 8bcGMP in eight indo 1-loaded myocytes. Diastolic cell length increased by 0.6±0.1 μm (P<.01; basal length, 116.4±6.9 μm).
The increase in diastolic cell length induced by 8bcGMP was independent of activation of the myocyte by electrical stimulation. In three quiescent (electrically unstimulated) myocytes, addition of 8bcGMP (50 μmol/L) resulted in an increase in diastolic length of 1.0±0.3 μm.

### Transient Positive Inotropic Effect of 8bcGMP

In 7 (54%) of 13 myocytes exposed to 8bcGMP (50 μmol/L) at 25°C and 0.5 Hz, a transient positive inotropic effect preceded the negative inotropic effect of 8bcGMP. This group of 13 myocytes includes the 8 indo 1-loaded myocytes described above and an additional 5 indo 1-unplated myocytes. Fig 4 shows the most dramatic example of such a biphasic response to 8bcGMP. The tracings below, on an expanded time scale, show that the initial transient increase in twitch amplitude and in the velocity of shortening was accompanied by an increase in amplitude of the indo 1 fluorescence transient. With continued exposure to 8bcGMP, however, twitch duration and twitch amplitude progressively decreased and resting length increased, whereas the indo 1 transient remained at an elevated level. This sustained increase in systolic indo 1 fluorescence was observed in all indo-loaded cells that had a transient positive inotropic response to 8bcGMP. Nevertheless, the predominant effect of 8bcGMP in all cells was to reduce the myofilament response to Ca²⁺, since after the first 4- to 6-minute exposure, cell shortening was reduced despite an augmented Ca²⁺ transient. There were no obvious differences between cells that exhibited a transient positive inotropic response and those that did not when considering, for example, the time of isolation or of study, the baseline contractile behavior, or the animals from which the cells were derived. In the 7 myocytes in which a transient positive inotropic effect was observed, the mean maximal percent increases in contractile parameters (5.0±0.6 minutes after addition of 8bcGMP) are shown in Fig 5.

### Effect of 8bcGMP at 2 Hz and 35°C

The contractile effects of 8bcGMP were also studied under more physiological conditions (a temperature of 35°C and stimulation frequency of 2 Hz). A similar pattern of contractile response to 8bcGMP was observed under these conditions as at 25°C and 0.5 Hz, with reductions in twitch amplitude and twitch duration and an increase in resting length but no change in shortening velocity (Table). However, the magnitude of negative inotropic effect was significantly smaller at the higher stimulation frequency and higher temperature (7.7±2.2% reduction in twitch amplitude versus 14.7±4.1% reduction, P<.05). Diastolic cell length also increased significantly less at 2 Hz and 35°C than at 0.5
Hz and 25°C (+0.5±0.1 versus +1.0±0.3 μm, P<.05). A transient positive inotropic effect was also observed in five of the nine myocytes studied at the higher temperature and stimulation frequency (data not shown).

**8bcGMP and Myofilament Response to Ca**

The phase-plane diagrams ("loops") of instantaneous cell length versus indo 1 fluorescence for the twitch contractions shown in Fig 2 are illustrated in Fig 6A. The relaxation trajectory of the loop (bold line denoting descending portion of loop) is shifted right and down by 8bcGMP, indicating a reduction in relative myofilament response to Ca²⁺. The effects on twitch contraction of this reduction in myofilament response are that (1) the cell shortens less despite a similar peak systolic Ca²⁺ level, (2) cell length is greater at comparable levels of intracellular Ca²⁺ throughout the twitch, and (3) twitch relaxation (or relengthening) begins at a slightly higher intracellular Ca²⁺ level. In the time domain, the change in relaxation is manifested as relaxation earlier in time at a given intracellular Ca²⁺ level (Fig 2). Identical changes in phase-plane diagrams were observed in all eight indo 1-loaded cells exposed to 8bcGMP (50 μmol/L).

Fig 6B shows the phase-plane diagram for the experiment illustrated in Fig 4. It is evident that, even at the time of the maximal positive inotropic effect (4 minutes), the trajectory during twitch relaxation for the augmented twitch lies to the right and below the control (basal) trajectory, indicating reduction in the relative myofilament response to Ca²⁺. With continued exposure to 8bcGMP, the decrease in twitch amplitude is accompanied by a further rightward and downward shift in relaxation trajectory. Thus, the main difference between a monophasic (negative inotropic) response to 8bcGMP (Fig 6A) and a biphasic response (transient positive followed by a negative effect, Fig 6B) is the occurrence during the biphasic response of an increase in cytosolic Ca²⁺; the relative myofilament response to Ca²⁺ is reduced in both cases.
Negative Inotropic Effects of 8-Bromo-cGMP in Isolated Rat Cardiac Myocytes

