Contributions of $[Ca^{2+}]_i$, $[P_i]_i$, and pH_i to Altered Diastolic Myocyte Tone during Partial Metabolic Inhibition

Hiroshi Ikenouchi, Osami Kohmoto, Mischelle McMillan, and William H. Barry
Division of Cardiology, Department of Medicine, University of Utah School of Medicine, Salt Lake City, Utah 84132

Abstract

Ischemia may cause increased or decreased distensibility of the left ventricle, but the cellular mechanisms involved have not been clarified. We examined the possible contributions of changes in intracellular inorganic phosphate, pH, and Ca^{2+} concentrations to altered diastolic function in cultured myocytes subjected to partial metabolic inhibition. Paced cultured embryonic chick and adult rabbit ventricular myocytes superfused with 20 mM 2-deoxyglucose (2DG) exhibited an increase in end-diastolic intracellular free calcium concentration ($[Ca^{2+}]_i$) and an upward shift in end-diastolic cell position. These results indicate that glycolytic blockade increases diastolic and systolic calcium in paced ventricular myocytes, and that this elevated diastolic calcium influences the extent of diastolic relaxation. In contrast, paced ventricular myocytes superfused with 1 mM cyanide (CN) exhibited a similar increase in end-diastolic $[Ca^{2+}]_i$ but a decrease in end-diastolic cell position and amplitude of motion. Although changes in ATP contents were similar in both groups (2DG, −29.9%; CN, −40.1%), alterations of intracellular pH and inorganic phosphate concentrations were different. In 2DG-treated cells, pH_i did not decrease significantly (7.18±0.04 to 7.12±0.11, $n = 14$) but in the CN group it decreased markedly within 6 min (7.18±0.04 to 6.76±0.11, $n = 11$, $P < 0.01$). Intracellular inorganic phosphate decreased slightly in the 2DG group (−14.8%, NS) but increased in cells exposed to CN (45.7%, $P < 0.02$). We conclude that while a prominent increase in diastolic $[Ca^{2+}]_i$ occurs in rapidly paced ventricular myocytes exposed to either inhibitors of glycolysis or oxidative phosphorylation, the effects of this increase in $[Ca^{2+}]_i$ on diastolic distensibility may be influenced by intracellular accumulation of metabolites that decrease the sensitivity of myofilament to $[Ca^{2+}]_i$. (*J. Clin. Invest.* 1991. 88:55–61.) Key words: cardiac myocytes · indo-1 · sodium cyanide · 2-deoxyglucose · diastolic distensibility

Introduction

It is recognized that in patients with coronary heart disease, ischemia produced by rapid atrial pacing can cause a transient decrease in left ventricular diastolic distensibility (1, 2). In canine coronary artery constriction models, an analogous "demand" ischemia has also been demonstrated to cause an upward shift in the left ventricular pressure–volume curve consistent with an increase in diastolic myocardial stiffness (3, 4). Several mechanisms to account for this diastolic dysfunction have been postulated, including chamber interaction and/or effects of the pericardium (5–7), and asynchronous left ventricular relaxation (8–11). It has also been suggested that increased stiffness during demand ischemia is due to a rise in diastolic intracellular free calcium concentration ($[Ca^{2+}]_i$) in ischemic ventricular myocytes, resulting in increased diastolic force development by myofilaments, and thus incomplete relaxation (4, 12–15). However, during "supply" ischemia, as occurs with complete coronary artery occlusion, which would also be expected to increase diastolic $[Ca^{2+}]_i$, diastolic distensibility is initially increased (16). This paradox has been recognized (17) but factors that could account for it have not been elucidated. Paulus (18) has recently suggested that the differences in the extent of accumulation of metabolites that alter Ca^{2+} sensitivity of contractile elements could influence the effect of an increase in $[Ca^{2+}]_i$ caused by ischemia on diastolic distensibility. It has been demonstrated that intracellular pH (pH_i) and/or intracellular inorganic phosphate ($[P_i]_i$) can affect Ca^{2+} sensitivity of myofilaments (19–21). We have therefore investigated the effects of partial metabolic inhibition on $[Ca^{2+}]_i$, $[P_i]_i$, and pH_i in isolated cultured ventricular myocytes. Our results indicate that increases in these metabolites can influence the effects of an increased diastolic $[Ca^{2+}]_i$ on diastolic distensibility.

Methods

Chick ventricular myocyte culture. Layer cultures of spontaneously contracting ventricular myocardial cells were prepared from 10-d-old chick embryos with a modification of previously described techniques (22, 23). Ventricles were minced and placed in Ca^{2+} −Mg^{2+} free Hanks' solution with 0.25 mg/ml of trypsin at 37°C for four cycles of 7 min each. The supernatant was discarded and the cells were resuspended in culture medium consisting of 6% heat-inactivated FCS, 40% medium 199 (Gibco Laboratories, Grand Island, NY), 0.1% penicillin-streptomycin antibiotic solution, and 54% balanced salt solution containing (in mM): 116 NaCl, 1.0 NaH_2PO_4, 0.8 $MgSO_4$, 26.2 $NaHCO_3$, and 5 glucose. The cell suspension was diluted to 4×10^5 cells/ml and placed in plastic petri dishes containing glass cover slips. Cultures were incubated in a 5% CO_2, 95% air atmosphere for 3 d at 37°C. All studies were performed on cells after 3 d of culture.

Dissociation of adult ventricular myocytes. Adult rabbit myocyte isolation was performed by a modification of the method of Haddad et al. (24). Hearts were removed from albino rabbits (2–2.5 kg) anesthe-

Address correspondence to Dr. William H. Barry, Division of Cardiology, University of Utah Medical Center, 50 North Medical Drive, Salt Lake City, UT 84132.

Received for publication 7 August 1990 and in revised form 31 December 1990.

