Pyruvate augments calcium transients and cell shortening in rat ventricular myocytes

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Pyruvate augments calcium transients and cell shortening in rat ventricular myocytes. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H8–H17, 1998.—Pyruvate has been shown to be a metabolic inotrope in the myocardium. In millimolar concentrations, it has been shown to increase both myocardial phosphorylation potential and the cytosolic [NADH]-to-[NAD+] ratio. To determine if changes in these parameters can alter intracellular Ca2+-concentration ([Ca2+]i) and hence contractile function, Ca2+-transients and cell shortening (CS) were measured in isolated rat ventricular myocytes superfused with a physiological N2-hydroxyethylpiperezine-N2-ethanesulfonic acid buffer (11 mmol/l glucose) with and without additional pyruvate, L-lactate, acetate, or isoproterenol. The addition of 5 mmol/l pyruvate resulted in a 33% increase in CS and a 39% increase in systolic [Ca2+]i. These pyruvate effects were 70% of those observed with 100 mmol/l isoproterenol. The mitochondrial monocarboxylate transport inhibitor α-cyano-4-hydroxycinnamate (250 μmol/l) strongly inhibited pyruvate inotropy, suggesting a substantial obligatory coupling between pyruvate inotropism and its oxidation by the mitochondria. A possible role of the cytosolic [NADH]-to-[NAD+] ratio was assessed by comparing the effects of 20 mmol/l L-lactate to those of equimolar pyruvate. In contrast to 20 mmol/l pyruvate, excess L-lactate failed to appreciably increase CS or systolic [Ca2+]i. The findings imply that, at levels substantially above 5 mmol/l, a portion of pyruvate inotropism might be due to extreme cytosolic [NADH]-to-[NAD+] ratios. This study is the first evidence that augmented [Ca2+]i transients are most likely the mechanism of cardiac pyruvate inotropism.

mitochondria; sarcoplasmic reticulum

due to its relatively limited energy stores, the heart is highly dependent upon exogenous energy substrates to maintain normal contractile function. Pyruvate, a key glycolytic intermediary metabolite, has been demonstrated to enhance myocardial energetic stability and to exert positive inotropic effects in the heart (4, 21, 22, 36–38). These effects have been observed in both in vivo (21, 23, 36, 37) and in vitro (4, 22, 28) heart preparations and also in normoxic (4, 20, 21, 22, 28, 36) as well as stunned (8, 23, 37) myocardium. Although the exact mechanism of this pyruvate inotropism is unknown, it is thought that pyruvate exerts its inotropic effect by enhancing the cytoplasmic phosphorylation potential. Myocardial phosphorylation potential is a determinant of the Gibbs free energy change for ATP hydrolysis (ΔG) and has been used as an index of intracellular energetization; the phosphorylation potential is stoichiometrically related to the creatine kinase system, cellular ion pumps, contractile proteins, and other ATP-dependent phosphorylating mechanisms (12, 29). Pyruvate-induced increases in phosphorylation potential have been reported in both in vivo (21, 37) and in vitro (4, 28) preparations and appear to be species independent.

The causal relationship between pyruvate inotropism and its effects on cellular energetics has not been established. Zweier and Jacobus (38) reported that pyruvate-induced augmentation of ventricular function in the isolated perfused rat heart is accompanied by an increase in the phosphocreatine-to-Pi ratio, flux of ATP synthesis from ADP, and other measures of cellular energization. They hypothesized that the increase in ΔG observed with pyruvate may improve sarcomplasmic reticulum (SR) Ca2+-adenosinetriphosphatase (ATPase) efficiency, leading to greater SR release of Ca2+, and subsequently greater contractile function. In digitoxin-lysed rat cardiomyocytes, Wimsatt et al. (33) reported a clear association between sarcomplasmic reticular Ca2+ uptake rate and both the cytosolic energy level (ΔG) and the cytosolic free ATP-to-ADP ratio. Kammermeier et al. (18) calculated the free energy requirements of several transport proteins in rat myocardium and theorized that the SR Ca2+-ATPase required the highest Gibbs free energy. They also reported that this SR Ca2+ transporter is sensitive to reductions (<45 kJ/mol) in the free energy of ATP hydrolysis. Mallet and Bünger (22) recently observed that pyruvate-induced increases in the cytosolic phosphorylation potential ([ATP]/[ADP][Pi], [CrP]/[Pi]; CrP is creatine phosphate and brackets denote concentration) in the isolated working guinea pig heart were associated with increased SR Ca2+ loading and left ventricular contractile function. These authors proposed that myocardial inotropism due to pyruvate was mediated metabolically and that the improved SR Ca2+ handling reflected a response to an increased cytosolic energy level, i.e., a rise in the cytoplasmic [ATP]/[ADP][Pi] (22).

Despite the recognized positive inotropic effect of pyruvate in the intact heart, and its potential ability to improve SR Ca2+ handling, direct experimental evidence for improved Ca2+ handling in response to pyruvate is not yet available. In an attempt to quantitate the Ca2+ transient in relation to contractile function, we examined the isolated rat cardiomyocyte. This single myocyte model can be rigorously controlled with regard to its metabolic and hormonal status. The purpose of the present study was 1) to determine whether the inotropic effect of pyruvate can be elicited in the isolated ventricular myocyte and, if so, 2) to
delineate the associated changes in SR Ca$^{2+}$ handling using Ca$^{2+}$ transient measurements. The effects of pyruvate were quantitatively compared with those of acetate (freely diffusible mitochondrial substrate without cytoplasmic redox effects), L-lactate (a cytosolic reductant in contrast to pyruvate), and submillimolar concentrations of α-cyano-4-hydroxycinnamate (HC; an inhibitor of the pyruvate transporter on the inner mitochondrial membrane).

