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# Reversal of effects of acidosis on contraction of rat heart myocytes by CGP-48506

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## 1. ABSTRACT

In experiments reported here, we tested the ability of CGP-48506 to reverse the depressed cardiac contractility associated with hypercapnic acidosis in isolated rat cardiac myocytes. CGP-48506 is a cardiotonic agent that directly and specifically promotes the actin-cross-bridge reaction. Myocytes superfused at pH 6.8 demonstrated a significantly reduced extent of cell shortening, but an increase in the peak amplitude of the $Ca^{2+}$ transient. Moreover, cells in acidosis showed small, but significant, decreases in time to peak shortening to 50% relaxation. Superfusion of the cells with 3, 7, and 10 micro-molar CGP-48506 restored the inhibited contractility as a function of concentration with no significant effects on the $Ca^{2+}$-transient. Moreover, 10 micro-molar CGP-48506 completely reversed the depressed myocyte contraction associated with an increase in time to peak shortening and time to 50% and 75% relaxation. Our results indicate that the depression of contractility associated with acidosis is due to a reduced myofilament response to $Ca^{2+}$, which can be overcome by agents working downstream from troponin C through a direct effect on the actin-myosin interaction.

## 2. INTRODUCTION

In experiments reported here we have tested whether a cardiotonic agent with direct effects on the actin-myosin reaction is able to reverse the effects of acidosis on contraction of isolated cardiac myocytes. Our rationale for these experiments was based on: 1) evidence that the decrease in the response of the myofilaments to increased intracellular $Ca^{2+}$ during acidosis is the main mechanism for the decline of force production (1), and 2) the unique properties of CGP-48506 to directly and specifically affect the myofilament response to $Ca^{2+}$ with much less of an effect on cardiac relaxation than other agents working through this mechanism (2,3).

Although acidosis inhibits the $Ca^{2+}$ current, the $Ca^{2+}$ pump activity of the sarcoplasmic reticulum (4) , and the $Na^{+}$-$Ca^{2+}$ exchange mechanism (5), direct measurements of intracellular $Ca^{2+}$ have shown that $Ca^{2+}$ delivery to the myofilaments actually becomes larger during acidosis (1). Apart from displacement of $Ca^{2+}$ from buffer sites including troponin C (6), it is known that acidic pH leads to an increase of intracellular $Ca^{2+}$ indirectly

through changes in cytoplasmic $Na^+$ via $Na^+$-$H^+$ mechanism (5). This increased $Na^+$ in turn induces an elevation of intracellular $Ca^{2+}$ via the $Na^+$-$Ca^{2+}$ exchange mechanism. The increase in cytoplasmic $Ca^{2+}$ leads to increased $Ca^{2+}$ loading of SR and hence increases release from SR. Yet, despite this increase in cytoplasmic $Ca^{2+}$, force falls, most likely due to an altered response of the myofilaments to $Ca^{2+}$. Mechanisms by which a fall in pH could depress systolic cardiac force generation involve both Ca-dependent and cross-bridge binding dependent activation of the myofilaments. Acidic pH is likely to decrease the ability of oxygens in the metal binding loop of the regulatory site of TnC to coordinately bind $Ca^{2+}$ (7). Moreover, transmission of the Ca-binding signal to TnI or TnT also appears to play an important role in the reduction of myofilament response to $Ca^{2+}$ associated with acidosis (8). A fall in pH is also well known to depress the force generating capability of cardiac myofilaments at concentrations of $Ca^{2+}$ at which TnC is saturated (1,6, 9). This effect, which suggests that the actin-cross bridge reaction itself is pH sensitive, has two important consequences. The first is that Ca-induced activation of myofilament force generation is depressed, and the second is that the ability of cross-bridges to activate the thin filament is also depressed.

