THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 14, Issue of April 8, pp. 10485–10491, 1994
Printed in U.S.A.

Response of Human Fibroblasts to Hypertonic Stress

CELL SHRINKAGE IS COUNTERACTED BY AN ENHANCED ACTIVE TRANSPORT OF NEUTRAL AMINO ACIDS*

(Received for publication, August 9, 1993, and in revised form, November 29, 1993)

Valeria Dall'Asta, Patrizia A. Rossi, Ovidio Bussolati, and Gian C. Gazzola‡

From the Istituto di Patologia Generale, Università degli Studi di Parma, Via Gramsci 14, I-43100 Parma, Italy

Regulatory volume increase (RVI) has been studied in cultured human fibroblasts (CHF) incubated in a complete hypertonic growth medium (400 mosmol/kg). After the initial cell shrinkage induced by hypertonic treatment, cells recover their volume almost completely within 3 h. This RVI response is associated with a marked increase of the cell content of free amino acids. The cell content of potassium increases only slightly. Chromatographic analysis of the intracellular amino acid pool shows that the RVI-associated increase in cell amino acids is mainly a result of changes in the L-glutamine content. The intracellular accumulation of the analog 2-methylaminoisobutyric acid, a specific substrate of transport system A, is increased in CHF undergoing RVI. Hypertonic treatment causes an immediate and sustained cell hyperpolarization, as demonstrated by changes in the *trans*-membrane distribution ratio of L-arginine and in the fluorescence of the potential-sensitive dye bis-1,3,-diethylthiobarbiturate-trimethineoxonol. Because of cell hyperpolarization, at the end of RVI the *trans*-membrane gradient of the sodium electrochemical potential is higher than that of the control. The increase in the extracellular potassium concentration ($[K^+]_{out}$ = 40 mM) abolishes the hyperpolarization induced by hypertonic treatment and delays volume recovery. Cycloheximide suppresses RVI at a high but not at physiologic $[K^+]_{out}$. It is proposed that CHF counteract hypertonic shrinkage through an enhanced accumulation of substrates of transport system A sustained, initially, by an increase in the energy available for transport and, subsequently, also by the synthesis of new site A carriers.

Cells counteract volume perturbations by complex mechanisms, collectively indicated as regulatory volume decrease (RVD)[1] and regulatory volume increase (RVI) (for review see Refs. 1–3). In mammalian cells these mechanisms involve changes of the intracellular concentrations of either inorganic ions or organic osmolytes. In contrast to inorganic ions, the organic osmolytes are called "compatible" or "nonperturbing" (4) since changes in their intracellular concentration scarcely affect the function of cell macromolecules.

Results obtained in our laboratory (5), as well as by other authors (6, 7), have pointed to sodium-dependent amino acid transport as a volume regulatory mechanism in mammalian cells. In particular, we demonstrated that the membrane transport of amino acids and methylamines through system A exerts an active role in the RVI of cultured human fibroblasts (5). Several observations supported that conclusion. (i) In these cells RVI is observed only in the presence of substrates of system A (*e.g.* glutamine, proline, betaine, or sarcosine). (ii) A synthetic, nonmetabolizable substrate of transport system A, the analog 2-methylaminoisobutyric acid (MeAIB), plays a permissive role for RVI comparable to that of natural substrates. (iii) Cell shrinkage triggers a cycloheximide-sensitive increase of the transport capacity (V_{max}) of system A.

These results prompted us to characterize the role of amino acids in the volume regulatory phenomena that follow hypertonic treatment of cultured human fibroblasts. To conform to physiological conditions, hypertonic treatment was carried on in complete growth medium. In this report we (i) show that cell volume recovery results from an increase in the intracellular amino acid pool while the cell content of potassium does not markedly rise; (ii) identify the amino acids primarily involved in these changes as L-glutamine and other neutral amino acid substrates of system A or in metabolically related compounds; and (iii) demonstrate that in human fibroblasts RVI and the associated enhancement in the accumulation of neutral amino acids are caused by a rapidly ensuing hyperpolarization and by a slower, cycloheximide-inhibitable mechanism. It is suggested that both phenomena lead to an increase in the intracellular amino acid pool through an enhanced operation of the electrogenic, highly concentrative transport system A.

EXPERIMENTAL PROCEDURES

Cell Culture

Human foreskin fibroblasts were obtained from a 15-year-old donor. Cells were routinely grown in 10-cm diameter dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 4 mM L-glutamine, and 5.5 mM glucose. The conditions of culture were: pH 7.4, atmosphere 5% CO_2 in air, temperature 37 °C. The experiments were made on fibroblast subcultures resulting from 3×10^4 cells seeded onto 2-cm^2 wells of disposable 24-well trays (Nunc) and incubated for 3–4 days in 1 ml of growth medium. The culture medium was always renewed 24 h before the experiment. Cells were used at a density of 35 ± 5 μg of protein/cm^2. The cell number was estimated assuming 3.2×10^6 cells/mg of protein.[2]

Incubations

All of the incubations were performed in a medium reconstituted from single components. This formula was based on DMEM but for the

* This study was partially supported by Consiglio Nazionale delle Ricerche Target Projects "Applicazioni Cliniche della Ricerca Oncologica" and "Biotecnologie e Biostrumentazione," Roma. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Istituto di Patologia Generale, Università di Parma, Via Gramsci, 14, 43100 Parma, Italy. Tel.: 39-521-291-521; Fax: 39-521-980-388.

[1] The abbreviations used are: RVD, regulatory volume decrease; RVI, regulatory volume increase; MeAIB, 2-methylaminoisobutyric acid; DMEM, Dulbecco's modified Eagle's medium; I-DMEM, incubation DMEM); NPS, ninhydrin-positive substances; HPLC, high performance liquid chromatography; bisoxonol, bis-1,3,-diethylthiobarbiturate-trimethineoxonol; $\Delta\tilde{\mu}_{Na}$, *trans*-membrane gradient of sodium electrochemical potential; R_{Arg}, *trans*-membrane distribution ratio of arginine at the steady state; R_{Na}, *trans*-membrane distribution ratio of sodium at the steady state.