**MATERIALS AND METHODS**

**Myocyte isolation.** Studies were conducted in single, Ca$^{2+}$-tolerant myocytes isolated from adult rat hearts by enzymatic dispersion (16). Male Wistar rats (300–350 g) were anesthetized with pentobarbital sodium (60 mg/kg ip) and heparinized (500 units ip). Hearts were rapidly excised and retrogradely perfused at a constant pressure of 70 mmHg on a Langendorff apparatus with a physiologic N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) buffer containing (in mmol/l): 118 NaCl, 4.7 KCl, 25 HEPES, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 11 glucose, and 1.0 CaCl$_2$. pH was adjusted to 7.0. All perfusion solutions were continually gassed with 100% oxygen. After 10 min of perfusion with standard HEPES, the buffer was switched to a nominally Ca$^{2+}$-free HEPES buffer (supplemented with minimal essential medium amino acid solution; Gibco-BRL, Gaithersburg, MD) for 5 min. The relaxed heart was subsequently perfused with HEPES buffer supplemented with 1 mg/ml collagenase (type II; Worthington) and 0.5 mg/ml hyaluronidase (Sigma) for 20 min. During the final 5 min of collagenase infusion, Ca$^{2+}$ was incrementally added back to a final concentration of 0.5 mmol/l. The partially digested heart was then removed from the cannula, minced, and placed in a shaking water bath (37°C) with 10 ml partially digested heart was then removed from the cannula, minced, and placed in a shaking water bath (37°C) with 10 ml of fresh HEPES-collagenase buffer for 30 min. The resulting cell suspension was filtered through a 250-µm nylon mesh, washed twice with fresh enzyme-free buffer, and resuspended in HEPES buffer (1.0 mmol/l CaCl$_2$, pH = 7.4) to a final concentration of 0.5–1.0 mg/ml total protein. Only preparations yielding >70% rod-shaped cells were used for experiments. All cells were used within 6 h of isolation and were kept at 37°C until the time of experimentation.

**Measurement of myocyte shortening.** Measurements of cell length and myocyte shortening were obtained with a video system and edge detection software. An aliquot of cells was placed in the recording chamber (model RC-24; Warner Instruments, Hamden, CT) on the stage of an inverted microscope. A high-speed video camera (CCD no. 400; Pulnix, Sandy, UT) was connected to the side port of the microscope. Contractions of single myocytes were displayed on a video monitor and recorded on videotape. The stored data were processed off-line by a video edge detector (Crescent Electronics, Sandy, UT) to measure motion along the longitudinal cell axis of the contracting myocyte. The output of the edge detector (temporal resolution of 60 Hz) was further analyzed with pClamp software (Axon Instruments, Foster City, CA).

**Measurement of intracellular Ca$^{2+}$.** Myocytes were loaded with the fluorescent Ca$^{2+}$ indicator fura 2-acetoxyethyl ester (Molecular Probes, Eugene, OR) at a concentration of 2 µmol/l for 10 min at 37°C. This loading protocol was used to minimize mitochondrial dye loading and fluorescence (25). Dye-loaded cells were washed twice to remove excess fura 2, centrifuged at ~400 revolutions/min for 1 min, and resuspended in fresh HEPES buffer. Cells were allowed a minimum of 30 min to deesterify the membrane-permeant acetoxymethyl ester form of the fluophore to its membrane-impermeant potassium salt. Aliquots of the loaded cell suspensions were placed in a 0.5-ml recording chamber (model RC-24; Warner Instruments, Hamden, CT) on the stage of an inverted microscope. The floor of the recording chamber consisted of a 22 × 22-mm glass coverslip that was coated with laminin to enhance cell adherence. The myocytes were left undisturbed for 5–10 min to allow them to settle and adhere to the coverslip. Cells were then superfused at 0.8 ml/min with HEPES buffer (pH = 7.4), which was continually gassed with 100% oxygen. The temperature of the buffer and recording chamber was maintained at 37 ± 1°C throughout the experiment.

The optical system for recording myocyte fluorescence with rapid time resolution consisted of a Nikon Diaphot inverted microscope with high ultraviolet transmission optics connected to the Photocam 2 software package (Nikon, Melville, NY). Ultraviolet light from a 75-W xenon arc lamp was split by a rotating optical chopper, passed through two monochromatic filters set at 340 and 380 nm, and directed to the fura 2-loaded myocytes on the stage of the microscope. The emitted light was detected with a photomultiplier (Photon Technologies International, South Brunswick, NJ) at 510 nm, and the signals were processed using the Photocam-2 software. The 340- to 380-nm ratio could be recorded with millisecond resolution. The free cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) was calculated using the method of Grynkiewicz, Poenie, and Tsien (13) according to the equation

$$[\text{Ca}^{2+}]_i = K_d \cdot (R - R_{\text{min}})/(R_{\text{max}} - R)$$

where $R$ is the ratio of cellular fluorescence with excitation at 340 and 380 nm recorded at an emission wavelength of 510 nm, $R_{\text{min}}$ is the fluorescence ratio at zero Ca$^{2+}$, and $R_{\text{max}}$ is the limiting ratio at saturating Ca$^{2+}$ concentrations (2.5 mmol/l). The dissociation constant, or $K_d$, was 200 nmol/l, as calculated by 10.2 ± 0.3 on October 30, 2017 http://ajpheart.physiology.org/ Downloaded from