In the search for agents with an ability to overcome depressed myocardial function in acidosis, agents that directly affect myofilament response to $Ca^{2+}$ (Ca-sensitizers) are especially interesting (10,11). These drugs, in principle, can increase force with little change or even a fall in levels of systolic $Ca^{2+}$. Other agents, such as β-adrenergic agonists, phosphodiesterase (PDE) inhibitors, and cardiac glycosides, all increase cellular $Ca^{2+}$ and can precipitate dysfunction associated with $Ca^{2+}$ overload as well as arrhythmias. However, in some cases Ca-sensitizers have had problems of their own. For reasons not clearly understood, many of these agents also possess PDE inhibitory activity (10,11) and there is a possible impairment of relaxation. Among these agents, the benzodiazocine CGP-48506 stands out as having no detectable PDE III inhibitory activity as well as minimal effects on diastolic force generation under relaxing conditions (12, 3). In experiments reported here, we therefore tested whether these advantages of CGP-48506, which have been investigated in normal (12, 13) and chronically failing heart preparations (14, 15), are important in the acute depression of cardiac function associated with acidosis.

## 3. METHODS

### 3. 1. Myocyte isolation and loading with fura 2-AM or BCECF

We isolated ventricular myocytes essentially as previously described (3). Adult, male Sprague-Dawley rats weighing 200-420 g were pretreated with heparin (200 U) and anesthetized with pentobarbital sodium (50 mg/kg intra-peritoneal). Hearts were rapidly removed, weighed, and transferred to cold, nominally $Ca^{2+}$- free control solution of the following composition (in milli-molar) : 133.5 NaCl, 4 KCl, 1.2 $NaH_2PO_4$, 1.2 $MgSO_4$, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 11 glucose, pH 7.4 containing bovine serum albumin (1 mg/ml ). The hearts were perfused through the aorta on a Langendorff apparatus at 37°C and at a perfusion pressure of 70 cm $H_2O$ for 5 min with $Ca^{2+}$-free control solution. The hearts were then perfused for 16.5 min/g wet weight with the above bovine serum albumin control solution containing collagenase D (0.3 mg/ml) and 5 micro-molar added $Ca^{2+}$ (actual $Ca^{2+}$ was higher inasmuch as we did not buffer the $Ca^{2+}$ ). At the end of perfusion period, the hearts were removed and placed into a dissection dish containing bovine serum albumin control solution with 50 micro-molar $Ca^{2+}$. After separation from the atria, ventricles were chopped and incubated in a 37°C water bath. They were gently triturated with a pipette every 1 min for 10 min. After the cell suspension was filtered and placed into centrifuge tubes, the cells were allowed to settle for 10 min. Thereafter the supernatant fraction was removed, and cells were resuspended in fresh bovine serum albumin control solution with 100 micro-molar $Ca^{2+}$. The procedure was repeated using fresh bovine serum albumin control solution with 200 micro-molar $Ca^{2+}$. The myocytes, which were kept at room temperature (22-23°C) until used, were transferred and allowed to settle in a chamber mounted on the stage of a Nikon inverted microscope. The cells were loaded at room temperature with fura 2-AM in loading solution (bovine serum albumin control solution containing 5% fetal calf serum, 0.1 milli-molar $Ca^{2+}$ and 5 micro-molar fura 2-AM (from 1 milli-molar stock in dimethyl sulfoxide) for 8 min or 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF, 9 micro-molar for 10 min). After loading, extra-cellular dye was washed out by perfusing with fresh pH 7.41 Tyrode's solution (equilibrated with 5% $CO_2$-95%$O_2$) and containing in milli-molar: 93 NaCl , 5 KCl, 20 $Na_2HCO_3$, 1 $Na_2HPO_4$, 1 $MgSO_4$, 20 sodium acetate, 1 $CaCl_2$, 10 glucose). The cells were field stimulated (0.5/s) with platinum electrodes placed close to the cell surface.

