Alterations in Cation Homeostasis in Cultured Chick Ventricular Cells during and after Recovery from Adenosine Triphosphate Depletion

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Abstract

Alterations in cation homeostasis during and after recovery from myocardial ischemia may account for some of the reversible and irreversible components of myocardial cell injury. To investigate possible mechanisms involved, we exposed cultured layers of spontaneously contracting chick embryo ventricular cells to media containing 1 mM cyanide (CN) and 20 mM 2-deoxyglucose (2-DG), and zero glucose for up to 6 h, and then allowed cultured cells to recover in serum-free culture medium for 24 h. Changes in Na, K, and Ca contents, ^{42}K uptake and efflux, ATP content, cell water content, and lactate dehydrogenase (LDH) release were measured, and compared with changes produced by exposure to 10^{-3} M ouabain and severe hypoxia. Exposure to CN and 2-DG caused marked increase in cell Na (sevenfold) and Ca (fivefold) contents, and a decrease in K content (one-fifth normal), coincident with ATP depletion to one-tenth normal levels. This produced only slight cell injury, evidenced by increased LDH release. Recovery for 24 h resulted in return to near normal values (expressed in nanomoles per milligram of protein) of Na, Ca, and ATP contents. However, there was failure of cell K content to return to normal, associated with a persistent reduced net uptake of ^{42}K, and an increase in the rate of ^{42}K efflux. These abnormalities in K homeostasis were associated with a decrease in cell volume and water content per milligram of protein. More marked ATP depletion (to $^1/_{100}$ normal values) was produced by hypoxia plus 2-DG and zero glucose, and was associated with much more severe cell injury manifested by LDH loss. Ouabain exposure resulted in a much greater Ca gain (20–30-fold), relative to increase in Na content, than did either CN and 2-DG or hypoxia; and ouabain effects were not reversible (after a 15-fold or greater increase in Ca content was produced) and were associated with significant LDH release. We conclude that these cells are resistant to cell injury caused by moderately severe Ca overload and ATP depletion produced by exposure to CN and 2-DG. However, metabolic inhibition of ATP production produces persistent abnormalities in K homeostasis, associated with functional abnormalities.

Introduction

Alterations in myocardial cell cation homeostasis during and after recovery from myocardial ischemia may account for some of the electrocardiographic (1), ^{201}Tl scintigraphic (2), and functional consequences (3) of coronary artery occlusion and subsequent reperfusion. Of particular possible importance is the control of intracellular Na concentration, which alters intracellular Ca via the Na-Ca exchange mechanism (4). For example, an elevation in intracellular Na concentration during ischemia has been proposed to produce calcium overload, especially during reperfusion of myocardium (5). Elevated Ca concentration in turn has been proposed as a mediator of irreversible cell injury via mechanical contracture, Ca-induced alterations in mitochondrial function, and/or by activation of proteases and phospholipases, with subsequent membrane structural damage and rupture (6).

Study of these cellular phenomena in intact tissue is difficult because of the presence of vascular and interstitial spaces which complicate interpretation of ion flux measurements; and the potential contributions of the vascular bed and nonmyocardial cells to the injury process (6). We have used cultured ventricular cells to examine changes in Na, K, and Ca contents, Na pump activity, and ATP contents, during and after recovery from ATP depletion induced by metabolic inhibition with cyanide, combined with 2-deoxyglucose (2-DG)[1]-zero glucose exposure. We have compared these changes with those produced by complete inhibition of the sodium pump with the cardiac glycoside, ouabain. Metabolic inhibition of ATP synthesis caused elevated Na and Ca contents, and reduced K and ATP contents. A comparable elevation in Na content produced by ouabain caused a much greater increase in Ca content, which was not reversible. The major effect of metabolic inhibition followed by recovery noted in these studies was reduced K^+ uptake and severe K depletion, associated with loss of cell water and cell shrinkage. This may be due to persistent activation of a K^+ channel, or a K-Cl cotransport system during severe metabolic inhibition.

Methods

Tissue culture. Spontaneously contracting layers of chick embryo ventricular cells were prepared as described previously (7). Briefly, ventricles from 10-d-old embryos were minced and placed in Ca-Mg-free Hanks' solution. The tissue was trypsinized in 10 ml of 0.025% trypsin in Ca-Mg-free Hanks' solution at 37°C for four cycles of 7 min each. The supernatant suspensions containing dissociated cells were placed in 20 ml of cold trypsin inhibitor medium and centrifuged at 2,000 rpm for 10 min. The supernatant was discarded and the cells were resuspended in culture medium consisting of 6% heat-inactivated fetal calf serum, 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 $MgSO_4$, 1.18 KCl, 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 25-mm circular glass coverslips. Cultures were incubated in

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1. *Abbreviations used in this paper:* CN, cyanide; 2-DG, 2-deoxyglucose; LDH, lactate dehydrogenase.

a 5% CO_2 atmosphere at 37°C. All studies were performed on cells after 3 or 4 d of culture.

Contractility measurement. Effects of interventions on contractile behavior of cultured cells were determined by measurement of the amplitude of motion of individual cells within the culture. A video motion detector system was utilized as described previously (7).