**Experimental protocol.** The protocols for the measurements of both [Ca$^{2+}$]$_i$ and cell shortening were identical, with the exception that fura 2-loaded cells were used for measurement of Ca$^{2+}$ transients and unloaded cells were used for myocyte shortening measurements. All cells were superfused with standard HEPES buffer (11 mmol/l glucose) while being stimulated at 0.5 Hz for 20 min before data acquisition. After this equilibration period, baseline recordings of either cell shortening or [Ca$^{2+}$]$_i$ were obtained. Recordings were obtained again <2 min later to ensure that steady-state values were obtained. Cells were then assigned to one of four treatments groups. All agents were added to the oxygenated HEPES buffer in the indicated concentrations and superfused over the myocyte for 10 min before obtaining measurements. Cells in group 1 ($n = 5$ at each dose) were exposed to either 2, 5, 10, or 20 mmol/l pyruvate. A subset of these cells ($n = 6$) was then washed for 10 min before exposure to 100 mmol/l isoproterenol. The cells in group 2 were exposed to a buffer containing either 20 mmol/l glucose ($n = 6$) or the 11 mmol/l glucose plus 10 U/l insulin ($n = 7$).

Group 3 was designed to assess the relationship between pyruvate inotropism and its uptake by the mitochondria. Control glucose cells were treated with 5 mmol/l acetate ($n =
or 250 µmol/l HC (n = 14). The hydroxycinnamate-treated cells were subsequently exposed to either 5 mmol/l pyruvate (n = 7) or 5 mmol/l acetate (n = 7). Hydroxycinnamate is a noncompetitive inhibitor of both the mitochondrial and sarcosomal monocarboxylate transporter; however, at the concentration used in the present study, it has been shown to inhibit mitochondrial pyruvate uptake, without significantly affecting sarcolemmal pyruvate transport (3). The reversibility of hydroxycinnamate was confirmed with a 10-min washout. Group 4 was used to examine the relationship between glycolysis and Ca\(^{2+}\) homeostasis in the normoxic myocyte. Cells were exposed to a glucose-free HEPES buffer for 10 min (n = 10). After this treatment, the inotropic effects of 5 mmol/l pyruvate were examined in a subset (n = 6) of these glucose-free cells. Cells in group 5 were used to examine the potential role of the cytosolic redox state in pyruvate inotropism. Cells (n $\geq$ 5/group) were superfused with HEPES buffer supplemented with either pyruvate (5 or 20 mmol/l), a cytosolic oxidant, or lactate (5 or 20 mmol/l), a cytosolic reductant, for 10 min.

Statistical analysis. All data are presented as means ± SE. Data were analyzed using a one-way analysis of variance (ANOVA) and Scheffe’s multiple comparison confidence intervals to detect group differences. Differences were deemed significant when P values < 0.05 were indicated.

RESULTS

Only 8% of the preparations yielded <70% viable myocytes as determined by morphology, i.e., intact sarcolemmal membranes and distinct sarcomere structure. In addition, cells that did not shorten vigorously during field stimulation were also excluded.

Effects of pyruvate on cell shortening and [Ca\(^{2+}\)]. Original recordings of [Ca\(^{2+}\)], and cell shortening from a representative myocyte are shown in Fig. 1. Recordings were obtained at 100 Hz under control conditions, after 10 min superfusion with 5 mmol/l pyruvate, and during superfusion with 100 nmol/l isoproterenol (2–3 min). Compared with control, both pyruvate and isoproterenol significantly increased [Ca\(^{2+}\)], and cell shortening. Although 5 mM pyruvate was ∼70% as effective as isoproterenol in augmenting systolic [Ca\(^{2+}\)] and contractility, the inotropy took considerably longer to develop. The effects of β-adrenergic stimulation with isoproterenol occur very rapidly, with maximal effects observed after 2–3 min of exposure. In contrast, metabolic augmentation of function with pyruvate took much longer to develop, not reaching a peak until 7–8 min of superfusion with 5 mmol/l pyruvate.

The effects of pyruvate on ventricular myocyte shortening are summarized in Fig. 2A. Control cells (n = 24) shortened 16.2 ± 1.7% of their resting cell length. The effects of subsequent treatments are expressed as a percentage of control shortening. Myocyte shortening in the presence of 2, 5, 10, and 20 mmol/l pyruvate measured 109 ± 1, 133 ± 5, 120 ± 2, and 116 ± 1% of control, respectively. The inotropic effects of 5, 10, and 20 mmol/l pyruvate were statistically significant (P < 0.05) when compared with control. In a subset of cells (n = 6) treated with the β-adrenergic agonist isoproterenol, cell shortening increased to 163% of control values, a statistically significant increase compared with both control and pyruvate-treated cells.