[2] V. Dall'Asta, unpublished results.

omission of arginine and lysine and the addition of vitamins from 100 × stock solutions of basal Eagle's medium vitamins. The osmolality of this medium (I-DMEM, incubation-DMEM) was 290–305 mosmol/kg. Hypertonic I-DMEM (395–405 mosmol/kg) was obtained by the appropriate addition of sucrose to I-DMEM. Before the experiments the cells were preincubated in isotonic I-DMEM for 2 h. During the preincubation and the various incubations all media were supplemented with 10% dialyzed fetal bovine serum. The osmolality of the solutions was checked routinely with a vapor pressure osmometer (Wescor 5500).

All of the experiments were performed using the cluster tray method for the measurement of solute fluxes in adherent cells (8) with appropriate modifications. Unless otherwise stated, incubations were terminated by rapidly rinsing the cell monolayers with ice-cold 0.1 M $MgCl_2$, and the cells were extracted as described below under "Determinations and Measurements." Extracted cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 M NaOH, and the protein content was determined directly in the well using a modified Lowry procedure as described previously (8).

Determinations and Measurements

Cell Volume—Cell volumes were estimated by measurements of cell water determined as the difference between urea and inulin distribution spaces. [^{14}C]Urea (0.5 μCi/ml, 0.5 mM final concentration) and [^{3}H]inulin (1 μCi/ml, 355 μCi/g) were added during the last 2 min of incubation. The incubations were terminated by rapidly removing the medium and carefully drying cell monolayers with blotting paper. Cell were covered with 0.5 ml of 0.1 M $MgCl_2$ which was then transferred into scintillation vials for the determination of radioactivity. Cell monolayers were finally dissolved with 0.5% sodium deoxycholate in 1 N NaOH for the determination of protein content. Cell volume, expressed in pl/cell, was calculated from cell water data expressed as μl/mg of protein. The volume of control cells was the mean of the values obtained at the times shown for each experiment in cells maintained in isotonic conditions. Each value of cell volume was calculated from six independent determinations. The variability of the cell volume of control cells did not exceed ± 10% of the mean value.

Intracellular Amino Acid Content and Concentration—Cell monolayers were extracted in a 5% solution of acetic acid in ethanol. The intracellular content of amino acids was determined as ninhydrin-positive substances (NPS) by the method of Law and Turner (9) and expressed as pmol/cell. The intracellular concentration of amino acids was calculated from the amino acid content and cell volumes determined in parallel cultures under the same experimental conditions.

The intracellular concentrations of the single amino acid species were determined by HPLC analysis with ion exchange chromatography (10). The chromatographic analysis was carried out with a Waters 440 model liquid chromatograph employing a column AA Interaction Lithium form. The eluent was a lithium citrate, lithium chloride buffer from pH 2.9 to 10.5. The analysis was carried out by detection of fluorescent products after postcolumn derivatization with a 0.3 M borate buffer, pH 10, containing orthophthalaldehyde.

Intracellular Ion Contents and Concentrations—Cell monolayers were fixed in place with ethanol (0.1 ml); the ethanol was allowed to dry, and the water-soluble pool was extracted in 2 ml of 10 mM CsCl. Potassium and sodium contents were determined with a Varian AA-275 atomic absorption spectrophotometer, using KCl and NaCl as standards, and expressed as pmol or as fmol/cell. Values of the intracellular concentrations of ions were calculated from the ion contents and cell volumes determined in parallel cultures under the same experimental conditions.

Evaluation of Concentrative Ability of Transport System A—The *trans*-membrane gradient of MeAIB reached in the presence of extracellular MeAIB was employed as an indicator of the concentrative ability of transport system A. The rationale for this approach derives from the consideration that MeAIB is a nonmetabolizable compound whose entry into the cell is restricted to system A. Its intracellular concentration, therefore, is the net result of system A operations and, possibly, of fluxes through diffusive routes. The intracellular MeAIB concentration was evaluated by determining the cell content of the nonmetabolizable analog after the addition of [^{14}C]MeAIB (0.1 mM, 0.5 μCi/ml) to the incubation medium. [^{14}C]MeAIB was allowed to equilibrate during the preincubation period (2 h) in isotonic I-DMEM. The extracellular concentration and specific activity of the probe were maintained constant thereafter throughout the subsequent treatments. At the end of the experiment, after three washes in 0.1 M $MgCl_2$, ethanol was added to each well and allowed to dry. Dried monolayers were extracted with 0.5 ml of water that was subsequently added to 4 ml of scintillation fluid and counted for radioactivity. The intracellular concentration was then calculated using appropriate values of cell volume, determined in parallel cultures.

Membrane Potential—Changes in membrane potential were estimated from the *trans*-membrane distribution of L-arginine, following the method described previously (11, 12). L-[^{3}H]Arginine (0.02 mM, 1 μCi/ml) was allowed to equilibrate during the preincubation period (2 h) in the modified arginine-free isotonic I-DMEM. The extracellular concentration and specific activity of labeled L-arginine were maintained constant thereafter throughout the incubation in hypertonic medium. After three washes with 0.1 M $MgCl_2$, ethanol was added to each well and allowed to dry; 2 ml of water was then added to each well. A fraction (0.5 ml) of the extracts was added to 4 ml of scintillation fluid and counted for radioactivity.

Qualitative changes in the membrane potential were detected as changes in the fluorescence intensity of the dye bis-1,3,-diethylthiobarbiturate-trimethineoxonol (bisoxonol), monitored with a LS-50 Perkin-Elmer spectrofluorometer as described previously (13). For these measurements the cells, grown on a coverslip, were put into a cuvette containing isotonic I-DMEM at 37 °C with constant stirring. Hypertonic conditions (400 mosmol/kg) were obtained by the addition of a sucrose solution containing bisoxonol. During these experiments serum was omitted, the medium was buffered with 20 mM HEPES/NaOH, and the extracellular concentration of bicarbonate was lowered from 44 to 24 mM. Dye was added from an ethanol stock solution to a final concentration of 200 nM. Fluorescence was recorded with excitation and emission at 530 nm (5-nm slit width) and 570 nm (7.5-nm slit width), respectively. The addition of 1 μM gramicidin D was employed to achieve cell depolarization.

Calculations

The membrane potential was calculated from the Nernst equation applied to the steady-state *trans*-membrane distribution ratio of L-arginine, as described previously (11, 12).