### 3.2. Measurement of $[Ca^{2+}]_i$ transients and $pH_i$

To measure the fura-2 signal, the cells were excited alternately at 340 and 380 nm wavelengths. The fluorescence emission was measured at 505 nm. The excitation light was transmitted to the cell under study by a 400 nm dichroic mirror beneath the microscope nosepiece and a 40 × Nikon oil-immersion objective lens. The emitted light was collected by the objective and transmitted to a multi-image module of the microscope. BCECF-loaded myocytes were alternately excited at 450 and 490 nm, and fluorescence was monitored using the same emission filter. The excitation light was transmitted to the myocyte under study by a 510 nm dichroic mirror. The signal was separated from long wavelength light (red, >600 nm) using a 580 nm dichroic mirror and passed to an emission mono-chromator, and then to a photomultiplier tube. The field of view was narrowed to a single cell using a field diaphragm mounted between the multi-image module and the photomultiplier tube. The background fluorescence was estimated each day of experiments by measuring and averaging the auto-fluorescence from at least seven cells of the same size. The appropriate ratio (340/380 for cells loaded with fura-2 or 490/450 for cells loaded with

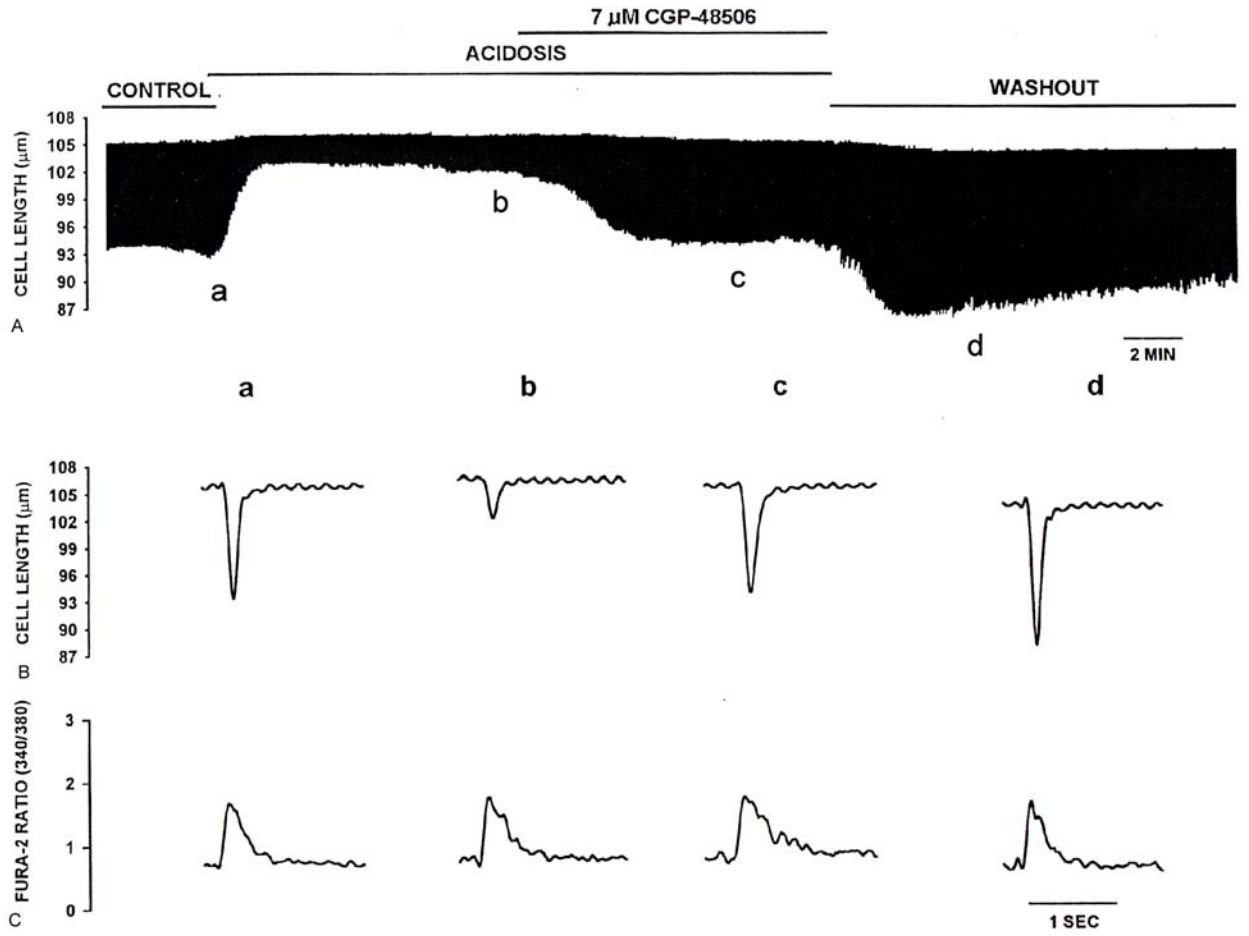


**Figure 1.** Data demonstrating the effect of 7 micro-molar CGP-48506 to restore contractions to control levels during a period of hypercapnic acidosis. A. Contractions on a slow time base demonstrating the severe reduction in extent of shortening with acidosis and the ability of CGP-48506 to restore contractility. B. Single contractions on a fast time base selected from steady-state points (indicated by the lower case letters) during the control period, acidosis, acidosis + CGP-48506, and washout. C. Single fura-2 transients determined on a fast time base simultaneously with the twitch contractions in panel B and demonstrating a constant peak amplitude of the $Ca^{2+}$ transient during the periods of acidosis, acidosis + CGP-48506.

BCECF) was calculated after subtracting the background fluorescence using the SPEX program. Fluorescence emission was collected for 3 ms at each wavelength, and the time between data points was 12 ms. Calibration of the BCECF signals was carried out as previously described (16).