Pyruvate also significantly increased the peak of the Ca\(^{2+}\) transient in the isolated ventricular myocyte. These data are summarized in Fig. 2B. In control cells (n = 24), peak systolic and diastolic Ca\(^{2+}\) concentrations measured 397 ± 6 and 76 ± 1 nmol/l, respectively. Diastolic Ca\(^{2+}\) concentrations were not significantly altered by any concentration of pyruvate. The addition

Fig. 1. Original recordings of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and cell shortening from a representative rat cardiac myocyte. Recordings were obtained at 100 Hz under control conditions and after 10 min of superfusion with 5 mmol/l pyruvate or 100 nmol/l isoproterenol. PYR, pyruvate; ISO, isoproterenol.
of 2 mmol/l pyruvate to the superfusion buffer did not significantly affect systolic Ca\(^{2+}\) concentration when compared with control conditions. However, when the pyruvate concentration was raised to 5 mmol/l, the peak systolic Ca\(^{2+}\) concentration increased to 552 ± 6 nmol/l, a 42 ± 6% increase compared with control values. These observations are consistent with the myocyte shortening data, which revealed a 33 ± 5% increase after the addition of 5 mmol/l pyruvate. Increasing the pyruvate concentration to 10 or 20 mmol/l was associated with no further increase in systolic Ca\(^{2+}\). In fact, systolic Ca\(^{2+}\) with these higher doses of pyruvate (524 ± 11 and 481 ± 6 nmol/l, respectively), while significantly increased compared with control, was significantly less (P < 0.05) than that in the presence of 5 mmol/l pyruvate. After a 10-min washout of pyruvate, Ca\(^{2+}\) concentrations in all cells returned to prepyruvate levels. Exposure to 100 nmol/l isoproterenol (n = 6) increased systolic Ca\(^{2+}\) concentrations to 703 ± 25 nmol/l, a significant increase when compared with both control and 5 mmol/l pyruvate. Isoproterenol also resulted in a slight, but significant, reduction in the diastolic Ca\(^{2+}\) concentration (68 ± 4 nmol/l).

Effects of supraphysiological glucose and insulin on [Ca\(^{2+}\)] and cell shortening. The experiments in group 2 tested whether the actions of pyruvate were possibly the result of providing metabolically starving cells with additional metabolic substrate. Increasing the glucose concentration from 11 to 20 mmol/l had no effect on either systolic (367 ± 12 nmol/l) or diastolic (81 ± 2 nmol/l) [Ca\(^{2+}\)], or on cell shortening (101 ± 2% of baseline values). In addition, systolic [Ca\(^{2+}\)], in the presence of 10 U/l insulin plus 11 mmol/l glucose remained near control values, i.e., 384 ± 9 and 82 ± 2 nmol/l during peak systole and diastole, respectively. Insulin did not significantly increase cell shortening compared with 11 mmol/l glucose alone (103 ± 3%).

Effects of monocarboxylate transport inhibition. The effects of the monocarboxylate transport inhibitor HC on cell shortening are summarized in Fig. 3A. Hydroxycinnamate at a dose of 250 µmol/l, a concentration that has been found to selectively inhibit the mitochondrial monocarboxylate transporter in the perfused heart (3), resulted in a 33 ± 5% reduction in myocyte shortening with 11 mmol/l glucose as the sole substrate. Although the addition of 5 mmol/l acetate, a weak-acid metabolic substrate whose entry into the mitochondria is largely independent of the monocarboxylate transporter, did not significantly affect cell shortening when compared with control (111 ± 6%), it completely reversed the

Fig. 2. Dose-response relationships of pyruvate with regard to myocyte shortening (A) and Ca\(^{2+}\) transients (B). In A, shortening data are expressed as a percentage of control shortening. *P < 0.05 vs. baseline and †P < 0.05 vs. 5 mmol/l pyruvate.

Fig. 3. Effects of 10-min treatment with α-cyano-4-hydroxycinnamate (HC, 250 µmol/l) on myocyte shortening (A) and Ca\(^{2+}\) transients (B) in the presence and absence of pyruvate (5 mmol/l) and acetate (ACE, 5 mmol/l). Shortening data in A are expressed as a percentage of control shortening. *P < 0.05 vs. control and †P < 0.05 vs. HC.
decrement in contractility induced by hydroxycinnamate (106 ± 1%). The addition of 5 mmol/l pyruvate to hydroxycinnamate-treated cells resulted in a slight, yet statistically significant, increase in cell shortening when compared with hydroxycinnamate alone (81 ± 3 vs. 67 ± 2%, P < 0.05). However, the magnitude of inotropy resulting from 5 mmol/l pyruvate was reduced by 58 ± 4% in the presence of hydroxycinnamate (P < 0.001).

The effects of hydroxycinnamate on the Ca2+ transient are summarized in Fig. 3B. Because systolic [Ca2+]i was similar among the various control groups, the values have been pooled (388 ± 13 nmol/l, n = 28). Hydroxycinnamate resulted in a significant decrease in systolic [Ca2+]i (from 393 ± 8 to 291 ± 10 nmol/l). Diastolic [Ca2+]i was not significantly different from control values after hydroxycinnamate. However, when these cells were then superfused with buffer supplemented with 5 mmol/l acetate, the effects of hydroxycinnamate again were reversed. Acetate significantly increased systolic [Ca2+]i in the presence of hydroxycinnamate from 291 ± 10 to 389 ± 10 nmol/l. In addition, transients measured in the presence of hydroxycinnamate plus acetate were not significantly different from those of control cells or those perfused with acetate alone (399 ± 4 nmol/l). The addition of 5 mmol/l pyruvate to cells treated with hydroxycinnamate resulted in a significant increase in systolic [Ca2+]i, when compared with hydroxycinnamate alone (324 ± 4 vs. 291 ± 10 nmol/l). However, these effects were 72 ± 7% less than those observed in cells treated with 5 mmol/l pyruvate alone (552 ± 12 nmol/l).