Ion content measurement. Atomic absorption spectroscopy methods were used to detect changes in cellular contents of Na, K, and Ca (7). Glass coverslips with an adherent layer of cells were washed for 30 s in 4°C in Hepes-buffered 140 mM choline Cl solution. The layer was scraped from the glass coverslip with a Teflon scraper that had been carefully washed in deionized water. Small (5 ml) Teflon sample vials were carefully washed in deionized water, and 0.75 ml of 33% nitric acid extract solution was added to the vials. The cells were washed off the Teflon scraper into this extract media, where they were digested. The nitric acid (GFS Chemicals, Columbus, OH) was double-distilled in Vycor glass and was spectrophotometrically pure. Prewashed plastic pipettes were used and the acid was stored in a contaminant-free Teflon bottle.

Samples of acid-digested cells were analyzed for Na, K, and Ca using a Varian AA 1275 atomic absorption spectrophotometer in conjunction with a Varian GTA 95 microprocessor-controlled graphite furnace and a programmable auto-sampler (Varian Associates, Palo Alto, CA). The details of this procedure have already been described (7). Quantities were analyzed per mg cell protein, determined by the Lowry method (8).

Potassium uptake and efflux measurements. Previously reported methods for K uptake determination using ^{42}K were employed (9). For 24 h before their use for ^{42}K uptake, glass coverslips with attached myocytes were exposed to medium containing L-[4,5-^{3}H, N]leucine (0.2 μCi/ml) to label cell protein. For ^{42}K uptake measurement, glass coverslips were loaded into Lucite baskets, care being taken not to damage the cells. The cells on 8–10 coverslips were equilibrated in Hepes-buffered medium (pH 7.35) at 37°C. By means of a thin wire handle, a basket containing coverslips was then immersed in isotope uptake medium (2 μCi/ml) at 37°C. After the desired uptake period, 1 or 2 min, coverslips were removed from the uptake medium and washed for 15 s each in 2 50-ml vol of balanced salt solution at 4°C to remove extracellular tracer.

After washing as described above, the layer was scraped off the coverslip, and the cells were placed in 2 ml of a solution containing 1% sodium dodecyl sulfate and 10 mM sodium borate. Over a 2-h period, complete cell dissolution was accomplished. A 1.6-ml aliquot of the dissolved cell mixture was placed in 15 ml of Aquasol liquid scintillation fluid (New England Nuclear, Boston, MA). From a quarter of the samples, 0.2 ml was used for determination of protein content. Simultaneous counting of ^{42}K and ^3H counts in dissolved cells from each coverslip was performed with a liquid scintillation spectrometer. From the ratio of ^3H counts per minute per milligram of protein for the culture, the K uptake per milligram of protein for each coverslip was determined.

For ^{42}K efflux measurements, cells were labeled to equilibrium with ^{42}K (2 h). After washing to remove extracellular label, fractional efflux of ^{42}K was measured into 2-ml volumes of efflux solutions, using 30-s efflux intervals. We have used a similar approach to determine fractional ^{45}Ca efflux in these cells (7).

ATP contents. ATP was measured in these experiments using a luciferase assay (10). Cells attached to glass coverslips were frozen in liquid nitrogen, and were subsequently scraped off the coverslip into 1 ml of 100 mM Tris, 0.1 mM EGTA solution at 0°C. After homogenization, half the sample was boiled for 2 min and the other half of the sample was set aside for subsequent protein determination. The boiled samples were centrifuged at 3,000 rpm for 20 min at 4°C, and after centrifugation, 10 μl of the supernatant was added to 1,990 μl of distilled water. ATP determination was then performed using the ATP constant light strength kit (Boehringer-Mannheim Diagnostics, Houston, TX), quantifying light emission with a liquid scintillation counter (Packard Instruments Co., Inc., Downers Grove, IL). ATP standards were prepared by adding known amounts of ATP stock solution. Protein was determined by the Lowry method (8), and ATP concentration expressed as nanomoles per milligram of protein.

Metabolic inhibition and recovery. ATP depletion was produced by exposure of cells to 1 mM cyanide (CN) and 20 mM 2-DG, in zero-glucose Hanks solution for periods ranging up to 6 h. Cells were subsequently removed from the CN–2-DG solution and placed in serum-free culture medium (11) for a subsequent 24 h to allow myocyte recovery, without fibroblast proliferation. Measurements were performed either during exposure to CN and 2-DG for the indicated time period; immediately after removal from CN and 2-DG and return to normal medium (early washout, 15 s); or after 24 h of recovery in serum-free culture medium. In a few experiments, cells on single coverslips in Leighton tubes were exposed to 20 mM 2-DG, zero-glucose plus severe hypoxia (Po_2 < 1 mm Hg) obtained by gassing with 5% CO_2, 95% nitrogen gas passed through a 5% alkaline pyrogallol solution for the indicated time periods. Control cells were kept in serum-free medium for 24 h, without exposure to metabolic inhibitors.

Sodium pump inhibition. Prolonged inhibition of the Na-K ATPase sodium pump was produced by exposure to 10^{-3} M ouabain for periods up to 6 h. Potassium uptake, Na, K, and Ca contents, and ATP contents were measured during exposure to ouabain for the indicated time periods, and after 24 h of recovery in serum-free culture medium without glycoside.

Lactate dehydrogenase (LDH) release. A lactate dehydrogenase kit (procedure no. 340-UV) was utilized (Sigma Diagnostics, St. Louis, MO). A 50-μl sample of culture medium was diluted into 2.85 ml of 0.1 M K phosphate buffer (pH 7.5), and allowed to sit for 20 min at 25°C. Na pyruvate (100 μl of 22.7 mM solution) was added, and the absorbance change at 340 nm was measured. LDH release was measured by the change in LDH concentration in medium with time, normalized per milligram of cell protein in the culture.

Statistical analysis. Significance of differences between means in different groups was assessed by analysis of variance.