The *trans*-membrane gradient of the Na^+ electrochemical potential $\Delta\tilde{\mu}_{Na}$, expressed as kJ/mol and defined as

$$\Delta\tilde{\mu}_{Na} = \Delta\mu_{Na} + F \cdot \Delta\psi \qquad \text{(Eq. 1)}$$

was calculated, as described (14), from the steady-state *trans*-membrane distribution of L-arginine and the intracellular Na^+ concentrations, as follows.

$$\Delta\tilde{\mu}_{Na} = RT \cdot \ln(R_{Na} \cdot R_{Arg}) \qquad \text{(Eq. 2)}$$

where

$$R_{Na} = \frac{[Na]_{out}}{[Na]_{in}} \text{ and } R_{Arg} = \frac{[Arg]_{in}}{[Arg]_{out}}$$

Materials

Fetal bovine serum and culture medium (DMEM) were purchased from Life Technologies, Inc. [^{14}C]urea (4.2 Ci/mol), [*methoxy*-^{3}H]inulin (355 mCi/g), 2-[1-^{14}C]methylaminoisobutyric acid, and L-[2,3-^{3}H]arginine hydrochloride (55 Ci/mmol) were obtained from Du Pont de Nemours, Bad-Homburg, Germany. Bisoxonol was purchased from Molecular Probes, Eugene, OR. Ethanol was obtained from Carlo Erba, Milan Italy; MeAIB was from Aldrich-Europe, Milan, Italy. Sigma was the source of all other chemicals.

RESULTS

Cell Content of Potassium and NPS during RVI of Cultured Human Fibroblasts—The results presented in Fig. 1, *upper panel*, demonstrate that cultured human fibroblasts, incubated in complete growth medium, effectively regulate cell volume after hypertonic stress. This result was expected since hypertonic treatment was carried on in the presence of extracellular amino acids which are known to be necessary for RVI in these cells (5). Although the absolute values of cell volume exhibited a certain degree of variability from experiment to experiment, the pattern of volume changes was substantially reproducible. Upon the substitution of hypertonic medium (400 mosmol/kg) for isotonic medium (300 mosmol/kg) a rapid cell shrinkage was observed. No consistent volume recovery was detectable up to 20 min of incubation in hypertonic conditions. After 60 min of incubation, a RVI response became progressively evident which

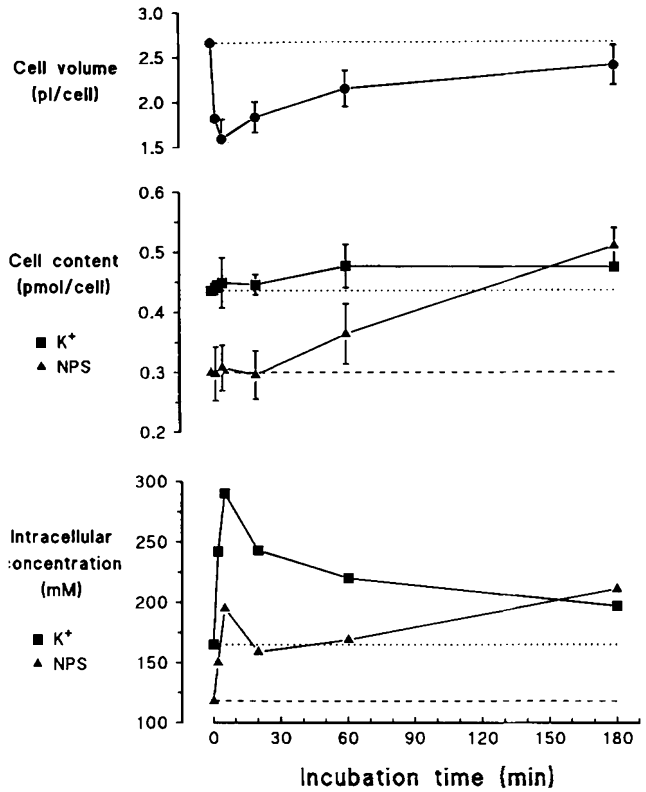

FIG. 1. **Changes in cell volume and intracellular osmolytes during RVI of cultured human fibroblasts.** Cell monolayers were preincubated for 2 h in isotonic modified I-DMEM. At the end of this period the medium was replaced with either isotonic or hypertonic I-DMEM, and the cell volume was determined after the indicated times of incubation (*upper panel*). *Points* are the means of six independent determinations within one representative experiment with the S.D. indicated. The *dotted line* represents the cell volume of control cells. Another series of cell monolayers was treated in parallel as described for the *upper panel*. The cell contents of potassium (■) and NPS (▲) were determined after the indicated periods of incubation (*middle panel*). *Points* are the means of three independent determinations with the S.D. indicated when greater than size of the *point*. The experiment was repeated three times with similar results. *Dotted* and *dashed lines* represent, respectively, the cell contents of potassium and NPS of control cells. The *lower panel* shows the intracellular concentrations of potassium and NPS calculated from data of cell content and volume reported in the *upper* and *middle panels*. *Dotted* and *dashed lines* represent, respectively, the intracellular concentrations of potassium and NPS of control cells. For a description of the analytical procedures employed, see "Experimental Procedures."

restored the cell volume at a value close to the initial within 3 h.

In the first 20 min after exposure to hypertonic medium, neither cell potassium nor cell NPS changed significantly (Fig. 1, *middle panel*). A modest, although reproducible, increase in cell potassium was detectable after 60 min of incubation in hypertonic medium. A progressive increase of cell NPS was observed, however, after 20 min of incubation in hypertonic I-DMEM. NPS content was still increasing after 3 h of hypertonic treatment when it was more than 60% higher than control value.

The *lower panel* of Fig. 1 shows the intracellular concentrations of potassium and NPS which resulted from the observed values of cell volume and cell content of potassium and NPS reported in the *upper* and *middle panels* of the same figure. After hypertonic stress the intracellular concentrations of both potassium and NPS rose sharply to values almost 2-fold higher than those of control cells. The intracellular potassium concentration then fell steadily and attained values only slightly higher than control after 3 h of incubation in hypertonic conditions. In contrast, the NPS concentration was maintained at values higher than control throughout the period of volume recovery.

TABLE I

Intracellular concentrations of free amino acids in cultured human fibroblasts incubated in isotonic and hypertonic conditions

Cultured human fibroblasts were incubated for 4 h in the conditions indicated. At the end of the incubation, cells were extracted, and HPLC of the cell extracts was performed as described under "Experimental Procedures." Data obtained from the HPLC analysis were corrected for the intracellular volume determined in parallel cultures. The results of a representative experiment are expressed as intracellular concentrations of amino acids. The experiment was repeated twice, and comparable results were obtained.