After a 10-min washout of hydroxycinnamate, both cell shortening (103 ± 3%) and Ca2+ transients (375 ± 11 nmol/l) returned to baseline values. This reversibility is consistent with the previously observed noncompetitive interaction between pyruvate and hydroxycinnamate at the inner mitochondrial membrane monocarboxylate transporter (3).

Effects of glucose-free perfusion. The effects of glucose-free perfusion on myocyte shortening are summarized in Fig. 4A. Removal of glucose from the perfusate significantly reduced cell shortening to 84 ± 2% of control values. The addition of 5 mmol/l pyruvate to these cells increased myocyte shortening to 109 ± 2% of control, a statistically significant 29% increase when compared with glucose-free treatment.

The effects of glucose-free perfusion on Ca2+ transients are summarized in Fig. 4B. The removal of glucose from the superfusion media decreased systolic [Ca2+]i from 399 ± 4 to 340 ± 6 nmol/l, a statistically significant 16% reduction when compared with control. Although diastolic [Ca2+]i tended to increase in the absence of glucose (76 ± 1 vs. 86 ± 3 nmol/l), statistical significance was not achieved. Systolic [Ca2+]i in the absence of glucose was increased 22% (340 ± 6 to 415 ± 4 nmol/l) by the addition of 5 mmol/l pyruvate.

Comparison of pyruvate and lactate effects. The effects of 5 and 20 mmol/l pyruvate on cell shortening are compared with those measured in the presence of equimolar concentrations of L-lactate in Fig. 5A. Al-
Pyruvate has been shown to be a positive inotrope in numerous cardiac preparations. The inotropic effects have been demonstrated in both in vivo (21, 23, 36, 37) and in vitro (4, 22, 28) preparations and in normoxic (4, 20, 21, 22, 28, 36) and stunned (8, 23, 37) myocardium. Mallet and Bünger (22) reported that 5 mmol/l pyruvate, compared with equimolar L-lactate, increased left ventricular stroke work and dp/dt in the isolated working guinea pig heart, and this was associated with an increase in the caffeine-sensitive SR Ca\(^{2+}\) pool.

Dose-response effects of pyruvate as a metabolic inotrope. The present study is the first report of the inotropic effect of pyruvate in the isolated cardiac myocyte. The most pronounced inotropic effect of pyruvate was observed at a concentration of 5 mmol/l, a concentration ∼25–50 times higher than its physiological plasma level of 0.1–0.2 mmol/l. A lower dose of pyruvate altered neither systolic [Ca\(^{2+}\)] nor the extent of myocyte shortening, and higher concentrations provided no additional inotropy. Although the inotropic effect of 5 mmol/l pyruvate was not as large as that observed with β-adrenergic stimulation by isoproterenol, it represents a significant improvement when compared with the glucose control. These dose-response relations in the isolated cardiomyocyte are in agreement with a number of previous studies on pyruvate inotropism in the intact heart. These studies have determined the maximally effective pyruvate concentration to be between 5 and 10 mmol/l (4, 6, 19, 20, 22, 28). Although the inability of high glucose and insulin concentrations to augment [Ca\(^{2+}\)] or myocyte function may indicate that glycolytic flux is physiologically limited by phosphofructokinase control, the data also suggest that pyruvate inotropism is not related to simply providing the cells with additional glycolytic substrate. Saturation of the cell and mitochondria with acetate did not augment [Ca\(^{2+}\)] or cell shortening, again suggesting that these isolated myocytes are not in a state of substrate deprivation. Thus pyruvate inotropism appears to be a unique property of this monocarboxylate and predominantly linked to its mitochondrial metabolism.

**DISCUSSION**

The findings of the present study indicate that supraphysiological doses of pyruvate have a direct positive inotrop effect in isolated rat ventricular myocytes, an effect that is associated with increases in systolic but not diastolic [Ca\(^{2+}\)]. Remarkably, 5 mmol/l pyruvate was ∼70% as effective as β-adrenergic stimulation with 100 nmol/l isoproterenol in augmenting systolic [Ca\(^{2+}\)], and contractility in cardiomyocytes. On the basis of results with the monocarboxylate transport inhibitor HC, the effects of pyruvate on [Ca\(^{2+}\)] and cell shortening appear to be primarily dependent on its uptake and hence metabolism by the mitochondria. Pyruvate-induced changes in the redox potential of the cytoplasm may also be involved in pyruvate inotropism but to a substantially lesser degree. These results are the first evidence, albeit circumstantial, to indicate that function of the intact cardiomyocyte may be responsive to “energetic modulation” of [Ca\(^{2+}\)].