### 3.3. Measurements of cell shortening

To monitor cell shortening, myocytes were illuminated with red light using a special red filter (>600nm). The image of cells was collected by the 40 × Nikon objective and transmitted to the multi-image module, where it was separated from the fluorescence by a 580 nm dichroic mirror. Output from the camera was split and went to a VCR and to a video-edge detector used to measure cell length. The signal from the video-edge detector was connected to a TV monitor where the image of cell was projected. The cell length signal was recorded on an Astro Med chart recorder and simultaneously recorded with the fluorescence signals on the computer. After measuring $[Ca^{2+}]_i$ and cell shortening in control Tyrode's solution, pH 7.41, we changed to acidic Tyrode's solution, equilibrated with 15% $CO_2$-85%$O_2$, pH 6.77. When contractions reached steady-state in acidic Tyrodes, the myocytes were superfused with CGP-48506 solution, pH 6.78. The pH of the solution was monitored continuously using a pH probe. After cells achieved a steady-state contraction, they were washed with control solution, pH 7.41.

### 3.4. Materials

CGP-48506 was synthesized at the Department of Cardiovascular Chemistry Research, Ciba-Geigy. Stock solutions (20 milli-molar) of CGP 48506 were made in dimethyl sulfoxide and kept at -20°C until used. They were diluted with DMSO such that the final concentration of solvent in the experimental solutions was always 0.5%. Control solutions contained 0.5% DMSO. Collagenase type D was purchased from Boehringer Mannheim Corporation,

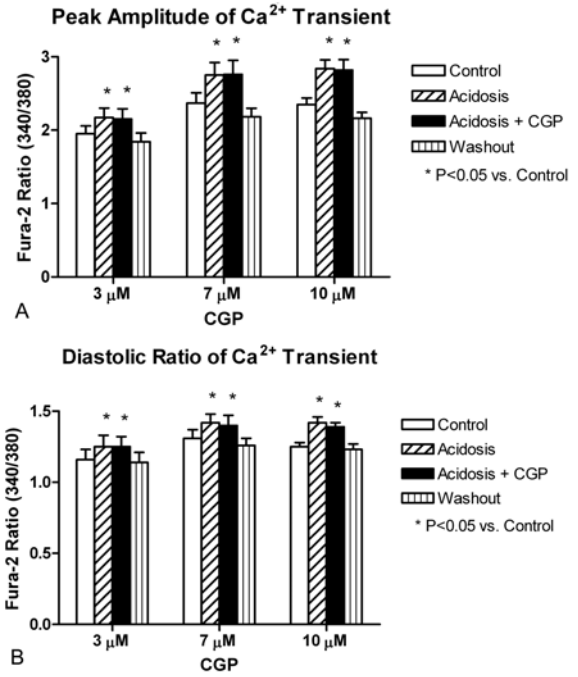


**Figure 2.** Histograms summarizing the effect of increasing concentrations of CGP-48506 on dynamics of the $Ca^{2+}$ transients during steady-state points in the control period, acidosis, acidosis + CGP-48506, and washout. A. Data demonstrating significant effects of acidosis to increase the peak amplitude of the $Ca^{2+}$ transient and the lack of effect of CGP-48506 on these changes. B. Data demonstrating significant effects of acidosis to increase the diastolic levels of $Ca^{2+}$ and the lack of effect of CGP-48506 on these changes.