Amino acid	Intracellular concentration	
	Isotonic	Hypertonic
	mM	
Asp	6.6	8.6
Thr	7.2	12.1
Ser	6.4	11.7
Glu	22.0	35.5
Gln	53.0	92.0
Gly	8.9	11.0
Ala	1.3	1.7
Val	1.8	3.1
Met	1.1	2.7
Ile	2.0	3.3
Leu	2.4	4.3
Tyr	0.7	1.4
Phe	1.9	3.2
Total	115.3	190.6

Composition of the Intracellular Amino Acid Pool in Fibroblasts Incubated in Hypertonic Medium—The results described above demonstrate that the intracellular amino acid pool underwent marked quantitative changes during RVI in cultured human fibroblasts. To identify which components of the pool were involved in these changes, a chromatographic analysis of intracellular amino acids was performed on cells maintained in isotonic medium and in cells incubated for 4 h in hypertonic I-DMEM. The results of this analysis (Table I) indicate that the intracellular concentrations of most of the amino acids increased in hypertonic conditions. The sum of intracellular concentrations of the individual amino acids was in good agreement with the NPS concentration in both isotonic and hypertonic conditions. The graphical representation shown in Fig. 2 depicts the difference between the intracellular concentrations of neutral and anionic amino acids measured in cells incubated in isotonic and hypertonic I-DMEM. L-Glutamine represented the major single component of the amino acid pool in isotonic conditions; moreover, the concentration of L-glutamine rose by over 70% in cells incubated in hypertonic conditions. Therefore, on a molar basis, L-glutamine was the single species whose intracellular concentration underwent the greatest change during RVI. The second largest change in intracellular concentration was exhibited by L-glutamate, an amino acid that cultured human fibroblasts derive mostly from L-glutamine (15).

Involvement of Changes in Amino Acid Transport in RVI—In cultured human fibroblasts the transport of L-glutamine occurs mainly through the Na^+-dependent systems A and ASC (16). Recent studies performed on these cells have demonstrated that system A is a secondary active transport mechanism (14), whereas system ASC behaves as an electroneutral exchange system (13). Therefore, it is conceivable that the activity of system A is a major factor in determining the intracellular concentration of L-glutamine. Indeed, results obtained by other authors have recently shown that the intracellular glutamine concentration depends upon the active influx of the exogenous amino acid (17). If this is the case, changes in system A trans-

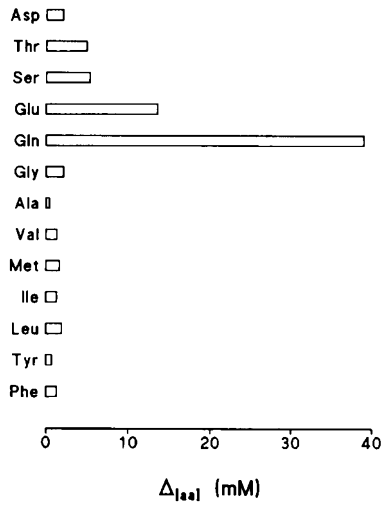

FIG. 2. **Increases in the intracellular concentrations of amino acids (*aa*) induced by RVI of cultured human fibroblasts.** The *bars* are the graphical representation of the differences detected between cells incubated in isotonic and hypertonic conditions calculated from the data reported in Table I.

port activity could be involved in the RVI-associated increase of intracellular glutamine.

In the experiment shown in Fig. 3, the cell content and the *trans*-membrane gradient of the amino acid analog MeAIB were determined as indicators of the concentrative activity of system A. Cells were loaded with labeled MeAIB during the preincubation period in isotonic I-DMEM and then transferred to hypertonic medium containing MeAIB at the same concentration and specific activity. After 3 h of incubation in hypertonic medium the cell content of the analog increased significantly; as a result, the *trans*-membrane gradient of MeAIB was 30% higher after a 3-h incubation in hypertonic than in control medium. Since MeAIB is a nonmetabolizable analog, the intracellular concentration of the amino acid only depends upon its *trans*-membrane fluxes. This conclusion is indeed in agreement with the higher influx of MeAIB determined in cells undergoing RVI compared with cells maintained in isotonic conditions (results not shown). Thus, the stimulation of MeAIB accumulation by hypertonic conditions suggests that cell shrinkage leads to an increase of the concentrative ability of system A.

Changes in Membrane and Sodium Potentials during Volume Regulatory Phenomena—Energization of the transport process mediated by system A depends upon $\Delta\tilde{\mu}_{Na}$ (14). RVI-associated changes in this parameter could therefore affect the operation of system A; such changes may stem from alterations of either the *trans*-membrane gradient of the sodium chemical potential or the membrane electrical potential.

The cell sodium content exhibited a significant although modest decrease upon incubation of cultured human fibroblasts in hypertonic medium (Fig. 4, *middle panel*); the decrease was maximal between 20 and 60 min, whereas after 3 h of incubation in hypertonic I-DMEM the cell sodium content approached control values again. However, because of cell shrinkage, the intracellular sodium concentration increased sharply upon hypertonic stress (Fig. 4, *lower panel*). At longer times of incubation in hypertonic conditions the intracellular sodium concentration was restored slowly to the control values.

The occurrence of changes of membrane potential during RVI was monitored in two independent, noninvasive ways. The first method employed the cationic amino acid L-arginine as a potential-sensitive probe (11, 12). The data obtained (Fig. 5) demonstrate that the cell content of L-arginine increased progressively in cells incubated in hypertonic medium. Maximal values