J. Clin. Invest.

0021-9738/91/07/0055/07 $2.00
Volume 88, July 1991, 55–61

1. *Abbreviations used in this paper:* BCECF, 2'7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF-AM, BCECF acetoxymethyl ester; $[Ca^{2+}]_i$, intracellular free calcium concentration; CN, cyanide; CP, creatine phosphate; 2DG, 2-deoxyglucose; indo-1 AM, indo-1 acetoxymethyl ester; pH, intracellular pH; $[P_i]_i$, intracellular inorganic phosphate.

tized with sodium pentobarbital (65 mg/kg, i.v.). The heart was immediately attached to an aortic cannula, and continuous retrograde coronary artery perfusion at 37°C by a pump (Masterflex; Cole-Parmer Instrument Co., Chicago, IL) was initiated at a coronary perfusion pressure of 60 mmHg. Sterile conditions were maintained. The heart was first perfused with nominally Ca^{2+}-free modified Krebs bicarbonate buffer solution for 5 min, immediately followed by 20–30 min of recirculating perfusion with the same solution containing 0.4 mg/ml collagenase (Class II; Worthington Biochemical Corp., Freehold, NJ), 0.4 mg/ml hyaluronidase (Type I-S; Sigma Chemical Co., St. Louis, MO) and 20 μM $CaCl_2$. Both cell isolation solutions contained (in mM): 91.7 NaCl, 30 KCl, 1.2 $MgSO_4$, 19 $NaHCO_3$, 1.2 $NaHPO_4$, 15 glucose, 20 taurine, and 0.5 adenosine, and were gassed with 5% CO_2 and 95% O_2 (pH 7.40). The heart was then detached from the cannula and the ventricles were cut into small pieces in the same solution containing 20 μM $CaCl_2$ and 1% albumin. The resulting suspension was then gently forced through a single 550-μm nylon sieve with a sterile glass wide bore (4 mm, i.d.) pipette into a 100-ml beaker to remove debris. The cell suspension was centrifuged twice at 250 rpm and then allowed to settle twice in incubator 37°C in the same solution with 2% albumin and 20 μM $CaCl_2$. The supernatant was discarded and the cells were resuspended in culture medium consisting of 5% heat-inactivated FCS, 95% MEM (Gibco Laboratories), 0.1% penicillin-streptomycin, and 0.1% gentamicin antibiotic solution. Cells were attached on cover slips with cell adhesive (Cell-Tak; Collaborative Research, Inc., Waltham, MA). Cultures were incubated in a 5% CO_2, 95% air atmosphere for 1 d at 37°C. All studies were performed on cells after 1 d of culture.

Solutions. The control superfusate for cultured chick and adult rabbit ventricular cells was a Hepes-buffered normal Tyrode solution (NT) containing (in mM): 137.0 NaCl; 3.7 KCl; 0.5 $MgCl_2$; 1.8 $CaCl_2$ (0.9 for rabbit); 5.6 glucose; and 4.0 Hepes (free acid) titrated to pH 7.35 with 2.1 mM NaOH. The 2-deoxyglucose (2DG) solution included 20 mM of 2DG without glucose in this solution, and cyanide (CN) solution included 1 mM of NaCN.

Simultaneous measurement of $[Ca^{2+}]_i$ and cell motion. $[Ca^{2+}]_i$ was measured with Ca^{2+} fluorescent dye indo-1 (25), as previously described by Peeters et al. (23). To prepare the indo-1 AM (indo-1 acetoxymethyl ester; Calbiochem Corp., La Jolla, CA), the method of duBell et al. (26) was used. First, 10 ml of FCS was mixed with 234 μl of 25% Pluronic F127 (BASF Wyandotte Corp., Parsippany, NJ; wt/wt in DMSO), and sonicated. Then 1,000 μl of fresh 1 mM indo-1 AM in DMSO was added to 9 ml of FCS-Pluronic mixture, sonicated to mix, and aliquoted into fifty 200-μl samples, which were stored frozen at −20°C in light proof containers. This "loading stock" of 100 μM indo-1 AM was diluted in culture medium or physiologic buffer to give appropriate indo-1 AM concentrations for cell loading.

Cover slips of cultured chick ventricular cells were incubated at 37°C in culture medium containing 5 μM indo-1 AM for 15 min and then washed in indo-1 free solution for 30 min. After dye loading, a cover slip was placed in the flow-through cell chamber and continuously superfused with NT. Approximately 5–10 s were required to exchange the solution in the cell chamber which was equipped with a clear glass bottom and mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo). Solution temperature in the cell chamber was 37°C. Dissociated adult myocytes were loaded with 5 μM indo-1 AM for 30 min. After dye loading, cells were superfused in the chamber as described above. All experiments were performed at 37°C.

The instrumentation used for fluorescence measurement has been described in detail (23). Briefly, the system uses a high-pressure Hg-arc lamp for the excitation source because it provides an intense emission peak at 360 mM. Further selection of this peak was made with narrow band-width interference filters at 360 Hz, and the cells were illuminated via epifluorescence optics using a Fluor ×40 objective lens (Nikon). The fluorescent light was collected by the objective lens and divided with a beam splitter to permit simultaneous measurement of both 400 and 500 nm wavelengths using two separate photomultiplier tubes. The ratio of emitted fluorescence (400/500 nm) was obtained on-line though an analogue divider circuit. Previous calibration studies in cultured chick ventricular myocytes (23) have shown that under control conditions end-diastolic $[Ca^{2+}]_i$ averages 328 nM, and peak systolic $[Ca^{2+}]_i$ averages 813 nM. In this work, we have not calibrated the $[Ca^{2+}]_i$ levels and have used the 400/500 fluorescence ratio as an indicator of changes in $[Ca^{2+}]_i$. When measuring $[Ca^{2+}]_i$ in rabbit myocytes, an adjustable rectangular window was used to restrict the optical image to the cell of interest, thereby minimizing background fluorescence from other cells and debris.

The image of the cells was obtained by illumination via the standard microscope light source passed through a 700-nm band-pass filter. This wavelength was long enough not to interfere with the fluorescence detection at 400 and 500 nm. Cell motion of cultured cells was detected with a custom-made video motion analyzer. Although the chick monolayers contracted spontaneously (average rate ~ 120/min), none of the adult rabbit cells used in this study exhibited spontaneous contractions, and therefore in these experiments electrical stimulation was used to pace both chick and rabbit myocytes. Constant current pulses (3–8 ms duration) were delivered via a glass capillary tube (70 μm tip diameter) filled with NT and positioned ~ 0.1 mm from the cell. Chick myocytes were paced at 3 Hz, and rabbit myocytes at 0.5–1.0 Hz.

Intracellular pH measurement. Intracellular pH (pH_i) was measured using the pH-sensitive fluorescent dye, BCECF (2',7'-bis-[2-carboxyethyl]-5[6]-carboxyfluorescein) as described previously (27, 28). A stock solution of BCECF AM (BCECF acetoxymethyl ester; Calbiochem) was prepared by dissolving 1 mg of the dye in 1 ml of DMSO. This was stored in 20 μl vials at −25°C in the dark. Cells were equilibrated for 40–60 min in NT containing 4 μM of the BCECF AM stock solution. After dye loading cells were washed with NT to remove extracellular dye. To measure pH_i with BCECF our fluorescence system was modified to provide dual excitation with rotating interference filters. Cells were sequentially excited at 500 nm and 440 nm (frequency = 180 Hz) while recording fluorescence emission at 530 nm. The ratio of emission intensity (500/440) served as the measure of pH_i. At the end of the experiment the emission ratio was calibrated in situ as described by Thomas et al. (29) by exposing cells to solutions of varying pH. Each solution contained 11 μM of the K^+-H^+ ionophore, nigericin (Calbiochem) and (in mM) 12.0 Hepes (titrated with 1 M KOH), 140.0 K^+ (K^+ adjusted to keep K^+ constant), 1.0 $MgCl_2$, and 11.0 glucose.