<table>
<thead>
<tr>
<th></th>
<th>8bcGMP (0.5 Hz, 25°C)</th>
<th>8bcGMP (2 Hz, 35°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Number of myocytes</td>
<td>13</td>
<td>.</td>
</tr>
<tr>
<td>Resting length, μm</td>
<td>109.3±8.4</td>
<td>110.3±8.3*</td>
</tr>
<tr>
<td>Twitch amplitude, % resting length</td>
<td>9.1±1.4</td>
<td>7.7±1.2*</td>
</tr>
<tr>
<td>Shortening velocity, μm/s</td>
<td>116.3±19.2</td>
<td>113.2±20.6</td>
</tr>
<tr>
<td>tPS, ms</td>
<td>226±15</td>
<td>188±12*</td>
</tr>
<tr>
<td>t50L, ms</td>
<td>346±23</td>
<td>285±17*</td>
</tr>
</tbody>
</table>

8bcGMP indicates 8-bromo-cGMP; Pre, baseline parameters; Post, maximal effect of 8bcGMP; tPS, time to peak shortening; and t50L, time to 50% relaxation. Values are mean±SEM.

*P<.05 for Pre vs Post values.

Fig 7 is a representative example of the effect of 8bcGMP (50 μmol/L) on the steady-state myofilament response to Ca2+ in an intact myocyte tetanized by stimulation at 10 Hz in the presence of thapsigargin (0.2 μmol/L). Despite the achievement of similar peak intracellular Ca2+ levels, in the presence of 8bcGMP steady-state cell shortening during the tetani was progressively decreased, indicating a reduced myofilament response to Ca2+. After prolonged superfusion with pure HEPES buffer, cell shortening returned to control levels. Similar results were observed in three other cells.

8bcGMP and Action Potential

No significant changes in duration of the cellular action potential were observed during external superfusion of 8bcGMP (50 μmol/L) in four myocytes. Electrophysiological parameters before and 10.5±0.5 minutes after exposure to 8bcGMP, respectively, were as follows: resting membrane potential, −71.5±2.7 and −70.5±3.4 mV; action potential duration at 50% repolarization, 19.1±3.9 and 21.2±3.1 milliseconds; and action potential duration at 90% repolarization, 26.4±2.6 and 26.6±2.8 milliseconds (all P=NS).

Effect of 8bcGMP on Cytosolic pH

To investigate whether the 8bcGMP-induced reduction in relative myofilament response to Ca2+ was a result of cytosolic acidification, experiments were carried out in myocytes loaded with SNARF-1. The typical negative inotropic effect of 8bcGMP (50 μmol/L) was not accompanied by a significant change in SNARF-1 fluorescence. In four SNARF-1–loaded myocytes, the maximum change in cytosolic pH after exposure to 8bcGMP was −0.02±0.01 pH units (P=NS).

Influence of a cGMP-Dependent Protein Kinase Inhibitor

Since 8bcGMP is thought to act predominantly via stimulation of PKG,46 we tested the effects of a specific inhibitor of PKG, KT5823,45 on the effects of 8bcGMP. Fig 8A shows that, in the presence of KT5823 (1 μmol/L for 10 minutes before the administration of 8bcGMP), the negative inotropic effect of 8bcGMP was abolished. In four experiments, after 10 minutes of exposure to 8bcGMP (50 μmol/L) in the presence of KT5823, the percent changes in contractile parameters compared with basal values after addition of KT5823 were as follows: twitch amplitude, +8.2±1.5%; time to peak shortening, −9.1±2.7%; and time to 50% relaxation, −9.9±2.2% (all P<.05 versus 8bcGMP alone). Diastolic cell length did not change significantly (+0.1±0.1 μm, P=NS).

Effect of 8bcGMP in Isoproterenol-Pretreated Myocytes

Previous studies have reported that both PKG and cAMP-dependent protein kinase (PKA) phosphorylate the same site on isolated troponin I, so that the effects of these protein kinases are nonadditive.20,21 Fig 8B shows an experiment in which 8bcGMP (50 μmol/L) was added to a myocyte pretreated with a submaximal dose (3 nmol/L) of isoproterenol.41 No negative inotropic effect was observed after the addition of 8bcGMP. In four experiments, the percent changes in contractile parameters compared with basal values after the addition of isoproterenol were as follows: twitch amplitude, +5.2±9.4%; time to peak shortening, −6.2±2.1%; and time to 50% relaxation, −7.8±2.8% (all P<.05 versus 8bcGMP alone). Diastolic cell length did not change significantly (−0.3±0.2 μm, P=NS).
Discussion

The possible role of cGMP in regulating myocardial contraction has received renewed interest recently because of observations that NO, released either by endothelial cells within the heart or within myocytes themselves, influences myocardial contractile behavior probably through the elevation of cGMP. Recent studies in isolated papillary muscles and isolated ejecting hearts suggest that NO or other cGMP-elevating interventions influence predominantly myocardial relaxation, causing an earlier onset of relaxation but with little effect on early contractile indices (eg, peak rate of force development). The mechanisms underlying these effects have been unknown. Previous studies of the contractile effects of cGMP have generally failed to analyze the time course of contraction and relaxation. Furthermore, most investigations of the effects of cGMP on myocardial subcellular processes have failed to correlate these data with changes in contractile behavior.