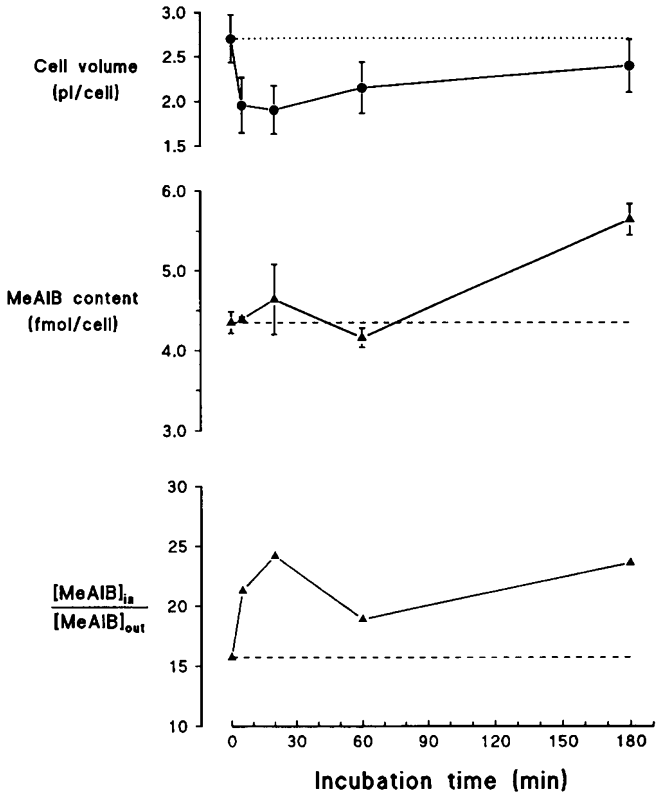

FIG. 3. **Changes in cell volume and intracellular MeAIB during RVI of cultured human fibroblasts.** Cell monolayers were preincubated for 2 h in isotonic modified I-DMEM containing 0.1 mM MeAIB. At the end of this period the medium was replaced with either isotonic or hypertonic I-DMEM (containing the same concentration of MeAIB), and the cell volume was determined after the indicated times of incubation (*upper panel*). *Points* are the means of six independent determinations within one representative experiment with the S.D. indicated. The *dotted line* represents the volume of control cells. Another series of cell monolayers was treated in parallel as described for the *upper panel*, with the difference that the various incubations were carried on in the presence of [^{14}C]MeAIB (0.1 mM, 0.5 μCi/ml). The cell content of MeAIB was determined after the indicated periods of incubation (*middle panel*). *Points* are the means of three independent determinations with the S.D. indicated when greater than size of the *point*. The experiment, repeated three times, yielded similar results. The *dashed line* represents the cell content of MeAIB of control cells. The *lower panel* shows the *trans*-membrane ratio of MeAIB calculated from the data of cell content and volume reported in the *upper* and *middle panels*. The *dashed line* represents the *trans*-membrane ratio of MeAIB of control cells. For a description of the analytical procedures employed, see "Experimental Procedures."

of cell-associated L-arginine were attained after 60 min of incubation in hypertonic conditions and maintained thereafter. The intracellular concentration of the cationic amino acid increased sharply as a consequence of both the increase in the cell content of L-arginine and cell shrinkage. The concentration of cell arginine was maintained at levels higher than those of control cells, thus indicating the occurrence of a prolonged cell hyperpolarization during RVI.

The information obtained from the cell levels of L-arginine has been confirmed by fluorometric assessments of membrane potential, employing the potential-sensitive dye bisoxonol (Fig. 6). The addition of sucrose to isotonic I-DMEM caused a marked decrease of fluorescence, pointing to a cell hyperpolarization (13). Note that the subsequent addition of gramicidin (expected to depolarize the cells) resulted in a net increase of fluorescence. The sucrose-induced change in fluorescence appeared to be immediate. Results obtained with the fluorescent probe and the changes described above for arginine levels indicate that the hyperpolarization induced by the hypertonic

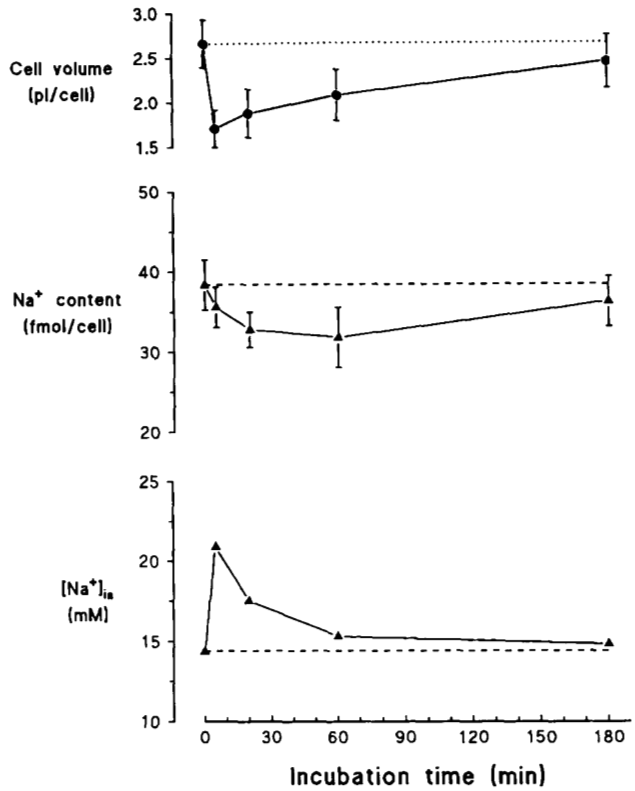

FIG. 4. **Changes in cell volume and cell sodium during RVI of cultured human fibroblasts.** Cell monolayers were preincubated for 2 h in isotonic modified I-DMEM. At the end of this period the medium was replaced with either isotonic or hypertonic I-DMEM, and the cell volume was determined after the indicated times of incubation (*upper panel*). *Points* are the means of six independent determinations within one representative experiment with the S.D. indicated. The *dotted line* represents the cell volume of control cells. Another series of cell monolayers was treated in parallel as described for the *upper panel*. The cell content of sodium was determined after the indicated periods of incubation (*middle panel*). *Points* are the means of three independent determinations with the S.D. indicated when greater than size of the *point*. The experiment, repeated three times, yielded similar results. The *dashed line* represents the cell sodium content of control cells. The *lower panel* shows the intracellular sodium concentration calculated from the data of cell content and volume reported in the *upper* and *middle panels*. The *dashed line* represents the intracellular sodium concentration of control cells. For a description of the analytical procedures employed, see "Experimental Procedures."