Measurement of ATP content. Cellular ATP content was measured during metabolic inhibition in these experiments by means of a luciferase assay (ATP Bioluminescence CLS; Boehringer Mannheim Corp., Indianapolis, IN) with modification of a previously described method (30). Cover slips of spontaneously contracting cultured chick myocytes were incubated in NT containing 20 mM 2DG or 1 mM CN for 6 min without pacing. Control cells or cells subjected to metabolic inhibition for 2, 4, or 6 min were frozen in liquid nitrogen, and scraped into 1 ml ice cold 0.7 N $HClO_4$. After homogenization and addition of 1 ml ice cold H_2O, the samples were divided equally for ATP assay and for protein assay. To the ATP sample, 60 μl of 1.5 M K_2HPO_4 was added followed by centrifugation at 3,000 rpm and 4°C for 20 min. After removal of the supernatant, 10 μl of the sample was added to 990 μl of distilled water and analyzed in a liquid scintillation counter using the ATP cls kit. A standard curve with known ATP concentrations was prepared to calculate the sample ATP concentrations. Protein was assayed by the Lowry method (31) and the final ATP concentration expressed as nanomoles per milligram protein.

Intracellular inorganic phosphate content. Cellular inorganic phosphate content was measured during metabolic inhibition in these experiment according to Chen's method (32). Cover slips of cultured myocytes were incubated in NT containing 20 mM 2DG or 1 mM CN for 2, 4, and 6 min. Control cells or cells subjected to metabolic inhibition were washed in ice cold HBSS and scraped into 1 ml ice cold 0.35 N $HClO_4$. After homogenization the samples were divided for P_i assay and for protein assay. For the P_i assay, samples were centrifuged at 1,300 rpm and 4°C for 10 min. Then, 750 μl of ascorbic acid-ammonium molybdate solution (mixture of 10% ascorbic acid in water and 0.42% ammonium molybdate in 1 N H_2SO_4 with a ratio 1:6) was added

to 250 μl of the supernatant. These samples were subsequently incubated at 37°C for 30 min. The phosphate content of samples was obtained by measuring absorbance at 820 nm with a spectrophotometer (Model 8452a; Hewlett-Packard Co., Palo Alto, CA). Standard curves with known phosphate concentration were prepared to calculate the sample inorganic phosphate concentration. Protein was assayed by the Lowry method and the final P_i concentration expressed as nanomoles per milligram protein.

Statistical analysis. Data were expressed as mean±SEM. Analysis of variance or paired Student's test was used in comparing changes in a group. A P value < 0.05 was considered significant.

Results

Effects of metabolic inhibition with 2DG on $[Ca^{2+}]_i$ and cell motion. To simulate demand ischemia, in which there is an increased work load placed on a heart coincident with a limitation (but not total elimination) of coronary perfusion, we exposed cultured myocytes to two different types of partial metabolic inhibition (2DG or CN) while cells were paced. Examples of changes in cell motion and $[Ca^{2+}]_i$ transients during superfusion with 2DG are shown in Fig. 1. During simple pacing, embryonic chick ventricular myocytes motion and $[Ca^{2+}]_i$ were quite constant (Fig. 1 *A*). Within 1–2 min after exposure to 2DG, diastolic $[Ca^{2+}]_i$ increased and diastolic cell position shifted upward, indicating an increase in diastolic "tone." The amplitude of $[Ca^{2+}]_i$ transients and cell motion decreased slightly. We were initially concerned that this response in cultured chick ventricular myocytes might not be typical of the response of adult myocytes, possibly because of a greater capacity for, and/or dependence on, glycolysis in immature ventricular cells. Therefore, we also examined the response of paced cultured adult rabbit ventricular myocytes. As shown in Fig. 1 *B*, a similar response to exposure to 2DG was observed. The changes in average normalized systolic and diastolic $[Ca^{2+}]_i$

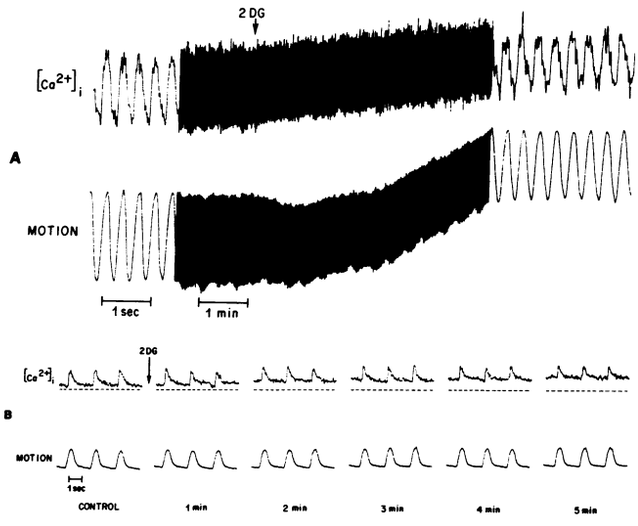

Figure 1. Examples of effects of exposure to 20 mM 2DG on $[Ca^{2+}]_i$ transients (ratio of 400/500 nm indo-1 fluorescence, *upper traces*), and motion (*lower traces*) in paced chick embryo ventricular cells (*A*), and in adult rabbit myocytes (*B*). In the rabbit myocytes $[Ca^{2+}]_i$ was measured only at 1-min intervals to decrease dye bleaching. Exposure to 2DG caused an increase in end-diastolic and peak-systolic $[Ca^{2+}]_i$ and cell position both in cultured chick myocytes and adult isolated adult rabbit myocytes.