Results

The changes in K^+ uptake, Na, K, and Ca contents, and ATP contents during exposure to CN and 2-DG, and after 24 h of recovery in serum-free cultured medium are shown in Table I. Exposure to CN and 2-DG induced moderately severe ATP depletion, with ATP contents falling from 32.9 to 3 to 4 nmol/mg of protein after 3–6 h of incubation. However, by 24 h of recovery ATP contents had returned to normal or near normal levels. Ca content increased markedly during metabolic inhibition with CN and 2-DG, rising from a control of 10.6 to 47.4 nmol/mg of protein at 6 h. However, after 24 h of recovery, Ca contents returned to normal values. Na contents also increased markedly during metabolic inhibition, rising from a control value of 107 to 752 nmol/mg of protein after 6 h of metabolic inhibition. However, by 24 h of recovery, Na content returned to normal. K contents showed a dramatic decline during metabolic inhibition with CN and 2-DG, from a control of 982 to 141 nmol/mg protein after 6 h of metabolic inhibition. However, unlike changes in Na, Ca, and ATP contents, K contents did not return to normal values after 24 h of recovery. Cells previously exposed to CN plus 2-DG for 3 and 6 h had K contents of 575 and 188 nmol/mg protein, respectively.

Changes in K uptake were associated with these marked changes in Na, K, and Ca contents. Ouabain inhibitable uptake of K was essentially completely suppressed within 1 h of exposure to CN and 2-DG, consistent with our previous finding (12). After recovery from exposure to CN and 2-DG for 1 h, there was a moderately good recovery of K uptake. However, cells treated for longer periods of time with CN and 2-DG

Table I. Effects of CN and 2-DG

		Control	1-h CN-2-DG	3-h CN-2-DG	6-h CN-2-DG
		nmol/mg protein			
Cation contents during exposure to CN-2-DG	Na	107±7 (26)	248±13 (29)	672±56 (31)	742±61 (18)
	K	982±43 (28)	481±19 (22)	365±25 (30)	141±29 (19)
	Ca	10.6±0.7 (12)	19.5±1.3 (13)	29.6±2.0 (12)	47.4±3.0 (13)
Cation contents after 24 h of recovery from CN-2-DG	Na	104±4 (24)	93±4 (33)	95±8 (30)	92±9 (17)
	K	1130±4 (31)	798±39 (35)	575±44 (27)	188±23 (19)
	Ca	12.3±1.0 (16)	11.6±0.5 (13)	9.6±0.5 (14)	10.8±0.5 (11)
ATP contents during exposure to CN and 2-DG	ATP	30.9±0.5 (6)	3.4±0.1 (6)	4.0±0.2 (6)	3.4±0.4 (6)
ATP contents after 24 h of recovery from CN and 2-DG	ATP	33.9±1.0 (6)	32.6±0.5 (6)	29.3±1.5 (6)	24.1±2.0 (6)
^{42}K uptake during exposure to CN and 2-DG	1 min	24.9±1.3 (18)	8±0.4 (17)	10.9±0.4 (15)	18.1±1.2 (17)
	2 min	43.5±2.1 (17)	12.2±0.5 (16)	14.8±0.9 (18)	21.0±1.0 (17)
^{42}K uptake after 24 h of recovery from CN and 2-DG	1 min	28.3±1.0 (15)	13.4±0.9 (17)	8.6±0.7 (17)	6.6±0.5 (15)
	2 min	50.3±2.7 (15)	23.2±1.9 (17)	14.6±1.5 (18)	7.2±0.8 (10)

All values are given as means±SEM (n).

showed persistent and marked depression of K uptake after 24 h of recovery. In addition, K uptake was not linear over a 2-min period, consistent with an increase in the rate of K efflux, and a decrease in the time constant for K exchange.

We were interested that K uptake early after washout of metabolic inhibitors and resupply of glucose did not reflect the ultimate degree of dysfunction in K homeostasis which was present after 24 h of recovery. This is illustrated in Fig. 1, which shows cellular K uptake immediately after washout of CN and 2-DG, and 24 h later. The K uptake values after washout after 1 h of CN and 2-DG exposure actually exceeded control values. In all cases (1-, 3-, and 6-h exposure), CN and 2-DG treatment followed by a 15-s recovery resulted in K uptake values that were significantly greater than the corresponding values after 24 h of recovery ($P < 0.001$).

As mentioned, the nonlinearity of the K uptake after recovery from prolonged metabolic inhibition, as well as the severe and relatively selective K depletion observed, suggested an increase in the permeability of the sarcolemma to K, with an increased K efflux rate. To test this hypothesis directly, we measured the fractional efflux of ^{42}K from cells labeled to equilibrium. The results are shown in Fig. 2. In addition to a marked decrease in total cell content of K the fractional rate of ^{42}K efflux was significantly greater in CN and 2-DG–treated cells allowed to recover for 24 h.