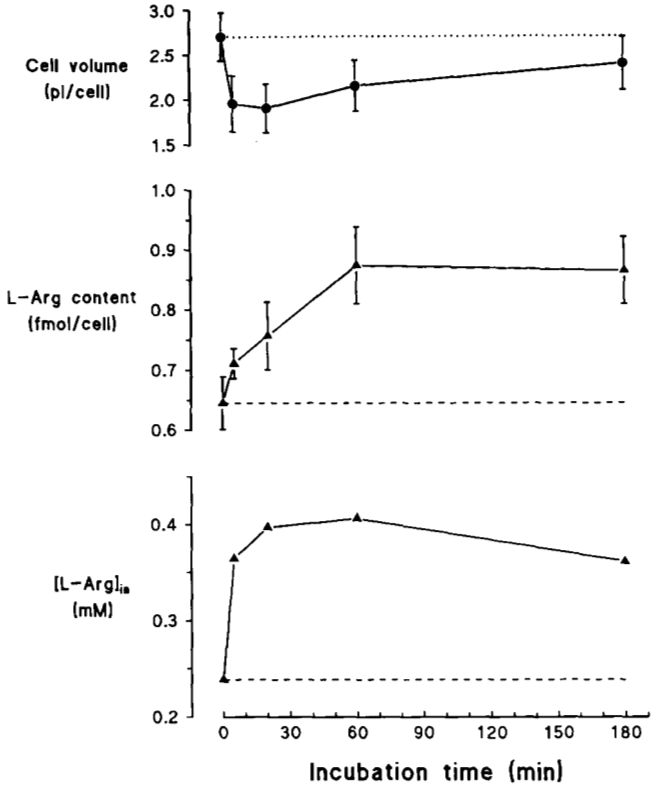

FIG. 5. **Changes in cell volume and intracellular L-arginine during RVI of cultured human fibroblasts.** Cell monolayers were preincubated for 2 h in isotonic modified I-DMEM containing 0.02 mM L-arginine. At the end of this period the medium was replaced either with isotonic or with hypertonic I-DMEM (containing the same concentration of L-arginine), and the cell volume was determined after the indicated times of incubation (*upper panel*). *Points* are the means of six independent determinations within one representative experiment with the S.D. indicated. The *dotted line* represents the cell volume of control cells. Another series of cell monolayers was treated in parallel as described for the *upper panel*, with the difference that the various incubations were carried on in the presence of L-[^{3}H]arginine (0.02 mM, 1 μCi/ml). The cell content of L-arginine was determined after the indicated periods of incubation (*middle panel*). *Points* are the means of three independent determinations with the S.D. indicated when greater than size of the *point*. The experiment, repeated three times, yielded similar results. The *dashed line* represents the control cell L-arginine content. The *lower panel* shows the intracellular concentration of L-arginine calculated from the data of cell content and volume reported in the *upper* and *middle panels*. The *dashed line* represents the intracellular L-arginine concentration of control cells. For a description of the analytical procedures employed, see "Experimental Procedures."

treatment ensued rapidly (at least in the time scale allowed by the "slow" behavior of the probes employed).

After 4 h of incubation in hypertonic conditions, cells have almost regained their original volume (see Figs. 1, 3–5; see also Table III). In this quasi-steady-state condition it was possible to calculate directly the value of membrane potential from the arginine *trans*-membrane distribution ratio (11, 12). From values of membrane potential and of R_{Na} obtained in the same conditions, the values of the *trans*-membrane gradient of sodium electrochemical potential have then been calculated (Table II). R_{Na} was comparable in hypertonic-treated and control cells (see Fig. 4). However, at the end of RVI, because of the hyperpolarization, the cells incubated in hypertonic medium exhibited a higher $\Delta\tilde{\mu}_{Na}$ than cells maintained in isotonic conditions.

These results demonstrate that the energy available for the transport process mediated by system A was increased in hypertonic conditions.

Effect of the Extracellular Concentration of Potassium on the Hyperpolarization Induced by Hypertonic Treatment—To identify experimental conditions that influence RVI-associated hyperpolarization, hypertonic treatment was performed at an increased extracellular concentration of potassium. Cells incubated in hypertonic I-DMEM at a $[K^+]_{out}$ of 40 mM exhibited a shrinkage comparable to that seen at the physiologic $[K^+]_{out}$ of 5 mM (not shown). Nevertheless, membrane potential, determined from the steady-state arginine distribution ratio after a 4-h incubation, was −64.7 mV in hypertonic I-DMEM at a $[K^+]_{out}$ of 40 mM, in comparison with −76.9 mV in cells incubated in hypertonic I-DMEM at the physiologic $[K^+]_{out}$ of 5 mM and −67.5 mV in cells maintained in isotonic I-DMEM. Spectrofluorometric data also demonstrated that no change in bisoxonol fluorescence was detected at an extracellular osmolality of 400 mosmol/kg provided that the hypertonic treatment was performed in the presence of increased $[K^+]_{out}$ (not shown). These results indicate that cell hyperpolarization, associated with hypertonic stress, was hindered by the increase in $[K^+]_{out}$.

Role of Hyperpolarization and Protein Synthesis in Volume Regulatory Phenomena and RVI-associated Accumulation of Amino Acids—A set of experiments was performed to evaluate

the role of RVI-associated hyperpolarization and of protein synthesis in the volume recovery processes. In these experiments the activity of system A was monitored following the redistribution of preaccumulated MeAIB.

If hyperpolarization was prevented by an increase in $[K^+]_{out}$, a substantial delay in RVI-associated responses was observed (Table III). Fibroblasts incubated in hypertonic I-DMEM at 5 mM $[K^+]_{out}$ had already recovered their volume and increased cell content of MeAIB after 2 h of incubation. On the contrary, neither volume recovery nor MeAIB gain was observed after 2 h of hypertonic treatment at 40 mM $[K^+]_{out}$. In this condition, the cell volume was comparable to that determined 30 min after hypertonic stress (1.987 ± 0.212 pl/cell), and the cell MeAIB was actually decreased in comparison with control. However, after 4 h of incubation in hypertonic I-DMEM at high $[K^+]_{out}$, the cell volume had been restored to values not significantly different from control. This delayed restoration of cell volume was accompanied by a recovery of cell MeAIB.

At 5 mM $[K^+]_{out}$ the inhibition of protein synthesis neither altered volume recovery nor suppressed the increase in cell MeAIB observed after 2 h of hypertonic treatment. At this time cell volume was not altered significantly by cycloheximide also at high $[K^+]_{out}$. After 4 h of hypertonic treatment, cycloheximide prevented the delayed volume recovery observed at high $[K^+]_{out}$ and lowered cell MeAIB at both $[K^+]_{out}$ values.

DISCUSSION

This paper concerns the role of amino acids in the RVI of cultured human fibroblasts. It should be stressed that in this contribution, at variance with the majority of earlier volume studies (for review see Refs. 1–3), volume perturbations and recovery phenomena occur in cells maintained in complete culture medium. Moreover, relevant cell parameters, such as volume itself or membrane potential, have been assessed in the same conditions employing noninvasive methods which do not perturb membrane functions significantly.