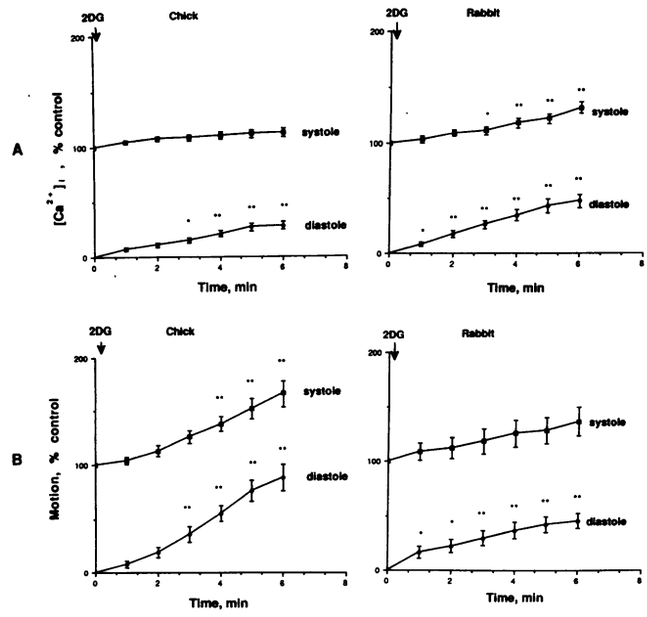

Figure 2. Average effects of exposure to 20 mM 2DG on peak-systolic and end-diastolic $[Ca^{2+}]_i$ (*A*) and peak-systolic and end-diastolic motion (*B*) in paced chick myocytes (*left*, $n = 13$, means±SEM) and in paced adult rabbit myocytes (*right*, $n = 7$). In both types of cells exposure to 2DG caused a comparable increase in peak-systolic and end-diastolic $[Ca^{2+}]_i$ and motion. Amplitude of $[Ca^{2+}]_i$ transients and motion decreased slightly in both groups.

and cell motion in 13 chick embryo myocytes and 7 isolated adult rabbit myocytes during exposure to 2DG are shown in Fig. 2. For this analysis, control end-diastolic $[Ca^{2+}]_i$ and cell position were assigned a value of 0, and control peak systolic calcium and motion a value of 100. Changes induced by exposure to 2DG were measured relative to this scale. 2DG caused increases in diastolic calcium and diastolic cell position. Usually diastolic cell position started shifting upward within 1–2 min both in chick and adult rabbit myocytes. Increases in systolic calcium and cell position also occurred, although they were somewhat less prominent. Thus, metabolic inhibition produced by 2DG caused a consistent increase in diastolic $[Ca^{2+}]_i$ and a corresponding decrease in myocyte distensibility in both cultured chick and adult rabbit ventricular myocytes.

To investigate the effects of 2DG on $[Ca^{2+}]_i$ and motion without a work load we exposed isolated adult rabbit ventricular myocytes to 2DG without pacing. An example of a typical experiment is shown in Fig. 3. After cessation of pacing the cell stopped beating and $[Ca^{2+}]_i$ and motion were stable in a resting state. After 2DG exposure, $[Ca^{2+}]_i$ and cell position did not increase. In Fig. 4 are shown average results obtained in five rabbit myocytes. These results indicate that the rate of beating is an important variable for the production of changes in $[Ca^{2+}]_i$ and diastolic cell distensibility during metabolic inhibition.

The effects of metabolic inhibition with cyanide. We also examined the effects of inhibition of oxidative phosphorylation with cyanide on $[Ca^{2+}]_i$ and motion. In Fig. 5 are shown examples of the effects of CN in chick (Fig. 5 *A*) and rabbit myocytes (Fig. 5 *B*). Inhibition of oxidative phosphorylation also produced a prominent increase in diastolic and systolic calcium,

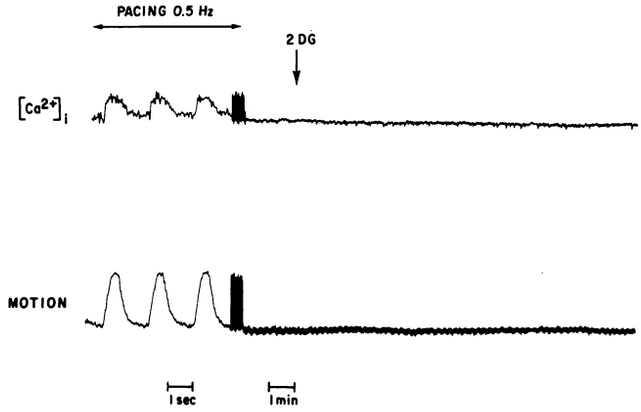

Figure 3. Effect of exposure to 20 mM 2DG on $[Ca^{2+}]_i$ and cell motion in a quiescent adult rabbit ventricular myocyte. After cessation of pacing, exposure to 20 mM 2DG caused no increase in $[Ca^{2+}]_i$ or increase in cell position.

but in contrast to effects of 2DG, in spite of the increase of $[Ca^{2+}]_i$, systolic and diastolic cell position shifted downward, and the amplitude of motion decreased, soon after exposure to CN. The effects of CN on average end-diastolic and peak-systolic cell positions and $[Ca^{2+}]_i$ are shown in Fig. 6. The increase in diastolic $[Ca^{2+}]_i$ was statistically significant, and the percent increase was similar to that produced by 2DG. However, end-diastolic cell position shifted downward significantly in both chick and rabbit myocytes.

Changes in intracellular ATP content in chick ventricular myocytes after metabolic inhibition with CN or 2DG are shown in Table I. The results indicate that each type of metabolic inhibition induced a decrease in ATP after 6 min exposure (2DG −29.9%, CN −40.1%), although CN induced a somewhat more rapid initial decline than did 2DG. Thus, the degree of ATP depletion induced did not appear sufficient to explain

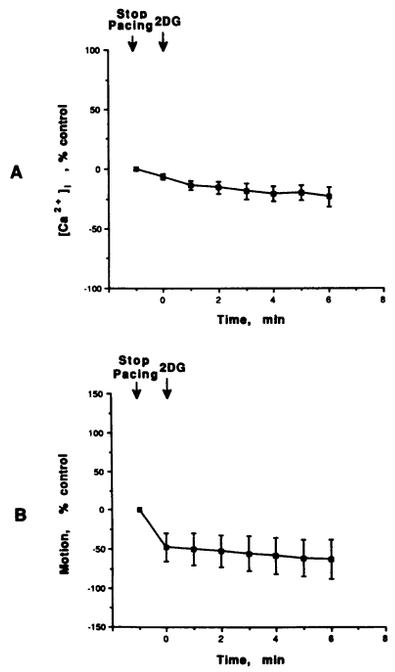

Figure 4. Average effects of exposure of resting rabbit myocytes to 20 mM 2DG on $[Ca^{2+}]_i$ (*A*) and motion (*B*). End-diastolic $[Ca^{2+}]_i$ and cell position decreased after cessation of pacing, and did not increase after exposure to 2DG (n = 5, means±SEM).