Pyruvate has been shown to be a positive inotrope in numerous cardiac preparations. The inotropic effects have been demonstrated in both in vivo (21, 23, 36, 37) and in vitro (4, 22, 28) preparations and in normoxic (4, 20, 21, 22, 28, 36) and stunned (8, 23, 37) myocardium. Mallet and Bünger (22) reported that 5 mmol/l pyruvate, compared with equimolar L-lactate, increased left ventricular stroke work and dp/dt in the isolated working guinea pig heart, and this was associated with an increase in the caffeine-sensitive SR Ca\(^{2+}\) pool. Intotropically effective pyruvate also doubled the cytosolic phosphorylation potential, substantially increased the [ATP]-to-[ADP] ratio, and also reduced the level of intracellular phosphate can-

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carboxylate-proton symporter (3, 14). In addition to the mitochondrial anaplerotic pyruvate carboxylase pathway and cytosolic transamination to alanine, there are two other major pathways that could provide the metabolic basis of the observed pyruvate inotropism (Fig. 6). The first pathway involves pyruvate reduction by lactate dehydrogenase to lactate, a reaction that can increase the cytosolic NAD$^+$-to-NADH ratio, and consumes one proton yielding L-lactate anion. At sufficiently high pyruvate levels, this reaction produces a significant increase in the cytosolic oxidation potential. The other, probably more important, mechanism entails the mitochondrial pathway and hence depends on the entry of pyruvate into the mitochondria via the distinct monocarboxylate transporter on the inner mitochondrial membrane. In the mitochondrial matrix, pyruvate is oxidatively decarboxylated to acetyl CoA plus CO$_2$ by the pyruvate dehydrogenase complex, producing intramitochondrial-reducing equivalents and (provided the pyruvate concentration is in the millimolar range) decreasing the mitochondrial NAD$^+$-to-NADH ratio (28). This augmentation of mitochondrial NADH reducing power may, via the tricarboxylic acid cycle enzymes and the electron transport chain, ultimately result in augmentation of the phosphorylation potential in the cytoplasm (28). It is likely that pyruvate exerts its effects on [Ca$^{2+}$], and contractility via one of these two or, alternatively, both of these metabolic pathways.

In an attempt to delineate these potential mechanisms, we altered the availability of mitochondrial metabolic substrate in the presence and absence of a submillimolar concentration of the monocarboxylate transport inhibitor HC. A 250 µmol/l dose of the compound is thought to inhibit predominantly, if not exclusively, the mitochondrial rather than the sarcolemmal pyruvate transporter (3), thus allowing differentiation between the cytosolic effects of pyruvate and those via mitochondrial metabolism. Two distinct populations of pyruvate transporters have been identified in cardiomyocytes: one on the sarcolemmal membrane and the other on the mitochondrial inner membrane (14, 26, 31). Although the reported Michaelis constant ($K_m$) values of these transporters for pyruvate are similar (0.5–3.0 mM; see Refs. 14, 26, 30, 31), the mitochondrial monocarboxylate transporter is extremely sensitive to inhibition by low doses of HC, with a reported inhibitory constant in rat heart of 6.3 µM (14, 15). In contrast, concentrations of ~3 mmol/l hydroxycinnamate are required to inhibit sarcolemmal pyruvate flux (32). At the concentration used in the present study (250 µmol/l), HC has been shown to selectively inhibit mitochondrial pyruvate uptake, without significantly affecting sarcolemmal transport (3, 9). Data from the present study indicate that a substantial portion of pyruvate-induced inotropism is dependent on its uptake by the mitochondria. Hydroxycinnamate reduced the effects of pyruvate on systolic Ca$^{2+}$ and cell shortening by 72 ± 7 and 58 ± 4%, respectively. However, the effects of acetate on cell shortening and Ca$^{2+}$ transients were not significantly altered by the presence or absence of hydroxycinnamate. These data confirm that hydroxycinnamate can selectively block mitochondrial uptake of pyruvate without inhibiting mitochondrial enzymes.

Although the exact linkage between pyruvate augmentation of the myocardial phosphorylation potential and the observed increase in peak systolic [Ca$^{2+}$], and contractility is not fully established, improved Ca$^{2+}$ handling by the SR is a distinct possibility. It has been hypothesized by previous investigators that an increase in the phosphorylation potential may allow the SR Ca$^{2+}$-ATPase to operate more efficiently (4, 38). This Ca$^{2+}$ transport ATPase has a high free energy requirement and is known to be sensitive to changes in the free energy of ATP hydrolysis, i.e., the myocardial phosphorylation potential (18). Improved SR Ca$^{2+}$-ATPase function may in turn lead to an increased end-diastolic SR Ca$^{2+}$ load. Furthermore, recent data from Janczewski et al. (17) indicate that augmentation of the SR Ca$^{2+}$ load increases the gain function of SR Ca$^{2+}$ release and also the peak of the Ca$^{2+}$ transient.

Alternative mechanisms. Pyruvate-induced augmentation of myocardial phosphorylation potential could alter function by another mechanism. Mallet and Bünger (22) observed that 5 mmol/l pyruvate increased left ventricular contractility and reduced P$_i$ concentration by 27% (6.3–4.6 mmol/l) in the normoxic isolated guinea pig heart. High levels of P$_i$ concentration have been reported to decrease SR Ca$^{2+}$ release secondary to inhibition of SR Ca$^{2+}$ uptake (34). One might therefore hypothesize that pyruvate inotropism may involve improved SR Ca$^{2+}$ loading resulting from a reduction in P$_i$ concentration. However, although exogenous administration of extreme concentrations of P$_i$ (20 mmol/l) has been demonstrated to inhibit Ca$^{2+}$ release, there is no evidence to suggest that reductions in P$_i$ concentration below physiological levels (2–5 mmol/l) augment Ca$^{2+}$ release.