Indianapolis, IN 46250. fura 2-acetoxymethyl ester was purchased from Molecular Probes, Eugene, OR.

### 3.5. Statistical analysis

All data were expressed as means ± standard error of the mean of n preparations. Statistical comparisons were made using repeated measures/analysis of variance.

## 4. RESULTS

Figure 1 shows representative responses to acidosis and 7 micro-molar CGP-48506 with acidosis on cell shortening during twitch contraction (A and B) and on fura-2 fluorescence ratio (C). Compared to control conditions at pH 7.41, cells at pH 6.77 demonstrated a rapid decline in cell shortening. The myocytes subsequently exhibited a slow, partial recovery of cell shortening. Superfusion with 7 micro-molar CGP-48506 with acidosis reversed the inhibited contractility. With a change to control solution at pH 7.41, myocytes showed an initial significant increase of cell shortening that was followed by return to contractions similar to those during the control period. Figure 1C shows representative examples of $Ca^{2+}$-transients of cells in acidosis and 7 micro-molar CGP-48506 with acidosis. As summarized in Figure 2A, on average acidosis induced a small, but significant increase of the peak amplitude of $Ca^{2+}$ and diastolic fura-2 ratio compared to control conditions. Superfusion with 7 micro-molar CGP-48506 during acidosis did not induce any significant changes in the $Ca^{2+}$ transients. After return of the cells to pH 7.4 solutions, $Ca^{2+}$ transients returned to the control levels.

Figure 2 summarizes the results of experiments testing effects of increasing concentrations of CGP-48506 on the peak amplitude of $Ca^{2+}$ transients (Figure 2A) and diastolic fura-2 ratios (Figure 2B). In each case, CGP-48506 did not affect the significantly increased the peak amplitude and diastolic levels of the $Ca^{2+}$ transients during acidosis. Data in Figure 3A summarize the effects of acidosis and superfusion with CGP-48506 on cell shortening. Compared to controls at pH 7.41, cells at pH 6.77 demonstrated a significantly reduced time to peak shortening (Figure 3B), which was reversed by 3, 7 and 10 micro-molar CGP-48506. Moreover, 10 micro-molar CGP-48506 completely reversed the depressed cardiac contraction. The effects of acidosis alone and acidosis in the presence of three different concentrations of CGP-48506 on dynamics of contraction are shown in Figure 3B-3D. Compared to controls at pH 7.41, cells at pH 6.77 demonstrated a significantly reduced extent of time to peak contraction (Figure 3B). Three different concentrations of CGP-48506 significantly prolonged the reduced extent of time to peak contraction. Figure 3C summarizes the effects of acidosis and superfusion with CGP-48506 on time to 50% relaxation. Acidosis significantly reduced time to 50% relaxation. Although 3 micro-molar CGP-48506 did not change time to 50% relaxation, 7 and 10 micro-molar CGP-48506 significantly increased the time to 50% relaxation from that occurring during acidosis. Figure 3D summarizes the effects of acidosis and superfusion of CGP-48506 on time to 75% relaxation. Although acidosis did not reduce time to 75% relaxation, there was a significant increase in time to 75% relaxation with 3, 7, and 10 micro-molar CGP-48506. In a few cells the diastolic cell length was either slow to reverse or did not completely reverse on washout of CGP or acidosis or this process was very slow. On average, however, diastolic length did return to the same levels as the control and treated myocytes. Diastolic cell length in micro-meter was 104.4 ± 2.3 (mean ± SEM) for controls, 107.2 ± 2.4 for acidosis, 106.8 ± 2.3 for acidosis + CGP, and 105.2 ± 2.4 for wash-out.