Exposure to severe hypoxia combined with 2-DG and zero

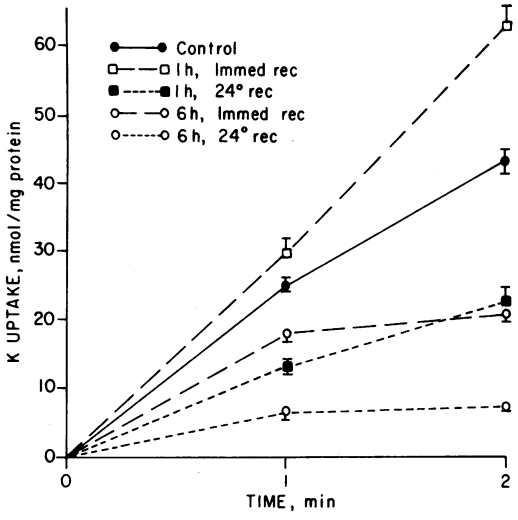


Figure 1. Changes in ^{42}K uptake early and late after recovery from exposure to CN and 2-DG. K uptake at 1 and 2 min is shown for control cells (*closed circles, solid line*); for cells exposed to CN and 2-DG for 1 h, then allowed recovery in normal media for 15 s (*open squares, dashed lines, "Immed rec"*), or for 24 h (*closed squares, dotted lines, "24° rec"*); for cells exposed to CN and 2-DG for 6 h, then allowed to recover for 15 s (*open circles, dashed lines, "6 h, immed rec"*), or for 24 hours (*open circles, dotted lines, "24° rec"*). Means±SEM are plotted, n = 15–18. After both 1 and 6 h CN and 2-DG exposure, K uptake early during recovery from ATP depletion was significantly greater ($P < 0.001$) than after 24 h of recovery.

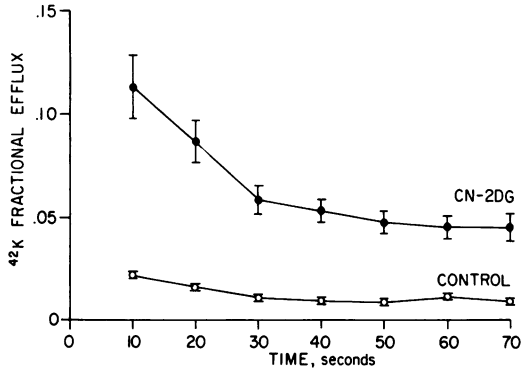


Figure 2. Effects of CN and 2-DG exposure on ^{42}K fractional efflux. Cultures were exposed to CN and 2-DG for 6 h, then allowed to recover in serum-free medium for 24 h. After labelling to equilibrium for 2 h in ^{42}K, fractional ^{42}K efflux was measured (see text). Relative to control cells not exposed to CN and 2-DG, efflux of ^{42}K was significantly increased in cells recovering from metabolic inhibition ($P < 0.001$). Points plotted are means±SEM, n = 10–12.

glucose produced more marked ATP depletion than CN–2-DG, as shown in Fig. 3. In addition, there was a much more marked failure of recovery of ATP contents after 24 h after hypoxia of 6 h duration. Failure of complete recovery of ATP contents in cells after recovery from severe hypoxia suggested that there was more irreversible injury during severe hypoxia, than during exposure to CN and 2-DG. This is confirmed in Fig. 4, which shows LDH release during exposure to CN and 2-DG, and exposure to severe hypoxia and 2-DG. There was significantly greater LDH release during severe hypoxia than during exposure to CN and 2-DG. Hypoxia (combined with 2-DG and zero glucose) caused changes in Na, K, and Ca contents, and K uptake that were similar to those observed after exposure to CN and 2-DG, although the degree of reversibility was somewhat less. For example, after 6 h of hypoxia and 2-DG, cell Na was 1,075±148, K 442±39, and Ca 40.9±2.8 (nmol/mg protein, means±SEM, n = 8–9). After 24 h of recovery, Na decreased to 199±29, K content was 585±42, and Ca content was 14.0±0.9. In view of the much more marked effects of hypoxia and 2-DG on ATP content and LDH release, relative to those produced by CN and 2-DG, an even more marked effect of 6 h of hypoxia on cation contents might have been expected. However, small changes in oxygen tension, difficult to monitor under these experimental conditions, can make a large difference in the degree of inhibition of oxidative phosphorylation (13, 14). This makes it somewhat difficult to achieve the same degree of ATP depletion in different experiments with the hypoxia methods utilized.

The effects of exposure to 10^{-3} M ouabain on ATP contents, Na, K, and Ca contents, and K uptake during ouabain exposure and after 24 h of recovery, are shown in Table II. Exposure to ouabain produced less severe ATP depletion than exposure to CN and 2-DG. However, recovery after 24 h was less complete even after only 1 and 3 h previous exposure to ouabain. Measurements of ion contents showed a marked gain in Na and loss of K during exposure to ouabain. Concomitantly there was an extraordinary gain in cell Ca content, with up to 40-fold increase in nanomoles per milligram of Ca noted after 3 h of exposure. During exposure to ouabain, there was also cell injury manifest as release of LDH (1,200 U/mg protein at 1 h, 1,700 U at 3 h, and 2,000 U at 6 h). After recovery in serum-free culture medium, there was partial recovery of Na and ATP contents, but K depletion persisted, even after only 1 h of previous exposure to ouabain. ^{42}K uptake returned to normal in cells recovered from 1 h of ouabain exposure, but remained depressed in 3- and 6-h ouabain exposure groups. Ca content remained extremely high in cells after recovery from ouabain exposure. Thus, the degree of Ca overload produced, and the extent to which it was reversible, differed depending on whether a comparable gain in Na and loss of K was produced by inhibition of the Na pump with ouabain, or by exposure to CN and 2-DG. Furthermore, the persistence of abnormal Na and K homeostasis in cells recovered from ouabain exposure is consistent with persistent abnormal sarcolemmal permeability to Na and K induced by Ca overload.