Fig. 6. Proposed highly simplified model to illustrate energetic control of SR function and contractility in cardiomyocytes. LAC, lactate; SR, sarcoplasmic reticulum; MCT, inner mitochondrial membrane monocarboxylate transporter; MAS, malate/aspartate shuttle; TLC, ATP translocase; TCA, tricarboxylic acid cycle.
Although it has been reported that 300 µmol/l hydroxycinnamate selectively reduces mitochondrial pyruvate uptake without inhibition of mitochondrial enzymatic or energetic function (3), the observed depression in myocyte contractile function after administration of hydroxycinnamate could be the result of some direct negative inotropic property of the compound. However, Ca$^{2+}$ transients and myocyte shortening in the presence of hydroxycinnamate plus acetate, a weak two-carbon fatty acid and mitochondrial substrate in which entry into the mitochondria is largely independent from the monocarboxylate transporter, were not significantly different from those in the presence of acetate alone, or glucose control cells, indicating that the functional effects of low-dose hydroxycinnamate are mediated by alterations in mitochondrial pyruvate utilization rather than direct effects on the contractile apparatus. Similar data were obtained using 0.2 mmol/l octanoate, a medium-chain-length fatty acid and oxidative substrate in which mitochondrial metabolism is independent of monocarboxylate transport (data not shown). On the basis of these data, it appears justified to conclude that glucose-incubated isolated myocytes are critically dependent on pyruvate oxidation for Ca$^{2+}$ homeostasis and contractility.

Glucose requirements. The experiments involving glucose-free superfusion of the myocyte provide insight not only into the mechanism of pyruvate inotropism but also into the metabolic control of cellular Ca$^{2+}$ handling. Removal of glucose from the perfusate, and the ensuing inhibition of glycolysis and glucose oxidation, resulted in a significant reduction in both systolic [Ca$^{2+}$]$_i$ and contractility. These observations are in contrast to the data obtained in the isolated heart in which global cardiac function can remain relatively constant for 30 min of substrate-free perfusion. This likely reflects a preference of the intact heart for endogenous triglycerides, which are readily utilized in the absence of glucose. In contrast, the intact isolated rat cardiomyocyte appears to be quite sensitive to removal of exogenous glucose substrate, perhaps because the endogenous triglyceride pools become depleted during the isolation and HEPES buffer incubation procedures. The data also suggest that basal SR Ca$^{2+}$ handling may be coupled to glycolytic ATP production. The potential of glycolysis to support basal SR Ca$^{2+}$ handling has recently been demonstrated in an SR vesicle preparation by Xu et al. (35). These SR vesicles actively transported Ca$^{2+}$ in the presence of glycolytic substrate but in the absence of oxidative phosphorylation, leading the authors to conclude that glycolysis is functionally coupled to basal SR Ca$^{2+}$ homeostasis. The present study is the first evidence that such an association between glycolysis and SR Ca$^{2+}$ handling might indeed be functional in the intact myocyte. Although the absolute magnitude of inotropy produced by pyruvate was reduced by the absence of glucose, pyruvate increased systolic [Ca$^{2+}$]$_i$ and cell shortening 22 and 29%, respectively, a significant increase compared with glucose-free perfusion.

Possible role of the cytosolic redox state. The fact that 250 µmol/l hydroxycinnamate did not completely block the effects of 5 mmol/l pyruvate could simply be due to incomplete blockade of the monocarboxylate transporter. However, another possible explanation could be that pyruvate exerted inotropic effects, at least in part, independent from mitochondrial metabolism. This hypothesis is supported by the observation that an equimolar concentration of lactate, which also has been reported to increase the myocardial phosphorylation potential (19, 22), did not produce the same increase in [Ca$^{2+}$]$_i$ and cell shortening as pyruvate. Due to its oxidizing effects in the lactate dehydrogenase reaction, high concentrations of pyruvate are known to increase the cytosolic NAD$^+$-to-NADH ratio and cytosolic oxidation potential (20, 28), whereas high concentrations of lactate function principally as a cytosolic reductant. At 5 mmol/l, lactate appears to moderately augment function in a normoxic myocyte preparation through its conversion to pyruvate by lactate dehydrogenase. However, support for the possible role of cytosolic redox state in pyruvate inotropism comes from the 20 mmol/l data. Pyruvate and lactate have greatly opposite effects on the cytosolic redox state at these extreme concentrations, and it has been estimated that the cytosolic NAD$^+$-to-NADH ratio differs by about three orders of magnitude under such conditions (5). Thus 20 mmol/l pyruvate acts as a strong oxidant in the cytosol, whereas 20 mmol/l lactate greatly reduces the cytosolic redox potential. Although lactate still resulted in a slight increase in systolic [Ca$^{2+}$]$_i$, myocyte shortening was actually reduced. Similar results have been reported by Cairns et al. (7), who observed that exposure of isolated rat cardiomyocytes to 20 mmol/l lactate resulted in a significant reduction in cell shortening. However, these authors did not examine lower, physiological concentrations of lactate, and all experiments were conducted at room temperature, rendering a direct comparison with the present study difficult. Additional evidence supporting the possible role of the cytosolic oxidation in pyruvate inotropism comes from Scholz et al. (28), who reported that, whereas 10 mmol/l pyruvate and lactate were equally effective at increasing mitochondrial NADH levels in working rabbit heart, pyruvate alone significantly increased cardiac contractility (left ventricular dP/dt).