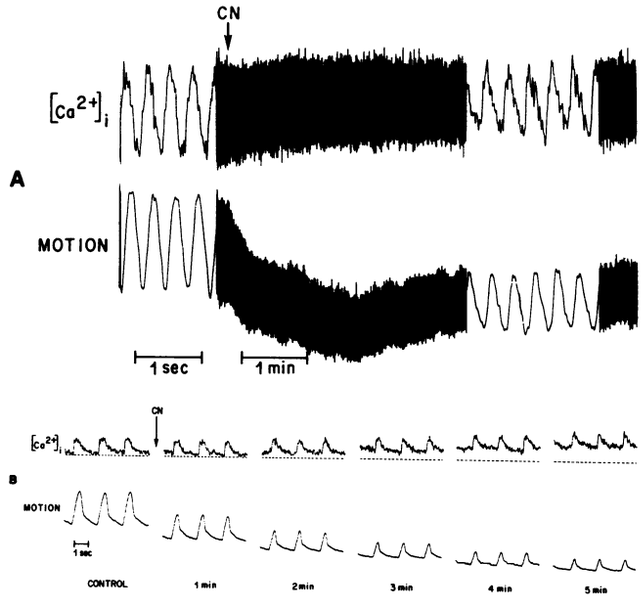

Figure 5. Example of effects of exposure to 1 mM CN on $[Ca^{2+}]_i$ transients (*upper trace*) and motion (*lower trace*) in paced chick myocytes (*A*) and an isolated rabbit myocyte (*B*). Inhibition of oxidative phosphorylation also produced a prominent increase in diastolic and systolic calcium, but decreased diastolic and systolic cell position and amplitude of motion.

these completely different changes in cell motion induced by CN or 2DG. To examine whether a change of Ca^{2+}-sensitivity of myofilaments during metabolic inhibition could be involved, possibly due to intracellular acidosis (33), we measured changes in pH_i during exposure to CN or 2DG.

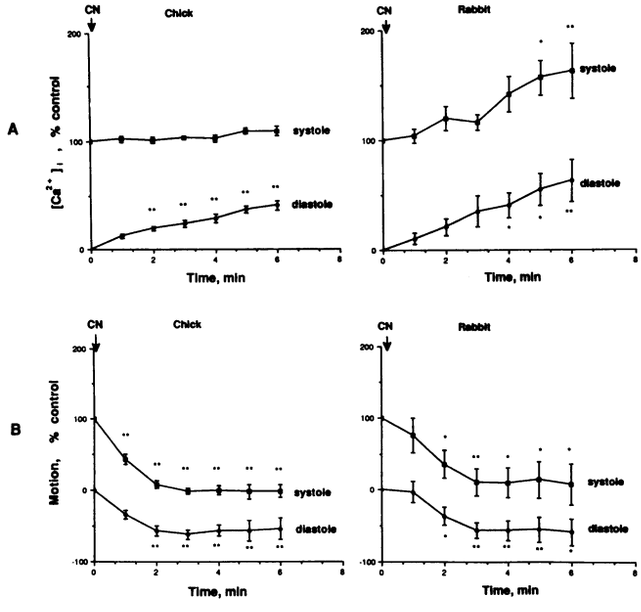

Figure 6. Average effects of exposure to 1 mM CN on systolic and diastolic $[Ca^{2+}]_i$ (*A*) and motion (*B*). $[Ca^{2+}]_i$ increased after CN exposure while motion decreased rapidly in both paced chick myocytes (*left*, n = 11) and paced rabbit myocytes (*right*, n = 6).

Table I. ATP Content during Metabolic Inhibition

Inhibitor	Time	ATP content	n	P
	min	*nmol/mg*		
2DG	0	34.6±2.7	21	
	2	32.9±4.0	12	NS
	4	25.9±2.4	12	<0.04
	6	24.3±1.4	9	<0.02
CN	0	34.6±2.7	21	
	2	31.6±2.1	12	NS
	4	20.2±1.2	12	<0.001
	6	20.7±1.3	12	<0.001

Means±SEM.

Intracellular pH during partial metabolic inhibition. Under control condition, during 3 Hz pacing the average intracellular pH in chick ventricular myocytes was 7.18±0.04 (Fig. 7), similar to previous reports of pH_i in ventricular myocytes (28, 34). After 15 min of control perfusion with NT solution, during which time pH_i was stable, cells were superfused with metabolic inhibitors. In cells exposed to 2DG pH_i did not fall significantly, but in cells superfused with CN cytosolic pH rapidly declined. Intracellular acidosis of this magnitude reduces contractility and reduces Ca^{2+} sensitivity of myofilaments in these cells (28). Thus, a difference in induced changes in intracellular pH may account in part for the differences observed in CN and 2DG effects on myocyte motion, in spite of a similar effect on $[Ca^{2+}]_i$.

Intracellular inorganic phosphate content. Intracellular inorganic phosphate (P_i) is another metabolite that can affect myofilament sensitivity to Ca^{2+}. Therefore we measured changes in $[P_i]_i$ in chick ventricular myocytes during metabolic inhibition with CN or 2DG. After 6 min of metabolic inhibition, $[P_i]_i$ decreased slightly in cells exposed to 2DG (−14.8%, NS) but increased in cells exposed to CN (+45.7%, $P < 0.02$, Fig. 8). These results indicate that changes in intracellular inorganic phosphate concentration may also contribute to the difference observed in CN and 2DG effects.

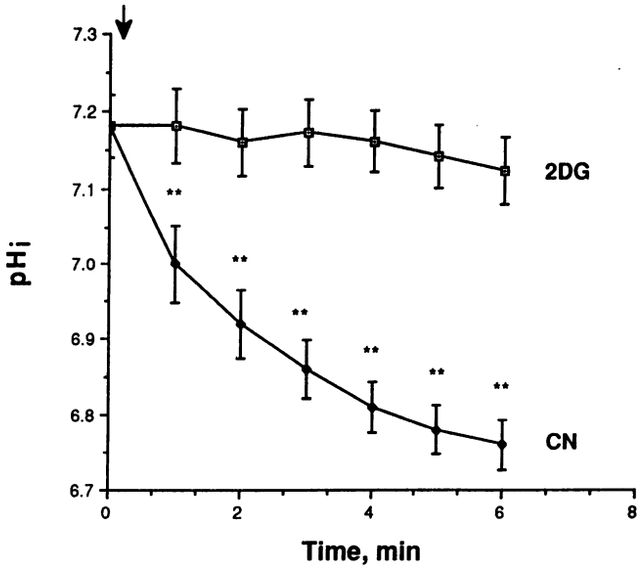

Figure 7. Effects of metabolic inhibition on intracellular pH (BCECF fluorescence) in paced chick embryo ventricular myocytes. Exposure to 20 mM 2DG caused slight decrease in intracellular pH, but this was not statistically significant (mean±SEM, $n = 14$). In contrast, exposure to CN rapidly caused a marked intracellular acidosis ($n = 11$; $^*P < 0.05$, $^{**}P < 0.01$).