To highlight the effects of CGP-48506 on myofilament response to $Ca^{2+}$ during acidosis, we have plotted the relation between fura-2 ratio and cell length in Figure 4. (17) The $Ca^{2+}$ - shortening loops illustrate the significant effect of CGP-48506 to restore systolic shortening and $Ca^{2+}$ sensitivity during a twitch contraction in acidosis. They also illustrate the significant effect of CGP-48506 in overcoming the effects of acidosis during the active contraction.

## 5. DISCUSSION

Our demonstration that treatment of cardiac myocytes with CGP-48506 is able to fully reverse the mechanical effects of acidosis with no change in the $Ca^{2+}$

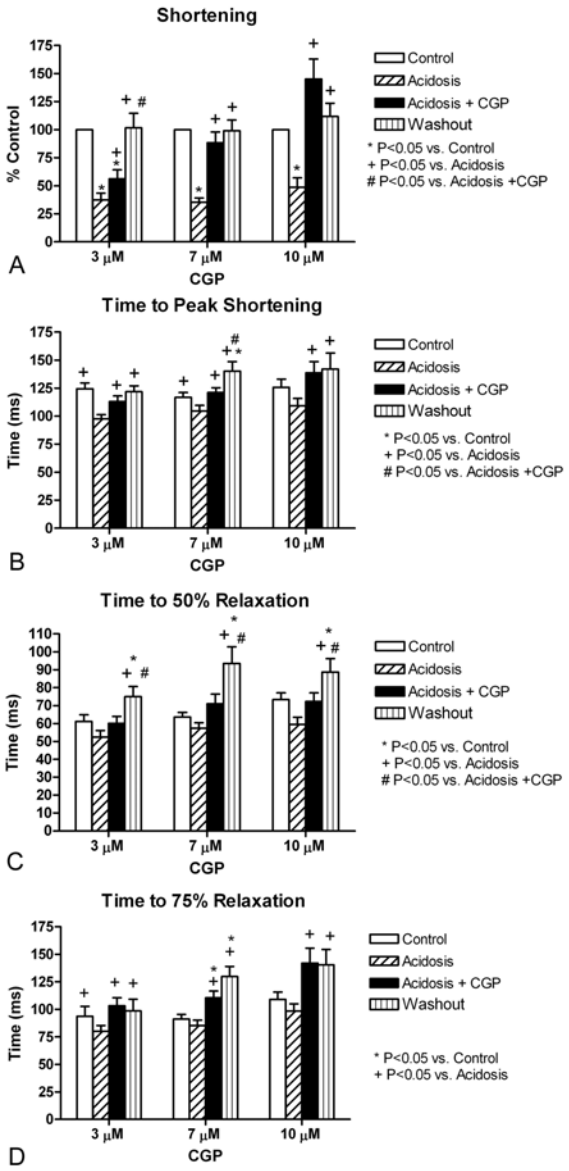


**Figure 3.** Histograms summarizing the effect of increasing concentrations of CGP-48506 on dynamics of length changes in twitches during steady-state points in the control period, acidosis, acidosis + CGP-48506, and washout. A. Data demonstrating significant effects of acidosis to depress the extent of shortening and the ability of CGP-48506 to restore contraction amplitude in a dose dependent manner. B. Data demonstrating significant effects of acidosis to depress the time to peak of shortening and the ability of CGP-48506 to restore time to peak shortening during acidosis. C. Data demonstrating significant effects of CGP-48506 to prolong time to 50% relaxation during acidosis. D. Data demonstrating a prolongation of the time to 75% relaxation in the presence of CGP-48506 during acidosis.