In the present study using isolated intact cardiac myocytes, we have correlated the contractile effects of 8bcGMP (used in threshold doses) with changes in intracellular Ca\(^2\+\). The main finding is that cGMP decreases the relative myofilament response to Ca\(^2\+\) and thus (1) induces a reduction in resting or diastolic tone, manifested as an increase in unloaded cell length, (2) induces an earlier onset of isometric twitch relaxation (or relengthening), and (3) causes a small decrease in isotonic twitch amplitude. Significant changes in action potential duration were unlikely, since Ca\(^2\+\) transients were unaltered, and were not observed in cells in which the action potential was recorded. 8bcGMP-induced reduction in relative myofilament response to Ca\(^2\+\) was confirmed both in twitch contractions, by phase-plane analysis, and in steady-state tetanic contraction. Reduction in diastolic tone (manifested as an increase in diastolic cell length) was also confirmed in quiescent myocytes. The pattern of change in relaxation induced by 8bcGMP in isolated intact myocytes was similar to that previously reported with other cGMP-elevating interventions (eg, endothelium-derived NO) in isolated papillary muscles and isolated ejecting hearts, suggesting that the doses we studied were physiologically relevant. cGMP-induced reduction in myofilament response to Ca\(^2\+\) was probably mediated through PKG, since 8bcGMP is thought to be a potent activator of PKG and since the negative inotropic effects of 8bcGMP were inhibited by KT5823, a relatively specific inhibitor of PKG. The inhibition constant (K_i) for PKG is reported to be \(\approx 0.2\) \(\mu\)mol/L with KT5823, whereas the K_i values for other protein kinases (eg, PKA and myosin light chain kinase) are reported to be >10 \(\mu\)mol/L. Other potential inhibitors of PKG, such as the compound \(N\)-(2-[methylamino]ethyl)-5-isoxquinolinesulfonic dihydrochloride (or H-8), tend to be even less specific and tend to inhibit several other protein kinases (particularly protein kinase C). The effect of 8bcGMP was not attributable to a reduction in cytosolic pH, which is recognized to reduce myofilament responsiveness to Ca\(^2\+\).

Fig 7. Representative example of effect of 8-bromo-cGMP (8bcGMP, 50 \(\mu\)mol/L) on steady-state myofilament Ca\(^2\+) response during tetanic contraction (10 Hz) of a myocyte pretreated with thapsigargin (0.2 \(\mu\)mol/L). At the top are Ca\(^2\+) transients and below are tetanic contractions after 5, 10, and 15 minutes of exposure to 8bcGMP and after a 30-minute washout, in each case compared with the baseline control (C) data. The amplitude of tetanic contraction is progressively decreased after exposure to 8bcGMP, without a corresponding decrease in peak intracellular Ca\(^2\+\).

Fig 8. Representative chart recordings showing effect of 8-bromo-cGMP (8bcGMP, 50 \(\mu\)mol/L). A shows a myocyte pretreated with cGMP-dependent protein kinase inhibitor KT5823 (1 \(\mu\)mol/L for 10 minutes); B, a myocyte pretreated with isoproterenol (ISO, 3 mmol/L). No reduction in twitch amplitude after the administration of 8bcGMP was observed in either case.
A change in myofilament responsiveness to Ca\(^{2+}\) is one of two general mechanisms that regulate myocardial contractile state, the other being a change in the cytosolic Ca\(^{2+}\) transient.\(^{40,51}\) Alteration in the myofilament response to Ca\(^{2+}\) may relate to several different mechanisms, including a change in binding of Ca\(^{2+}\) to the regulatory protein, troponin C, or a change in kinetics of actomyosin crossbridge turnover.\(^{51}\) Our studies do not allow elucidation of the precise nature of myofilament effect. However, the observation that unloaded shortening velocity was unaltered by 8bcGMP may suggest that maximal myosin ATPase activity was unchanged.\(^{52}\) Phosphorylation by PKG of isolated troponin I at possibly the same site as that phosphorylated by PKA has been reported.\(^{20,21}\) Phosphorylation of troponin I by PKA reduces the affinity of troponin C for Ca\(^{2+}\) and enhances dissociation of the troponin C/Ca\(^{2+}\) complex.\(^{53}\) The failure of the present study to demonstrate a further reduction in myofilament response to Ca\(^{2+}\) with 8bcGMP in isoproterenol-pretreated myocytes, using sub-maximal doses of isoproterenol, is consistent with PKG and PKA having a similar, but nonadditive, action on cardiac myofilaments, although it is not direct proof of this possibility. We are aware of one preliminary report that PKG induces a rightward shift of the tension/pCa relation in skinned single rat ventricular cells, compatible with reduction in Ca\(^{2+}\) sensitivity of troponin C.\(^{54}\) There is also a previous report by McClellan and Winegrad\(^{29}\) that cGMP increases maximum Ca\(^{2+}\)-activated force in hyperperfusable rat ventricular fibers, but only in the presence of dilute detergent. However, in light of recent evidence that dilute detergent solutions selectively damage cardiac endothelial cells and thus alter the release of diffusible agents that modulate myocardial contraction,\(^{17,23,33,50}\) the mechanism of the observed effects of cGMP in that report requires reinvestigation.