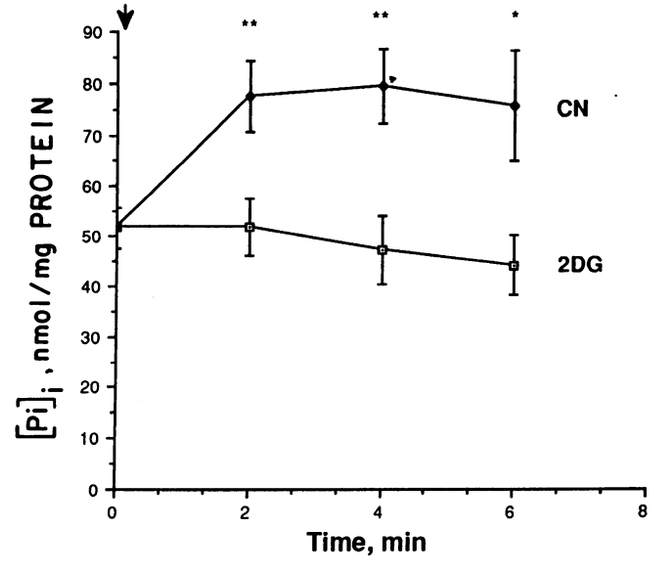

Figure 8. Effects of metabolic inhibition on intracellular inorganic phosphate concentration. Exposure to 20 mM 2DG decreased intracellular inorganic phosphate in cultured chick ventricular myocytes. In contrast, exposure to CN caused an increase in intracellular inorganic phosphate (means±SEM, $n = 11$; $^*P < 0.05$, $^{**}P < 0.01$).

Discussion

Effects of partial metabolic inhibition on $[Ca^{2+}]_i$. Our previous results (35) demonstrated that complete metabolic inhibition of cultured myocytes with CN plus 2DG caused an initial increase in diastolic $[Ca^{2+}]_i$, but within less than 1 min cells stopped contracting, probably due to failure to generate action potentials, and $[Ca^{2+}]_i$ then declined to below diastolic levels before starting to increase again. However, as shown in this work, partial metabolic inhibition combined with electrical pacing at rapid rates causes a rapid and progressive increase in diastolic (and systolic) $[Ca^{2+}]_i$. This difference in early effects of partial versus complete metabolic inhibition emphasizes the importance of Ca^{2+}-loading of myocytes on the changes in $[Ca^{2+}]_i$ observed. In cells that remain electrically excitable and are paced, $[Ca^{2+}]_i$ influx via the slow Ca^{2+} channel (36), and Na^+ influx which can influence $[Na^+]_i$ (37) and alter $[Ca^{2+}]_i$ via Na^+/Ca^{2+} exchange (38), probably to maintain Ca^{2+} loading of the cell. The importance of continuing excitation of myocytes in contributing to the increase in $[Ca^{2+}]_i$ during metabolic inhibition is further emphasized by Figs. 3 and 4, in which it is demonstrated that quiescent adult rabbit ventricular myocytes fail to show a rapid increase in $[Ca^{2+}]_i$ after metabolic inhibition with 2DG.

The magnitude and time course of the increase in diastolic $[Ca^{2+}]_i$ we have detected in paced ventricular myocytes subjected to metabolic inhibition is similar to that reported by

Kihara et al. (39), in the intact ferret heart loaded with aequorin, subjected to ischemia or hypoxia. However, it is slower than that reported by Lee et al. (40) in the indo-1 loaded rabbit heart during ischemia. This could be due to the fact that in the studies by Lorell et al. (41) a component of the indo-1 surface fluorescence signal may be due to endothelial cell $[Ca^{2+}]_i$ (38); and $[Ca^{2+}]_i$ in endothelial cells increases rapidly during hypoxia (Silverman, H. S., and M. D. Stern, personal communication) or metabolic inhibition (42).

Effects of increased diastolic $[Ca^{2+}]_i$ on diastolic distensibility. Our results demonstrate that although diastolic $[Ca^{2+}]_i$ increases comparably after exposure to either CN or 2DG, the effects of increased $[Ca^{2+}]_i$ on diastolic distensibility, reflected by end-diastolic cell position or length, differ. Exposure to CN results in increased distensibility (decreased tone), whereas 2DG causes decreased distensibility (increased tone). This difference in effects of $[Ca^{2+}]_i$ on distensibility under these conditions appears to be accounted for at least in part by the intracellular acidosis and increase in P_i that occur after exposure to CN but not 2DG. This is possibly due to the fact that increased production of lactic acid via increased glycolysis, and increased breakdown of creatine phosphate and ATP (releasing P_i), occur after exposure to CN, whereas 2DG inhibits glycolysis and serves as P_i "sink" within the cell as a consequence of phosphorylation of 2DG to 2DG-6-P (43). As shown in Fig. 2, in chick ventricular myocytes diastolic cell position increased within 6 min after exposure to 2DG to a position near the control peak systolic position, whereas the diastolic $[Ca^{2+}]_i$ increase was less marked. This is consistent with an increase in sensitivity of contractile elements to $[Ca^{2+}]_i$ under these conditions, possibly due to the slight, though not statistically significant, decrease in intracellular P_i noted under these conditions (Fig. 8). In rabbit myocytes, the degree of increase in $[Ca^{2+}]_i$ and change in diastolic cell position after 2DG were more concordant. A less marked alteration in P_i in rabbit myocytes might have occurred under these conditions after exposure to 2DG, but we were unable to measure P_i in the adult dissociated myocytes because of their relatively small number and heterogeneity.

The observation that hypoxia, or inhibition of oxidative phosphorylation with CN ("chemical hypoxia"), can produce a significant intracellular acidosis in myocardium is consistent with previous studies (44), as is the observation that this effect is opposed by glycolytic inhibition (44). Furthermore, Koretsune and Marban (21) have recently demonstrated that an increase in P_i and a decrease in pH_i, not a decrease in systolic $[Ca^{2+}]_i$, accounts for the negative inotropic effect of hypoxia in the ferret heart. Our results with CN confirm that the negative inotropic effect of hypoxia (or chemical hypoxia) cannot be ascribed to a decrease in peak systolic $[Ca^{2+}]_i$, and also indicate that a rise in P_i and/or a decrease in pH_i could be involved, by causing antagonism of the effects of $[Ca^{2+}]_i$ on myofilament force development during systole. In the study of Koretsune and Marban (21) NMR and F-BAPTA were used to detect changes in $[Ca^{2+}]_i$ in perfused ferret hearts subjected to hypoxia and no alterations of diastolic $[Ca^{2+}]_i$ or pressure were detected. However, diastolic $[Ca^{2+}]_i$ did increase significantly in our experiments in which indo-1 was used as a $[Ca^{2+}]_i$ indicator, and cells were rapidly paced. The reason for this difference is not clear. However, the degree and rate of depression of mitochondrial respiration produced in our experiments by CN was probably greater than that achieved by perfusing ferret hearts with physiologic buffer equilibrated with 10% O_2-90% N_2, and our experiments were performed at 37°C. Other factors relating to species differences, and $[Ca^{2+}]$ indicators, may be involved. In any case, our results indicate that accumulation of intracellular H^+ and P_i may alter the effects of $[Ca^{2+}]_i$ on diastolic as well as on systolic force development.