transient constitutes a significant advance and proof of principal with regard to the search for inotropic agents acting specifically at the actin-myosin interface. This concept has also been supported by studies employing other $Ca^{2+}$-sensitizers (18,19). Although CGP-48506 was not developed further as therapeutic agent, the search for such sarcomeric activators continues (10, 20). The mechanism by which CGP-48506 reversed the negative inotropic effects of acidosis must be considered in the context of current hypotheses regarding how and why elevated proton concentrations depress tension generation by cardiac myofilaments. Turning the force generating actin-myosin interaction of cardiac myofilaments on and off involves allosteric and steric mechanisms as well as short and long range cooperative mechanisms that could be affected by changes in pH (21). When solution pH is reduced, the relationship between intracellular $Ca^{2+}$ and contractile activity of cardiac myofibrils is affected in two ways. One is an inhibition of $Ca^{2+}$-induced (1,9) and cross-bridge induced (22-24) myofilament activation that results in a decrease in myofibrillar activity at sub-maximally activating levels of free $Ca^{2+}$. The other is a potential reduction in the number of force generating cross-bridges reacting with the thin filament (9) and a depression of the maximum force production at saturating levels of $Ca^{2+}$ (1).

The acidosis induced decrease in myofilament response to $Ca^{2+}$ is complex and involves a lower affinity of TnC for $Ca^{2+}$, but also alterations in thin filament Ca-signaling and thin filament activation by bound cross-bridges. With a drop in pH, the affinity of myofilament TnC binding sites for $Ca^{2+}$ is reduced (6). Yet, our earlier studies (3) demonstrated by direct measurements in skinned fiber bundles that the affinity of TnC for $Ca^{2+}$ was unaffected by CGP-48506. It seems more likely that CGP-48506 reverses the effects of acidosis on myocyte mechanics by mechanisms downstream from the thin filament regulatory proteins at the actin-myosin interface. An action of CGP-48506 at the actin-myosin interface was strongly suggested by our experiments showing that CGP could overcome a 2,3-butanedione monoxime (BDM) induced depression of shortening in isolated myocytes and depression of force generation by skinned fiber bundles (3). This is an important observation in the context of the present experiments in that both BDM and acidic pH depress strong cross-bridge interactions with actin.

Acidic pH may not only depress maximum force generation through this mechanism, but also alter activation of the myofilaments by strongly bound cross-bridges. One effect of bound cross-bridges is an increase in the affinity of TnC for $Ca^{2+}$ (25). A second action of strongly bound cross-bridges is to cooperatively activate near neighbor functional units, consisting of a stretch of actins under the control of a troponin-tropomyosin complex. The effect of acidic pH on this mechanism has been explicitly tested (22-24). In these experiments, force generated by myofilaments of detergent-treated single cardiac myocytes was activated in the virtual absence of $Ca^{2+}$ by varying the MgATP concentration. At low concentrations of MgATP, rigor links form and maximally activate the thin filament; force is generated by cross-bridges that contain nucleotide. As MgATP increases, fewer rigor links are present and activation by this mechanism wanes. At the acidic pH all of these studies reported a shift of the relation such that

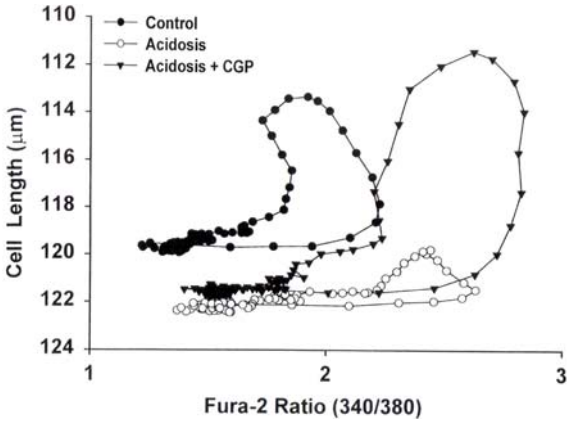


**Figure 4.** Relation between cell length and fura-2 ratio during entire contraction-relaxation cycle in single twitch during steady state in the control condition, during acidosis, and during acidosis + CGP-48506. The loops demonstrate the severe depression induced by acidosis in extent of shortening and in sensitivity to $Ca^{2+}$ as indicated by the difference in the falling phase of relation between fluorescence and cell length. The loop in the presence of acidosis + CGP-48506 demonstrates a restoration of extent of shortening and an enhanced myofilament responsiveness to $Ca^{2+}$.

acidosis reduced the ability of a particular level of MgATP to sustain thin filament activation. Evidence (25) demonstrating that both muscle tension and stiffness during the rigor state are depressed during acidosis suggests that a straight-forward interpretation of these experimental results is that acidic pH reduced the strength of the actin-myosin complexes, and reduced the ability of bound cross-bridges to cooperatively activate the thin filament. In view of the ability of CGP-48506 to directly activate the actin-myosin reaction, it is apparent that an important mechanism by which CGP-48506 relieves myocytes from the inhibition by acidic pH is by restoring the ability of cross-bridges to cooperatively activate the thin filament.