The responsiveness of cardiac myofilaments to Ca\(^{2+}\) may be altered by several intracellular molecules, including cAMP, inorganic phosphate, hydrogen ions, and protein kinase C.\(^{40,51,53,57}\) There is less strong evidence for a similar effect of myosin light chain kinase, inositol trisphosphate, and various phosphatases.\(^{49,53,58}\) The reduction in myofilament response to Ca\(^{2+}\) observed with 8bcGMP in the present study differs somewhat from most of these agents, in that no major additional effects on cytosolic Ca\(^{2+}\) were consistently found. In approximately half the cells studied, 8bcGMP did induce a small transient positive inotropic effect, attributable to an increase in cytosolic Ca\(^{2+}\). However, the predominant effect in all cells was a reduction in myofilament response to Ca\(^{2+}\). The mechanism of the rise in cytosolic Ca\(^{2+}\) in approximately half the cells was not elucidated but did not appear to be a result of any obvious difference in baseline morphology or function of these cells. One possibility is that it was a result of cGMP-mediated inhibition of a cAMP phosphodiesterase,\(^{14}\) with a consequent increase in cAMP levels and thus in sarcosomelal Ca\(^{2+}\) influx and in sarcoplasmic reticulum Ca\(^{2+}\) cycling in these cells. The pattern of change in twitch contraction during the positive inotropic effect, with an increase in twitch amplitude and shortening velocity and a decrease in twitch duration, was consistent with this possibility.\(^{41}\) We did not, in the present study, investigate the influence of 8bcGMP pretreatment on β\(_1\)-adrenergic agonist–induced changes in myocardial contraction or the effect of cGMP pretreatment on responses to other agonists. It is possible that cGMP could have different actions under these conditions. For example, some (though not all) of the studies referred to earlier have suggested interaction between cGMP- and cAMP-mediated mechanisms.\(^{1,6,8,13,14,59}\)

The implications of these findings for cardiac pump function are speculative at this stage. A similar pattern of change with cGMP-elevating interventions has recently been reported in isolated ejecting hearts.\(^{24,29}\) In vivo, a hastening of relaxation and reduction in diastolic tone could enhance both cardiac filling and coronary perfusion by increasing the diastolic time interval and improving subendocardial perfusion, respectively.\(^{60}\) However, any direct myocardial relaxant effects could be overwhelmed by the complex hemodynamic effects of associated changes in heart rate, loading, and neurohumoral state. The relevant physiological stimuli that raise myocardial cGMP in vivo are not established. Intracardiac release of NO, either by endothelial cells or by myocytes themselves, would be an attractive mechanism for modulating myocardial contraction independent of systemic effects, because of the very brief biologic life of NO.\(^{30}\) In conclusion, the present study demonstrates that cGMP decreases the relative myofilament response to Ca\(^{2+}\) in intact cardiac myocytes and suggests that cGMP could play a role in selectively modulating myocardial relaxation and cardiac diastolic tone.

Acknowledgments

Dr Shah was supported by the British Heart Foundation and the Medical Research Council of Great Britain.

References

28. Evans
22. 21. 24. Fort
27. 26.


McClellan GB, Winegrad S. Cyclic nucleotide regulation of the contractile proteins in mammalian cardiac muscle. J Gen Physiol. 1980;75:283-295.


McVor ME, Orchard CH, Lakatta EG. Dissociation of changes in apparent myofilibril Ca++ sensitivity and twitch relaxation induced by α-adrenergic and cholinergic stimulation in isolated ferrat cardiac muscle. J Gen Physiol. 1988;92:509-529.

8-bromo-cGMP reduces the myofilament response to Ca2+ in intact cardiac myocytes.
A M Shah, H A Spurgeon, S J Sollott, A Talo and E G Lakatta

Circ Res. 1994;74:970-978
doi: 10.1161/01.RES.74.5.970

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/74/5/970

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/