Relationship to effects of demand vs. supply ischemia on diastolic distensibility. Our results clearly indicate that accumulation of intracellular metabolites during an increase in $[Ca^{2+}]_i$ produced by limitation of ATP production can alter diastolic myocyte distensibility. It is possible that this or a similar mechanism accounts for the difference in effects of demand vs. supply ischemia on left ventricular compliance. A comparative study of 3-min periods of supply ischemia (coronary artery occlusion) and demand ischemia (coronary stenoses with pacing tachycardia) has revealed some metabolic differences that have the potential to affect diastolic compliance (45). Relative to demand ischemia, supply ischemia resulted in greater tissue acidosis (pH decreased −0.33 vs. −0.14 pH U) and a threefold greater decrease in subendocardial creatine phosphate (CP) content (decrease of 43 vs. 14 nmol/mg protein from a preischemic control value of 52). ATP content decreased to a small and similar extent (11% to 12%) during both type of ischemia. The greater decrease in CP could result in a substantially greater increase in cytosolic inorganic phosphate concentration during supply ischemia than during demand ischemia. Thus, the greater severity of acidosis and probable greater increase in cystolic inorganic phosphate during supply ischemia relative to demand ischemia may contribute to the observed differences in the functional effects of these types of ischemia.

It should be considered whether rigor, or myofilament crossbridge attachment due to severe ATP depletion, could account for decreased distensibility during these experimental conditions. In isolated myocytes this seems very unlikely, because only a moderate degree of depression of ATP (by 30–40%) occurs after exposure to 2DG at a time when a distensibility decrease occurs. Furthermore, in previous studies we have consistently found that rigor develops only when sufficient ATP depletion occurs to cause failure of excitability (35, 46). In the intact heart the situation is perhaps not so clear. Although the average ATP depletion in ventricular myocardium produced by demand ischemia is not severe, greater ATP depletion could conceivably occur in the subendocardium. Due to heterogeneity of blood flow in the intact heart, it is conceivable that during demand ischemia a small population of ventricular myocytes develops rigor due to severe ATP depletion, and that this diminishes distensibility of the entire ventricle. Although this is a theoretical possibility, based on findings in isolated myocytes we consider it also possible that the transient reversible decreases in left ventricular compliance during demand ischemia results, at a myocardial cell level, from altered $[Ca^{2+}]_i$ homeostasis.

Acknowledgments

We appreciate the assistance of Dr. Stephen Prescott and Eric Stroud in the inorganic phosphate measurements.

This work was supported by the National Institutes of Health Grant HL-30478.

References

1. Barry, W. H., J. Z. Brooker, E. L. Alderman, and D. C. Harrison. 1974. Changes in diastolic stiffness and tone of the left ventricle during angina pectoris. *Circulation.* 49:255–263.

2. Mann, T., B. R. Brodie, W. Grossman, and L. P. McLaurin. 1977. Effect of angina on the left ventricular diastolic pressure-volume relationship. *Circulation.* 55:761–766.

3. Serizawa, T., B. A. Carabello, and W. Grossman. 1980. Effect of pacing-induced ischemia on the left ventricular diastolic pressure-volume relations in dogs with coronary stenoses. *Circ. Res.* 46:430–439.

4. Momomura, S., A. B. Bradley, and W. Grossman. 1984. Left ventricular diastolic pressure-segment length relations and end-diastolic distensibility in dogs with coronary stenoses. *Circ. Res.* 55:203–214.

5. Ross, J., Jr. 1979. Acute displacement of the diastolic pressure-volume curve of the left ventricle: role of the pericardium and the right ventricle. *Circulation.* 59:32–37.

6. Lorell, B. H., I. Palacios, W. M. Daggett, M. L. Jacolis, B. Fowler, and J. B. Newell. 1981. Right ventricular distention and left ventricular compliance. *Am. J. Physiol.* 240:H87–H98.

7. Maruyama, Y., K. Ashikawa, S. Isoyama, H. Kanatsuka, E. Ino-Oka, and T. Takishima. 1982. Mechanical interactions between four heart chambers with and without the pericardium in canine hearts. *Circ. Res.* 50:86–100.

8. Waters, D. D., P. DaLuz, H. L. Wyatt, J. H. C. Swan, and J. S. Forrester. 1977. Early changes in regional and global left ventricular function induced by graded reductions in regional coronary perfusion. *Am. J. Cardiol.* 39:537–543.

9. Weigner, A. W., G. J. Allen, and O. H. L. Bing. 1978. Weak and strong myocardium in series: implication for segmental dysfunction. *Am. J. Physiol.* 235:H776–H783.

10. Brutsaert, D. L., F. E. Rademakers, and S. V. Sys. 1984. Triple control of relaxation: implications in cardiac disease. *Circulation.* 69:190–196.

11. Aoyagi, T., M. Iizuka, T. Takahashi, T. Ohya, T. Serizawa, S. Momomura, H. Sato, T. Mochizuki, H. Matsui, H. Ikenouchi, et al. 1990. Wall motion asynchrony prolongs time constant of left ventricular relaxation. *Am. J. Physiol.* 257:H883–H891.

12. Henry, P. D., R. Shuchleib, J. Davis, E. S. Weiss, and B. E. Sobel. 1977. Myocardial contracture and accumulation of mitochondrial calcium in ischemic rabbit heart. *Am. J. Physiol.* 233:H677–H684.

13. Ashraf, M., and P. M. Rahamathulla. 1984. Cardiac injury in short duration anoxia and modification by diltiazem, a calcium channel blocking agent. *J. Am. Coll. Cardiol.* 3:1237–1244.

14. Paulus, W. J., T. Serizawa, and W. Grossman. 1982. Altered left ventricular diastolic properties during pacing-induced ischemia in dogs with coronary stenoses: potentiation by caffeine. *Circ. Res.* 50:218–227.

15. Lavanchy, N., J. Martin, and A. Rossil. 1986. Effects of diltiazem on the energy metabolism of the isolated rat hearts submitted to ischemia: a ^{31}P-NMR study. *J. Mol. Cell. Cardiol.* 18:931–941.