The decline in K content without a corresponding equal increase in Na content 24 h after recovery from metabolic inhibition with CN and 2-DG, or after hypoxia, suggested to us that a loss of cell volume might be occurring under these conditions. To test this hypothesis, we measured the diameter of spheroidal myocardial cells released from the coverslips by trypsinization, and we measured cell water content with [^{3}H]*O*-methyl-glucose (15). The results are shown in Fig. 5, and indicate that inhibition of ATP production in these cells followed by 24 h of recovery results in a decrease in cell size and loss of cell H_2O. The changes in light microscopic appearance of these cells, and cells exposed to ouabain and allowed to recover for 24 h, are shown in Fig. 6. Loss of confluence, with

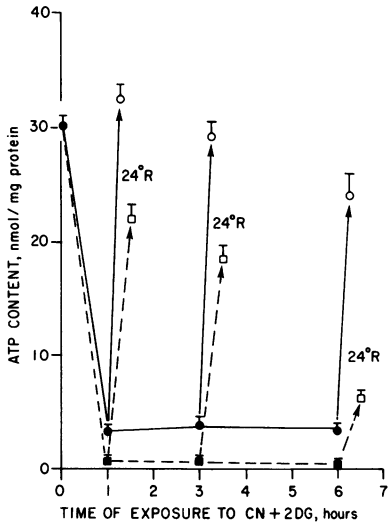


Figure 3. Effects of severe hypoxia, or CN, and 2-DG–zero glucose on ATP contents. Cells were exposed to CN and 2-DG or hypoxia (zero glucose) for indicated times. ATP contents (means±SEM, n = 4–7) are plotted during exposure to CN and 2-DG (*solid line, closed circles*) and after 24 h of recovery (*24° R, open circles*) in serum-free media. There was return to normal of ATP contents after recovery from 1 and 3 h of CN and 2-DG treatment. After recovery from 6 h of CN and 2-DG exposure, ATP contents were slightly below normal ($P < 0.05$). Hypoxia combined with 2-DG–zero glucose (*dashed line, closed squares*) produced more severe ATP depletion, and there was less recovery (*open squares*) particularly after 6-h exposure.

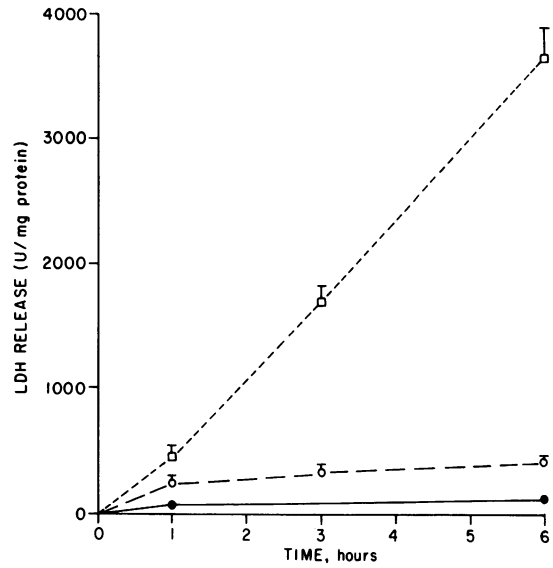


Figure 4. Effects of CN and 2-DG, or hypoxia, on LDH release. LDH release from control cells (*solid line, closed circles*) is given as means±SEM, n = 5–7. CN and 2-DG (*open circles, dashed line, n* = 8) caused an increased release of LDH relative to control, which reached 8% of total cellular LDH by 6 h. Severe hypoxia and 2-DG caused a much greater release of LDH (*open squares, small dashed line, n* = 9–11), with 73% of LDH released after 6 h.

Table II. Effects of Ouabain

		Control	1-h ouabain	3-h ouabain	6-h ouabain
		nmol/mg protein			
Cation contents during exposure to ouabain	Na	107±7 (26)	566±26 (21)	859±60 (20)	895±37 (20)
	K	982±43 (28)	543±27 (20)	223±24 (17)	158±14 (23)
	Ca	10.6±0.7 (12)	155±10 (22)	391±19 (20)	251±17 (23)
Cation contents after 24 h of recovery from ouabain	Na	104±4 (29)	405±13 (21)	388±15 (22)	550±42 (24)
	K	1130±41 (31)	137±8 (20)	146±20 (22)	161±18 (23)
	Ca	12.3±1.0 (11)	126±25 (20)	99±19 (23)	157±20 (24)
ATP contents during exposure to ouabain	ATP	33.6±1.8 (10)	16.6±0.7 (10)	12.9±1.3 (10)	15.3±1.1 (10)
ATP contents after 24 h of recovery from ouabain	ATP	34.5±1.0 (10)	20.2±1.5 (10)	13.9±0.9 (10)	13.4±1.4 (10)
^{42}K uptake during exposure to ouabain	1 min	32.9±2.5 (5)	23.4±1.0 (4)	21.2±1.5 (6)	15.7±1.0 (5)
	2 min	52.9±2.3 (6)	40.9±3.0 (5)	27.5±1.2 (6)	26.9±2.2 (5)
^{42}K uptake after 24 h of recovery from ouabain	1 min	28.3±2.6 (5)	36.2±1.3 (6)	22.4±0.5 (6)	15.8±0.6 (6)
	2 min	54.5±5.2 (6)	53.9±3.0 (6)	37.3±0.8 (6)	28.4±1.2 (6)

All values are given as means±SEM (n).

development of "gaps" in the culture layer presumably due to cell shrinkage, is apparent.