Recent observations suggest that Ca$^{2+}$ release via SR ryanodine receptors can be modulated by the redox state of the cytosol (2, 11, 27). Via a series of reactions involving transhydrogenation and possibly also glutathione reductase, augmentation of the NAD$^+$-to-NADH ratio by excess pyruvate might subsequently increase the NADP$^+$-to-NADPH and glutathione disulfide-to-glutathione ratios, respectively. Glutathione disulfide and the sulfhydryl oxidant thimerosal have been reported to increase the open probability of ryanodine receptors in SR vesicles obtained from rabbit skeletal muscle (2). Both effects are thought to be mediated by oxidation of critical sulfhydryl groups on the channel. Oxidation and reduction of ryanodine receptor sulfhydryl groups has been postulated to be a mechanism
underlying the gating of Ca$^{2+}$ release proteins (1). In the absence of direct measurements of glutathione in these single myocytes, it cannot be excluded that pyruvate, when applied to cardiomyocytes in extreme and nonphysiological concentrations, may act in part through such an oxidation mechanism to augment Ca$^{2+}$ release and subsequently improve myocardial contractility.

Possible role of intracellular pH. There is another aspect to be considered in explaining the disparities between pyruvate and lactate with respect to SR Ca$^{2+}$ handling and cell shortening. Both pyruvate and lactate are relatively weak organic acids and tend to reduce intracellular pH when administered extracellularly (10). This intracellular acidification is the result of proton cotransport when pyruvate or lactate enters the cell via the plasma membrane monocarboxylate transporter. However, equimolar doses of pyruvate and lactate are not expected to result in equivalent reductions in intracellular pH because the transport K$m$ is much lower for pyruvate than for lactate (3). Data from Wang et al. (30) have shown that exposure of isolated rat myocytes to even a modest extracellular lactate concentration (2 mmol/l) resulted in a 0.1-unit drop in intracellular pH by 0.24 pH units. Intracellular acidosis has been demonstrated to reduce the Ca$^{2+}$ sensitivity of the contractile elements and to reduce the maximal tension produced at a given [Ca$^{2+}$]i (24). Therefore, the disparity in contractility observed with the 20 mmol/l levels of pyruvate and lactate could be due to differences in intracellular pH and Ca$^{2+}$ sensitivity, parameters not assessed in the present study.

Limitations of the study. Myocardial phosphorylation potential, cytosolic oxidation-reduction state, and intracellular pH were not directly measured in the isolated cardiomyocytes, and this renders the current evidence for energetic control of SR function essentially circumstantial. However, the above discussion and interpretations are based on firmly established metabolic effects of pyruvate, lactate, acetate, and HC on cytosolic and mitochondrial [NAD$^+$]-to-[NADH] ratios and phosphorylation potentials in intact myocardium. Another potential weakness is the fact that measurements of cell shortening and [Ca$^{2+}$]i were not obtained simultaneously in the same single myocyte, and therefore the temporal relationships between these parameters could not be accurately established. Nevertheless, the isolated myocyte model does allow one to accurately assess [Ca$^{2+}$]i and myocardial contractility, without the inherent hemodynamic and pharmacological complexities of whole heart studies. The use of the isolated myocyte model also permits precise control of the extracellular milieu to which the cells are exposed, eliminating interference from endothelial metabolism and diffusional barriers.

Conclusions. The present study has shown that the positive inotropic effect of pyruvate can be demonstrated in single isolated rat ventricular myocytes and is most likely the direct result of augmentation of systolic [Ca$^{2+}$]. This effect requires millimolar concentrations of pyruvate, develops slowly within 10 min, and is fully reversible. In addition, the HC results strongly suggest that the major component of pyruvate inotropism is dependent on its uptake by energetically competent mitochondria. As the mitochondria are essential for the maintenance of cellular energetics and ionic homeostasis, our [Ca$^{2+}$]i transient findings imply enhanced SR Ca$^{2+}$ handling in response to increased cytosolic phosphorylation potential as the fundamental mechanism of pyruvate inotropism. However, the results also are consistent with the hypothesis that a minor portion of the inotropic effect of pyruvate may be related to an increase in the cytosolic oxidation potential.

Although receptor regulation (β-adrenergic) and protein regulation (phospholamban) of contractility and SR function have been well characterized and are the primary means of control of cardiac contractility physiologically, this is the first report to support the hypothesis of a direct causal relationship between [Ca$^{2+}$]i modulation by cellular energetics and cardiac inotropy. Although it appears to be a powerful mechanism, increasing myocyte contractility 33% in the present study, it may only be of marginal physiological significance. Pyruvate-induced augmentation of contractility was only observed at concentrations 10–20 times higher than those found physiologically in the plasma. The concept of pyruvate as a metabolic inotrope in the heart may, however, have clinical relevance and applicability, particularly in the treatment of stunned myocardium and other types of contractile dysfunction in which inotropic support at the cost of myocardial energetics may not be desirable.

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