16. Serizawa, T., W. M. Vogel, C. S. Apstein, and W. Grossman. 1981. Comparison of acute alterations in left ventricular relaxation and diastolic chamber stiffness induced by hypoxia and ischemia. *J. Clin. Invest.* 68:91–102.

17. Apstein, C. S., and W. Grossman. 1987. Opposite initial effects of supply and demand ischemia on left ventricular diastolic compliance: the ischemia diastolic paradox. *J. Mol. Cell. Cardiol.* 19:119–128.

18. Paulus, W. J. 1990. Upward shift and outward bulge: divergent myocardial effects of pacing angina and brief coronary occlusion. *Circulation.* 81:1436–1439.

19. Kusuoka, H., M. L. Weisfeldt, J. L. Zweier, W. E. Jacobus, and E. Marban. 1986. Mechanism of early contractile failure during hypoxia in intact ferret heart: evidence for modulation of maximal Ca^{2+}-activated force by inorganic phosphate. *Circ. Res.* 59:270–282.

20. Eisner, D. A., A. C. Elliott, and G. L. Smith. 1987. The contribution of intracellular acidosis to the decline of developed pressure in ferret hearts exposed to cyanide. *J. Physiol. (Lond.)* 391:99–108.

21. Koretsune, Y., and E. Marban. 1990. Relative roles of Ca^{2+}-dependent and Ca^{2+}-independent mechanisms in hypoxic contractile dysfunction. *Circulation.* 82:528–535.

22. Barry, W. H., C. A. F. Rasmussen, Jr., H. Ishida, and J. H. B. Bridge. 1986. External Na-independent Ca^{2+} extrusion in cultured ventricular cells. Magnitude and functional significance. *J. Gen. Physiol.* 88:394–411.

23. Peeters, G. A., V. Hlady, J. H. B. Bridge, and W. H. Barry. 1987. Simultaneous measurement of calcium and motion in cultured ventricular cells. *Am. J. Physiol.* 253:H1400–H1408.

24. Haddad, J., M. L. Decker, L. Hsieh, M. Lesch, A. M. Samarel, and R. S. Docker. 1988. Attachment and maintenance of adult rabbit cardiac myocytes in primary cell culture. *Am. J. Physiol.* 255:C19–C27.

25. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 265:3440–3450.

26. duBell, W. H., C. Philips, and S. R. Houser. 1988. A technique for measuring cytosolic free Ca^{2+} with indo-1 in feline myocytes. *In* Biology of Isolated Adult Cardiac Myocytes. W. Clark, editor. Elsevier Science Publishing Co., New York. 187–201.

27. Rink, T. J., R. Y. Tsien, and T. Pozzan. 1982. Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell Biol.* 95:189–196.

28. Kohmoto, O., K. W. Spitzer, M. A. Movsesian, and W. H. Barry. 1990. Effects of intracellular acidosis of $[Ca^{2+}]_i$ transients, transsarcolemmal Ca^{2+} fluxes, and contraction in ventricular myocytes. *Circ. Res.* 66:622–632.

29. Thomas, J. A., R. N. Buchshaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 81:2210–2218.

30. Ishida, H., O. Kohmoto, J. H. B. Bridge, and W. H. Barry. 1988. Alteration in cation homeostasis in cultured chick ventricular cells during and after recovery from adenosine triphosphate depletion. *J. Clin. Invest.* 81:1173–1181.

31. Lowry, D. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

32. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* 28:1756–1758.

33. Fabiato, A., and F. Fabiato. 1978. Effects of pH on the myofilaments and sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *J. Physiol. (Lond.)* 276:233–255.

34. Eisner, D. A., C. G. Nichols, S. C. O'Neill, G. L. Smith, and M. Valdeolmillos. 1989. The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *J. Physiol. (Lond.)* 411:393–418.

35. Barry, W. H., G. A. Peeters, C. A. F. Rasmussen, Jr., and M. J. Cunningham. 1987. Role of changes in $[Ca^{2+}]_i$ in energy deprivation contracture. *Circ. Res.* 61:726–734.

36. duBell, W. H., and S. R. Houser. 1989. Voltage and beat dependence of the Ca^{2+} transient in feline ventricular myocytes. *Am. J. Physiol.* 257:H746–H759.

37. Cohen, C. J., H. A. Fozzard, and S. S. Sheu. 1982. Increase in intracellular sodium ion activity during stimulation in mammalian cardiac muscle. *Circ. Res.* 50:651–662.

38. Brill, D. M., H. A. Fozzard, J. C. Makielski, and J. A. Wasserstrom. 1987. Effect of prolonged depolarizations on twitch tension and intracellular sodium activity in sheep cardiac purkinje fibers. *J. Physiol. (Lond.)* 384:355–375.

39. Kihara, Y., W. Grossman, and J. P. Morgan. 1989. Direct measurement of changes in intracellular calcium transients during hypoxia, ischemia and reperfusion of intact mammalian heart. *Circ. Res.* 65:1029–1044.

40. Lee, H., R. Mohabir, N. Smith, M. R. Franz, and W. T. Clusin. 1988. Effects of ischemia on calcium dependent fluorescence transients in rabbit hearts containing indo-1; correlation with monophasic action potential and contraction. *Circulation.* 78:1047–1059.

41. Lorell, B. H., C. S. Apstein, M. J. Cunningham, F. J. Schoen, E. O. Weinberg, G. A. Peeters, and W. H. Barry. 1990. Contribution of endothelial cells to calcium-dependent fluorescence transients in rabbit hearts loaded with indo-1. *Circ. Res.* 67:415–425.

42. Eki, Y., and G. A. Peeters. 1990. Metabolic inhibition increases $[Ca^{2+}]_i$ in endothelial cells. *Clin. Res.* 38:404A. (Abstr.)

43. Pirolo, J. S., and D. G. Allen. 1986. Assessment of techniques for preventing glycolysis in cardiac muscle. *Cardiovasc. Res.* 20:837–844.

44. Allen, D. G., P. G. Morris, C. H. Orchard, and J. S. Pirolo. 1985. A nuclear magnetic resonance study of metabolism in the ferret heart during hypoxia and inhibition of glycolysis. *J. Physiol. (Lond.)* 361:185–204.

45. Momomura, S., J. S. Ingwall, J. A. Parker, P. Sahagian, J. J. Ferguson, and W. Grossmann. 1985. The relationships of high energy phosphates, tissue pH and regional blood flow to diastolic distensibility in the ischemic dog myocardium. *Circ. Res.* 57:822–835.

46. Kohmoto, O., and W. H. Barry. 1989. Mechanism of protective effects of calcium channel blockers on energy deprivation contracture in cultured ventricular myocytes. *J. Pharmacol. Exp. Ther.* 248:871–878.