Given the changes in cell H_2O content and ion contents observed after 24 h of recovery from CN and 2-DG exposure (Table I and Fig. 5), the corresponding changes in ion concentrations can be estimated. These are shown in Table III. After recovery from 1 h of exposure to CN and 2-DG, concentrations of Na and K return to near normal values. Although K concentration was preserved after recovery from 3 h of exposure to CN and 2-DG, there was an increase in Na concentration; and after 6 h there was a marked decrease in K concentration, associated with a further increase in Na concentration.

That reasonably normal Na and K concentrations were preserved after 24 h of recovery from 1 h of exposure to CN and 2-DG is consistent with our observation that spontaneous contraction of individual cells, although less vigorous than in control cells, were present. For example, we measured the maximum amplitude of contraction in each of 15 high power fields in cultures after 24 h of recovery, and obtained the following results (μm, means±SEM, n = 15): control, 0.50±0.05; 1-h CN and 2-DG, 0.36±0.14; 3 h, 0; 6 h, 0. Thus, no spontaneous contractile activity was present after recovery for 24 h from 3–6 h of metabolic inhibition.

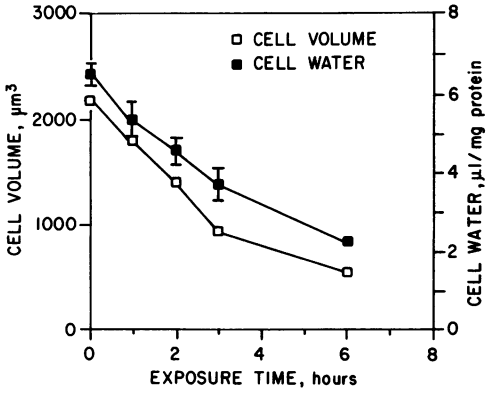
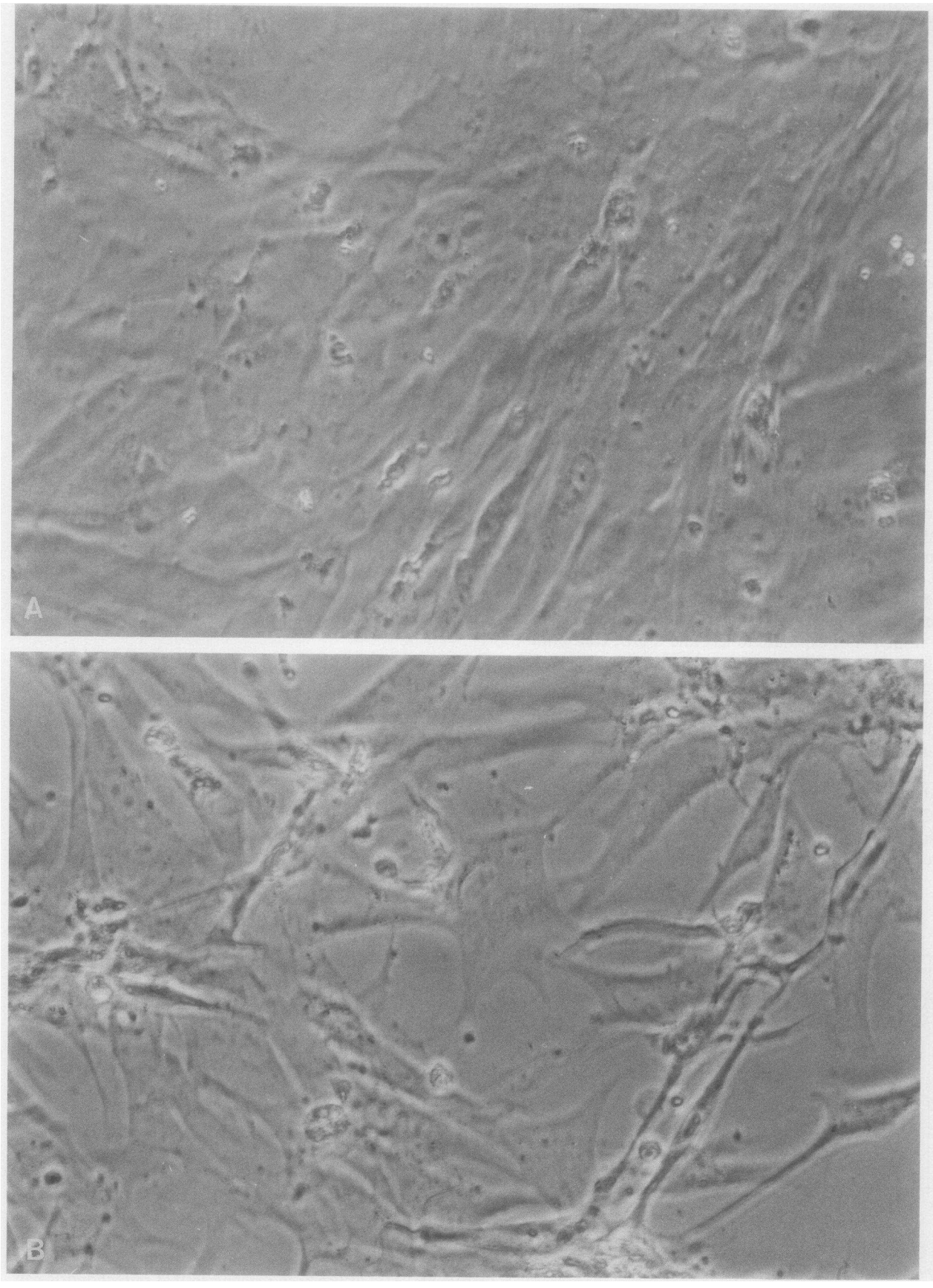


Figure 5. Cell volume (*open squares*, μm^3, n = 20) and cell H_2O (*closed squares*, μl/mg protein, n = 6) after recovery from metabolic inhibition with CN and 2-DG for the time indicated on the abscissa, and then allowed to recover for 24 h in serum-free medium (means±SEM).