The results described here confirm earlier findings (5) that cultured human fibroblasts are able to exert a RVI, even without a previous incubation in hypotonic medium (the so called "RVI-after-RVD protocol," see Refs. 1–3). The RVI competence of cultured human fibroblasts is strictly dependent on the presence of methylamines or selected amino acids (such as L-glutamine or MeAIB) in the extracellular medium (5). The present study adds data about the behavior of the cell content of ions during RVI; these data indicate that only a very limited increase in the cell content of potassium is detectable during the development of RVI. This increase in potassium content, however, is relatively smaller than the parallel regulatory increase of cell volume, and, as a consequence, the intracellular potassium concentration falls progressively during RVI. Changes in the potassium content, therefore, do not account for the volume recovery in shrunken cultured human fibroblasts. In contrast, the cell content of NPS increases by almost 70% during RVI. Moreover, the intracellular concentration of NPS is maintained at values much higher than control and actually increases throughout RVI. These results demonstrate that NPS constitute the prominent osmolytes involved in the RVI of cultured human fibroblasts.

The content of NPS of shrunken fibroblasts could be increased through activation of either membrane transport or metabolic processes. The results shown in Fig. 3 indicate that when cells undergo RVI in the presence of the nonmetabolizable analog MeAIB, the cell content of this amino acid increases. This observation can be explained only by an increase in the net uptake of the amino acid. Since MeAIB is a specific substrate of transport system A, this result indicates that system A is involved in RVI of cultured human fibroblasts.

System A is a secondary active transport mechanism, and hence the *trans*-membrane gradients of substrates established by this agency depend upon $\Delta\tilde{\mu}_{Na}$ (14). Values of $\Delta\tilde{\mu}_{Na}$ are higher at the end of RVI than they are in control cells maintained in isotonic medium (Table II). This increase in $\Delta\tilde{\mu}_{Na}$ is a result solely of the hyperpolarization that occurs immediately after hypertonic stress and is maintained throughout RVI. The mechanism of this hyperpolarization is not obvious, since electroneutral transport pathways have been so far involved in the response of other biological models to hypertonic stress (for review see Refs. 1–3). The change in membrane potential increases the energy available for the transport process mediated by system A and appears to be important for the volume recovery since, when hyperpolarization is inhibited by an increase in $[K^+]_{out}$, the increase in cell amino acids and the volume recovery itself are significantly delayed. After 4 h of incubation in hy-

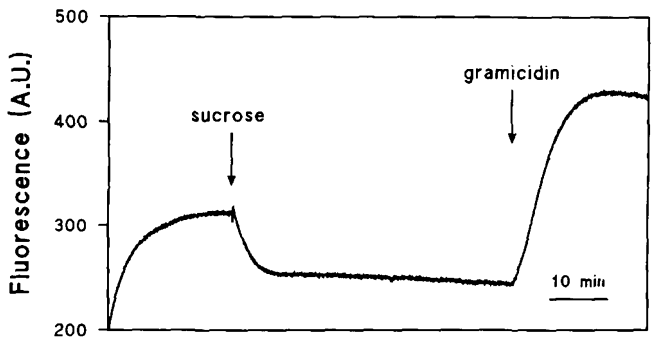

FIG. 6. **Changes in bisoxonol fluorescence upon incubation of cultured human fibroblasts in hypertonic I-DMEM.** Cultured human fibroblasts, grown on a coverslip, were incubated in a cuvette containing 2 ml of isotonic modified I-DMEM (serum absent) in the presence of the fluorescent dye bisoxonol (200 nM). When a stable base line was reached, the incubation medium was rendered hypertonic (*arrow*) by the addition of a bisoxonol-containing sucrose solution. The addition of gramicidin was performed from a 1,000-fold stock solution in ethanol. The experiment was repeated four times with comparable results.

TABLE II

Values of R_{Na}, R_{Arg}, $\Delta\psi$ and $\Delta\tilde{\mu}_{Na}$ in cultured human fibroblasts incubated in isotonic and hypertonic conditions

Three sets of cultured human fibroblasts were incubated in parallel for 4 h in the indicated conditions. Cell volumes and the cell contents of sodium and L-arginine were determined at the end of the incubation and employed to calculate *trans*-membrane distribution ratios (see "Experimental Procedures"). Data are the means of six independent determinations with the S.D. indicated. The level of significance was assessed with a two-tailed *t* test for unpaired data. The values of membrane potential and of *trans*-membrane gradient of sodium electrochemical potential were calculated as described (see "Calculations").

Condition	$[Na]_{in}$	R_{Na}	$[Arg]_{in}$	R_{Arg}	$\Delta\psi$	$\Delta\tilde{\mu}_{Na}$
	mM		*mM*		*mV*	*kJ/mol*
Isotonic I-DMEM	14.09 ± 1.1	10.81	0.226 ± 0.020	11.3	−64.2	12.37
Hypertonic I-DMEM	14.51 ± 1.2	11.09	0.348 ± 0.032[a]	17.4	−75.7	13.55

[a] $p < 0.01$

TABLE III

Cell volume and MeAIB redistribution in hypertonically stressed cultured human fibroblasts: effects of increase in $[K^+]_{out}$ and of cycloheximide

Cultured human fibroblasts were incubated for 4 h in a modified I-DMEM in which sodium chloride was lowered to 120 mM, and choline chloride was present at a concentration of 35 mM ($[K^+]_{out}$ = 5 mM). Cell volume, cell content, and intracellular concentration of MeAIB were determined (see "Experimental Procedures") at the end of this period (control) or after a further incubation in hypertonic conditions (modified I-DMEM supplemented with 100 mosmol/kg of sucrose) at normal or high $[K^+]_{out}$ (choline chloride replaced by KCl to yield a final $[K^+]_{out}$ of 40 mM) in the presence or in the absence of cycloheximide (18 μM). Data are the means of four independent determinations with the S.D. indicated in a single experiment. The level of significance was assessed with a two-tailed *t* test for unpaired data. The experiment was repeated twice with comparable results.