There is also evidence that CGP-48506 may have an effect on tension cost (ratio of tension developed to ATP hydrolysis) during acidosis. Both maximum tension and maximum ATPase rate of cardiac myofilaments falls with a drop in pH. However, the fall in maximum tension is greater than that of maximum ATPase activity. For example, Ebus et al (26) reported that maximum tension at pH 6.2 was 54% of that at pH 7.0, whereas maximum ATPase activity at pH 6.2 was 80% that at pH 7.0. Thus, acidic pH may not only reduce maximum tension by a reduction in numbers of strongly bound cross-bridges, but also by affecting tension cost, the ratio of tension:ATPase rate. CGP-48506 has been reported to reduce tension cost in muscle strips from human myocardium (13). This inotropic mechanism is of some advantage in the case of the acidotic state of heart muscle in which energy consumption is down during a time of reduced energy supply. Reduced energy consumption is undoubtedly an important protective mechanism in that heart cells are spared from entering into a rigor state and a condition of $Ca^{2+}$ overload with electrical and mechanical arrhythmias.

An understandable concern with the use of agents that sensitize the myofilaments to $Ca^{2+}$ is that they may impair relaxation (10). In our studies, the reversal of the effects of acidosis could be obtained in a dose range (up to 7 micro-molar) in which there was no change in time to half maximal relaxation and only a slight (~10%) increase in time to 75% relaxation. These results generally agree with the data of Palmer and Kentish (2), who used flash photolysis of the caged $Ca^{2+}$ chelator, diazo-2, to demonstrate that the intrinsic relaxation rate of myofilaments was unaffected by concentrations of CGP-48506<10 micro-molar. Thus, our findings demonstrate the potential that the promotion of the actin-myosin interaction by CGP-48506 may not only be therapeutically useful, but not interfere with relaxation as has been a concern with $Ca^{2+}$-sensitizers increasing $Ca^{2+}$-binding to troponin C. In the case of studies with ejecting rabbit hearts, Slinker et al. (27) reported that at the same level pressure generating capability that CGP-45906 and elevated extra-cellular $Ca^{2+}$ had no significant effect on relaxation, whereas EMD-57033 slowed relaxation, and dobutamine hastened relaxation. On the other hand, Senzaki et al. (28) have reported that EMD 57033 improved contractility with no impaired relaxation in hearts of dogs stressed by tachycardia induced heart failure. Similar effects were reported in studies with both EMD-57033 and CGP-48506 in isolated myocytes from dog hearts in failure following tachycardia (15).

Results of clinical trials employing agents with effects at the level of the cardiac sarcomere have been summarized elsewhere (29). However, there is evidence for a new potential use of these agents in cardiomyopathies linked to mutations in sarcomeric proteins. These myopathies generally demonstrate a reduction in myofilament response to $Ca^{2+}$, and a typical example is a mouse knock-in model expressing a mutant form of cardiac troponin T with deletion of a critical lysine (30,31). Treatment of these mice with pimobendan an agent with combined $Ca^{2+}$ -sensitizing and PDE III inhibition rescued cardiac and myofilament function and increased survival (32). Rescue could not be obtained by treatment of the mice with a pure phosphor-diesterase inhibitor or a beta-adrenergic receptor blocker. The action of pimobendan is at the thin filament, most likely troponin C, and it will be of some interest to determine whether agents such as CGP-48506, which promote the reaction of cross-bridges with thin filament will have similar effects on inherited and acquired cardiac disorders. Fortunately, new compounds are under development with promise for the future (33).

## 6. ACKNOWLEDGEMENTS

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