Discussion

A variety of factors have been postulated as being important in the induction of irreversible cell injury during and after recovery from ischemia, including ATP depletion (16), an increase in cell $[Ca^{2+}]$ (17), alteration of membrane phospholipids (18), and damage induced by oxygen-free radicals (19, 20). However, the relative importance of these various mechanisms has not been ascertained (6). In isolated cultured myocardial cells, some toxic metabolites released during ATP depletion, including free radicals and lysophospholipids, would be expected to diffuse away from the cells into the much greater volume space of the culture media (6). In addition, components of the injury process attributable to endothelial cells and neutrophils are not present. However, severe myocyte ATP depletion, and/or Ca overload, can be produced in this system, and the effects studied.

Our results indicate that moderate Ca overload (increase in Ca content by approximately fivefold) during incubation in media containing CN and 2-DG for 3–6 h, associated with a decrease in ATP content to ~ $^1/_{10}$ normal, can be reversed, with return of Ca and ATP contents, and cell Na content to normal or near-normal levels after 24 h of recovery. This degree of Ca^{2+} overload and ATP depletion produces little cell injury, estimated by release of LDH. These results are consistent with the findings of Murphy et al. (21) who found that a 5–10-fold increase in cultured heart cell Ca content (45% in mitochondria), produced by inhibition of the Na pump, was reversible. Also, it has been recognized that mitochondria can tolerate considerable Ca^{2+} loading and still maintain their electron transport capacity (22). However, when more severe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B

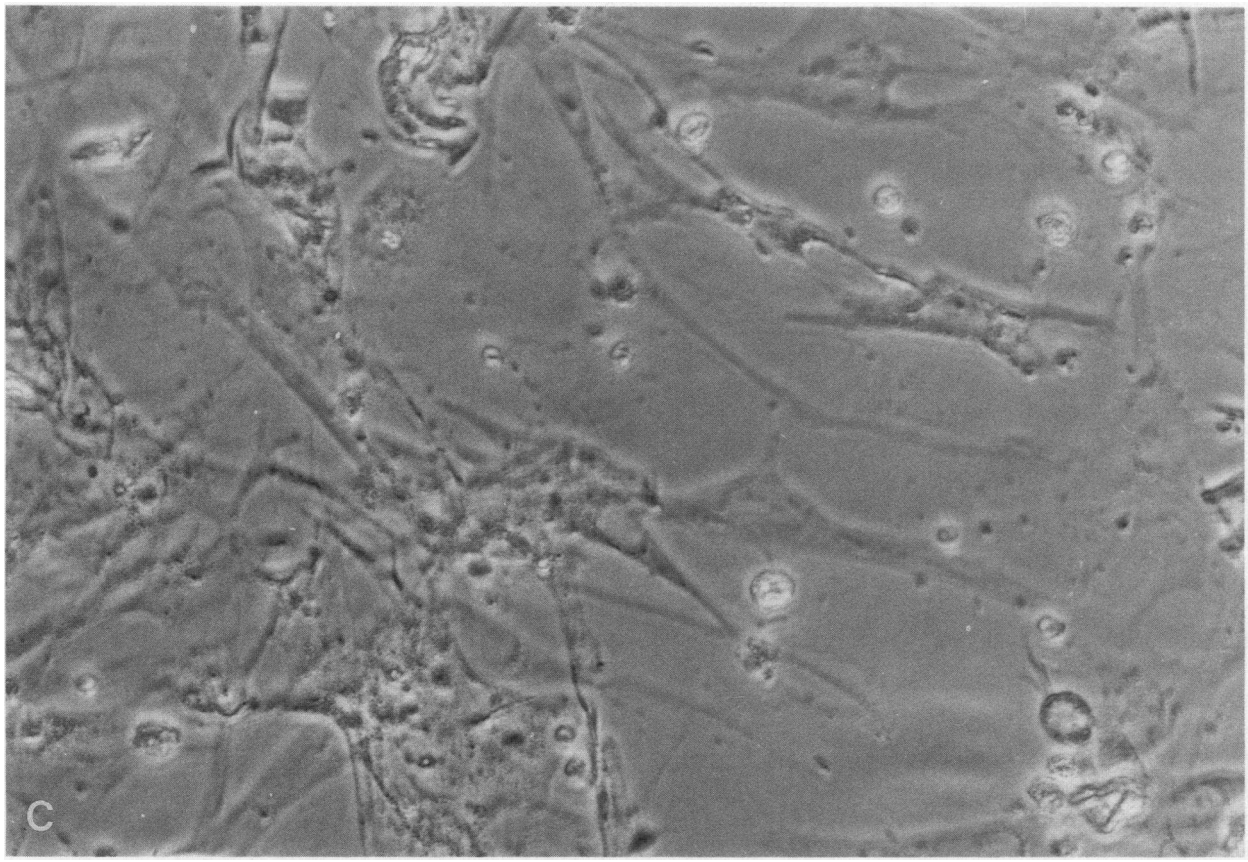

Figure 6. (*Opposite page*) Photomicrographs of cultured ventricular cell layers under control conditions (*A*); after exposure for 6 h to CN and 2-DG for 6 h, followed by 24 h of recovery in serum-free medium (*B*); (*above*) and after exposure to ouabain for 6 h, followed by 24-h recovery (*C*). The magnification was identical in all (total width of field = 60 μm). After recovery from metabolic inhibition, or Na pump inhibition, confluency was lost, and many myocytes appeared injured, with a granular cytoplasm, particularly in ouabain-treated cultures.