Hypertonic treatment	$[K^+]_{out}$	Cell volume	MeAIB content	$[MeAIB]_{in}$
	mM	*pl/cell*	*fmol/cell*	*mM*
None (control)	5	2.51 ± 0.14	5.10 ± 0.30	2.03
Cycloheximide absent				
2 h	5	2.43 ± 0.12	5.71 ± 0.13[a]	2.48
2 h	40	2.00 ± 0.15[b]	3.97 ± 0.19[b]	1.90
4 h	5	2.44 ± 0.18	7.24 ± 0.10[b,c]	2.96
4 h	40	2.30 ± 0.20	4.91 ± 0.12[c]	2.13
Cycloheximide present				
2 h	5	2.41 ± 0.30	5.73 ± 0.46[a]	2.38
2 h	40	2.18 ± 0.15[a]	4.70 ± 0.36	2.15
4 h	5	2.70 ± 0.16	6.62 ± 0.14[b,c]	2.45
4 h	40	2.11 ± 0.15[b]	4.63 ± 0.13[a]	2.20

[a] $p < 0.05$ *versus* control.
[b] $p < 0.01$ *versus* control.
[c] $p < 0.01$ *versus* corresponding value at 2 h of incubation.

pertonic conditions, however, RVI becomes evident even in cells maintained at high $[K^+]_{out}$, provided that protein synthesis is not inhibited. Thus, distinct mechanisms contribute to the production and maintenance of higher than normal intracellular levels of amino acids in different phases of the RVI response. In the first 2 h of hypertonic treatment an enhanced accumulation of neutral amino acids is achieved through an increased net uptake associated with an increase in $\Delta\tilde{\mu}_{Na}$. This phase of RVI is not dependent upon protein synthesis, since cycloheximide, which does not interfere with RVI-associated hyperpolarization (not shown), does not affect volume recovery. Subsequently, however, a delayed, cycloheximide-sensitive mechanism for RVI becomes evident. This mechanism is referable to the increase in the capacity of system A, induced by hypertonic stress and dependent upon protein synthesis, already described in cultured human fibroblasts (5) and in bovine kidney cells (6). The increase in $\Delta\tilde{\mu}_{Na}$ and protein synthesis fully account for RVI in cultured human fibroblasts since when both are suppressed no volume recovery occurs, and cells remain shrunken.

The results presented here demonstrate that an enhanced accumulation of substrates of system A plays a pivotal role in the RVI of cultured human fibroblasts. Among these amino acids, L-glutamine has a peculiar relevance. This can be explained by several arguments. First, glutamine is a good substrate of system A in cultured human fibroblasts (16). Second, the medium employed has a very high concentration of glutamine (4 mM), although it lacks other good substrates of system A such as L-proline and L-alanine. Third, a previous study has shown that the intracellular concentration of glutamine is regulated by the active uptake of the extracellular amino acid (17); therefore it is probable that glutamine can be accumulated according to $\Delta\tilde{\mu}_{Na}$ through system A. Fourth, L-glutamine is also a good substrate of system ASC (16). Given the exchange nature (13) and the broad spectrum of substrates (18) of this agency, a change in the intracellular concentration of L-glutamine can directly affect the intracellular concentrations of a variety of other neutral amino acids. Fifth, in cultured human fibroblasts intracellular L-glutamine is the major source for the synthesis of L-glutamate (15), the second highest component of the intracellular amino acid pool. In summary, the operation of system A can influence the intracellular concentrations of most amino acids by determining the intracellular concentration of L-glutamine. Although the high concentration of glutamine in the external medium employed here may overemphasize the role of this amino acid in RVI, it should be noted that L-glutamine is one of the most prevalent amino acids in the fasting human plasma. This amino acid, therefore, may represent a key compound for the regulation of cell volume *in vivo* as well as *in vitro*.

Acknowledgments—We acknowledge gratefully the contribution of Prof. R. Marchelli and Dr. R. Bortolini for the chromatographic analysis of intracellular amino acid pool and Prof. Philip C. Laris for helpful discussions.

REFERENCES

1. Hoffmann, E. K., and Simonsen, L. O. (1989) *Physiol. Rev.* **69,** 315–382
2. Sarkadi, B., and Parker, J. C. (1991) *Biochim. Biophys. Acta* **1071,** 407–427
3. Spring, K. R., and Hoffmann, E. K. (1992) in *The Kidney: Physiology and Pathophysiology* (Seldin, D. W., and Giebish, G., eds) pp. 147–169, Raven Press, New York
4. Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982) *Science* **217,** 1214–1220
5. Gazzola, G. C., Dall'Asta, V., Nucci, F. A., Rossi, P. A., Bussolati, O., Hoffman, E. K., and Guidotti, G. G. (1991) *Cell Physiol. Biochem.* **1,** 131–142
6. Soler, C., Felipe, A., Casado, F. J., McGivan, J. D., and Pastor-Anglada, M. (1993) *Biochem. J.* **289,** 653–658
7. Bode, B. P., and Kilberg, M. S. (1991) *J. Biol. Chem.* **266,** 7376–7381
8. Gazzola, G. C., Dall'Asta, V., Franchi-Gazzola, R., and White, M. F. (1981) *Anal. Biochem.* **115,** 368–374
9. Law, R. O., and Turner, D. P. J. (1987) *J. Physiol.* **386,** 45–61
10. Marchelli, R., Dossena, A., Palla, G., Audhuy-Peaudecerf, M., Lefeuvre, S., Carnevali, P., and Freddi, M. (1992) *J. Sci. Food Agric.* **59,** 217–226
11. Bussolati, O., Laris, P. C., Nucci, F. A., Dall'Asta, V., Longo, N., Guidotti, G. G., and Gazzola, G. C. (1987) *Am. J. Physiol.* **253,** C391–C397
12. Bussolati, O., Laris, P. C., Nucci, F. A., Dall'Asta, V., Longo, N., Guidotti, G. G., and Gazzola, G. C. (1989) *Am. J. Physiol.* **256,** C930–C935
13. Bussolati, O., Laris, P. C., Rotoli, B. M., Dall'Asta, V., and Gazzola, G. C. (1992) *J. Biol. Chem.* **267,** 8330–8335
14. Dall'Asta, V., Bussolati, O., Guidotti, G. G., and Gazzola, G. C. (1991) *J. Biol. Chem.* **266,** 1591–1596
15. Bannai, S., and Ishii, T. (1988) *J. Cell. Physiol.* **137,** 360–366
16. Dall'Asta, V., Rossi, P. A., Bussolati, O., Guidotti, G. G., and Gazzola, G. C. (1990) *Biochim. Biophys. Acta* **1052,** 106–112
17. Darmaun, D., Matthews, D. E., Desjeux, J. F., and Bier, D. M. (1988) *J. Cell. Physiol.* **134,** 143–148
18. Christensen, H. N. (1984) *Biochim. Biophys. Acta* **779,** 255–269