Ca overload was produced in our experiments by prolonged Na pump inhibition with ouabain, irreversible cell dysfunction and injury was produced and cell Na, Ca, and ATP levels were not restored, even after 24 h of recovery.

The reason why much more marked Ca overload occurred when Na loading was produced by ouabain, relative to that which occurred when Na loading was produced by CN and 2-DG, is not clear. However, a greater increase in Ca content relative to an increase in Na content was also found by Murphy et al. (23) in cultured heart cells treated with ouabain 10^{-4} M, as compared with those treated with rotenone and iodoacetic acid. This could be due to an inhibitory effect of ATP depletion on Ca influx via Na-Ca exchange (24, 25) resulting in less $[Ca^{2+}]_i$ being available for mitochondrial uptake. In addition, there may be greater preservation of the mitochondrial membrane potential, which contributes to Ca uptake (22), in the presence of ouabain. Ca uptake by mitochondria may also occur in association with phosphate, resulting in deposition of calcium phosphate in the mitochondrial matrix (21, 22). The extent to which this occurs during ouabain exposure, and during CN and 2-DG exposure, may be different.

Table III. Estimated Na and K Concentrations after Recovery from 1, 3, and 6 h of Metabolic Inhibition

	[Na]	[K]
	mM	
Control	15.9	146
1-h CN and 2-DG	16.9	145
3-h CN and 2-DG	25.7	155
6-h CN and 2-DG	38.3	78

The reversibility of Na and Ca overload after moderately severe ATP depletion produced by CN and 2-DG was much greater than the degree of recovery of K contents. K contents remained low after recovery from metabolic inhibition or hypoxia, and this K depletion was associated with a loss of cell volume and cell water. The mechanism for this K loss is not clear. It could be due to a decrease in K uptake, mediated by the Na-K ATPase Na pump, and/or an increase in K efflux. A decrease in the activity of Na-K ATPase after ischemia in intact ventricle has been reported by other investigators (26–28). Also consistent in our studies was a decrease in the K-uptake rate after recovery from metabolic inhibition. However, Na contents are restored toward normal (even though cell [Na] is high after recovery from 3 and 6 h of CN and 2-DG

exposure [Table III]), and this suggests that in our experiments, after recovery from ATP depletion, the Na pump remains able to function to some extent. The stores of ATP should certainly be adequate to supply the pump.

An increase in cell surface/volume due to cell shrinkage could produce an apparent increase in fractional ^{42}K efflux. However, the increase in K efflux that we have measured may be due to several other factors that could directly contribute to K depletion. Noma and colleagues (29, 30) have recently described an ATP-dependent K channel, which is activated when ATP levels within myocardial cells fall below 0.5 mM. This is a time-independent K channel which can markedly increase the conductance of the cardiac membrane to K (31). A similar ATP-sensitive K^+ channel has been described in frog skeletal muscle (32). Activation of this channel may account for the initial increase in extracellular K (33, 34) and shortening of the action potential (35) which are noted during ischemia. It is possible that persistent opening of this channel during ATP depletion, and failure of closure with resynthesis of ATP, contributes to the K loss noted after recovery from metabolic inhibition.

Recently, Piwnica-Worms et al. (36) have documented the presence of a K-Cl cotransport system in cultured chick embryo ventricular cells. This transport system can contribute to K influx or efflux. For example, it contributes to K uptake in spontaneously contracting, beating heart cells (37), and accounts for the component of K uptake that is not inhibited by ouabain in this preparation (38). K efflux via this transport system has been reported to be activated by metabolic inhibition in a number of different cell types (39), and may also be activated by increases in $[Ca^{2+}]_i$ (40). Activation of K-Cl cotransport in some cells is associated with a volume decrease, and net K loss (39, 40). Thus, activation of this K-Cl transport system during metabolic inhibition by ATP depletion and/or by an increase in $[Ca^{2+}]_i$, which occurs during metabolic inhibition in these cultured ventricular cells (41), could also contribute to the changes we have noted. It is not clear whether this K-Cl cotransport system is present in adult mammalian myocardial cells. Horres and co-workers (42) have proposed that it is, but that it may be obscured from detection by complex morphology in intact tissue. Consistent with this proposal is the finding by Silver and Houser (43) of significant non-ouabain-inhibitable ^{42}K influx in isolated feline adult ventricular myocytes.

These alterations in cation homeostasis may be of significance in recovery from ischemic injury. For example, Kimura et al. (44) have found that feline ventricular cells in the border zone of healed infarction have an increased intracellular Na^+ activity, and decreased K^+ activity compared with normal cells, and have suggested that these cells have persistent altered K and/or Na conductance. In addition, Dresdner et al. (45) have recently reported marked K depletion, with only slight increases in $[Na]_i$, in an ion-sensitive microelectrode study of severely ischemic canine subendocardial Purkinje cells 24 h after ligation of the left anterior descending coronary artery. They also noted a substantial reduction in combined Na^+ plus K^+ cytoplasmic activities from 121.4 mM in control cells, to 77.2 mM. These findings of a more marked K loss than Na gain and decrease in Na plus K concentrations, are similar to our results after recovery from 6 hours of metabolic inhibition, shown in Tables I and III.

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