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Glycine Protection of PC-12 Cells against Injury by ATP-Depletion

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A distinctive mechanism of cell injury during ATP depletion involves the loss of cellular glycine. The current study examined whether provision of glycine during ATP depletion can prevent injury in PC-12 cells, a cell line with neuronal property. In addition, we have examined the role played by glycine receptors in cytoprotective effects of the amino acid. It was shown that ATP depletion led to plasma membrane damage in PC-12 cells, which was ameliorated by 0.25–5mM glycine. Cytoprotective activity of glycine was shared by alanine, but not by glutamate or γ-aminobutyric acid (GABA). Of interest, strychnine, an antagonist of glycine receptor, was also protective. The results, while suggesting the involvement of glycine receptor in cytoprotection, indicate that chloride channel activity of the receptor is dispensable. Such a scenario is further supported by the observation that removal of extracellular chloride did not affect ATP depletion–induced cell injury or its prevention by glycine. In short, this study has provided the first evidence for glycine protection of cells with neuronal properties. Cytoprotection may involve the glycine receptor; however, it can be dissociated from its channel activity.

KEY WORDS: ATP-depletion; cell injury; glycine; ischemia; neuron.

INTRODUCTION

Ischemic diseases, including myocardial infarction, acute renal failure, and stroke in the brain, are main causes of morbidity and mortality in industrial countries. As a result of the decreased blood flow, cells in ischemic tissues are depleted of oxygen and metabolic nutrients, and lose their capacity for ATP production and become energy deprived. When cell ATP falls to a level that is inadequate to sustain cellular homeostasis, destructive processes are activated, culminating in cell injury and death (1–6).

Cell injury resulting from ATP depletion is mediated by multiple factors. Intracellular Ca^{2+} overload has long been recognized as a destructive event in ATP-depleted cells (7,8). Generation of free radicals has been documented in vivo during organ ischemia, as well as in vitro models of ATP depletion (9–11). Moreover, activation of hydrolytic enzymes, including DNases, phospholipases, and proteases, has been shown in ATP-depleted cells and is expected to execute the cells at the molecular level (5,6,12). In addition to these observations, recent studies have documented yet another mechanism that underlies cell injury by ATP depletion (13). This distinctive mechanism involves the loss of glycine from ATP-depleted cells, followed by plasma membrane breakdown, leakage of cytosol, and cell death by necrosis (14). Of significance, this type of injury is completely diminished by the presence of 0.25–1 mM

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glycine (13–17). In vivo, glycine has been shown to diminish tissue damage in ischemic organs including liver, intestine, and kidneys (18–23).

Despite these studies, there is no report on glycine protection of cells of neuronal origin or from the nervous system. Investigation of glycine effects in neuronal cells is further justified by the potential involvement of glycine receptors in the cytoprotective action of the amino acid (24–26). Glycine-sensitive injury in ATP-depleted cells has been defined as proteinaceous pores in the plasma membrane (16). In addition, pharmacological evidence suggests that development of the porous defects may be related to glycine receptors (24–26). To date, glycine receptors have been identified only in nervous systems (27). Examination of glycine protection in neuronal cells may therefore lay a background for pursuing the role of classical glycine receptors in cytoprotection by this amino acid.

With these considerations, we have examined the effects of glycine on ATP depletion–induced injury in PC-12 cells. Originally isolated from rat adrenal pheochromocytoma, PC-12 cells exhibit characteristics of neurons, including neurotransmitter secretion, electrical excitability, and neurite extension after nerve growth factor stimulation, and therefore have been widely used in neurobiological research (28–30). This study was designed to answer two specific questions: (i) does glycine protect PC-12 cells against cell injury resulting from ATP depletion; and (ii) is the glycine effect dependent on the activity of glycine-gated chloride channels? In other words, does glycine protect ATP-depleted cells through its regulation of chloride fluxes? Our results show that ATP depletion led to plasma membrane damage in PC-12 cells. This type of injury was significantly suppressed by glycine. Of interest, the glycine-receptor antagonist strychnine inhibited cell injury under ATP depletion as well. Moreover, when extracellular chloride was completely removed, cell injury and the protective effects of glycine were not modified. The results suggest that glycine protection is independent of the channel activity of glycine receptors and may be dissociated from chloride fluxes in ATP-depleted cells.

EXPERIMENTAL PROCEDURE

Cell Culture. PC-12 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI medium 1640 containing 10% heat-inactivated horse serum and 5% fetal bovine serum. The cultures were split every 4 days. Flasks and dishes for cell culture were coated with collagen. For experiment, cells were seeded at 0.25×10^6/well of 12-well plate and used after overnight growth.

ATP Depletion. Cells were rinsed twice with phosphate-buffered saline to remove culture medium. For experimental groups, cells were then transferred to glucose-free Krebs-Ringer bicarbonate solution (composition in mM: 115 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1 KH_2PO_4, 1.25 $CaCl_2$, and 1 $MgSO_4$; gassed with 5% CO_2). Carbonyl cyanide-m-chlorophenyl hydrazone (CCCP, 15 μM), a mitochondrial uncoupler, was added to the incubation solution to block ATP production. Free Ca^{2+} in the medium was buffered to 100 nM with 2.25 mM EGTA (31). The Ca^{2+} ionophore ionomycin was included in the buffer at 5 μM so that the cells were permeabilized to Ca^{2+}, and intracellular free Ca^{2+} was clamped at extracellular levels (32,33). Clamping of intracellular free Ca^{2+} at 100 nM during ATP depletion minimizes Ca^{2+}-dependent injury and facilitates the examination of cytoprotective effects of glycine (16,33). For control groups, cells were incubated in glucose-free Krebs-Ringer bicarbonate solution with 100 nM Ca^{2+}.

Measurement of LDH Release from Cells. Lactate dehydrogenase (LDH) is a cytosolic protein with a molecular weight of 136 kD. When plasma membranes of cells are broken, LDH is released into the incubation medium. We therefore measured cellular LDH release to indicate the breakdown of plasma membranes and cell injury. LDH was measured as the enzymatic activity catalyzing the reaction: pyruvate + NADH = lactate + NAD^+. NADH exhibits fluorescence at an excitation wavelength of 360 nm with emission at 450 nm. The velocity of decreases in Ex 360 nm/Em 450 nm fluorescence in the reaction indicates the conversion of NADH to the nonfluorescent NAD, therefore LDH activity. At the end of cell incubation, 100 μl of medium was subjected to brief centrifugation to remove cellular debris. Supernatant was added to 2.5 ml enzymatic reaction buffer (0.2 M potassium phosphate buffer, pH 7.5) containing 6 mM pyruvate and 0.02 mM NADH. Velocity of decreases in fluorescence at Ex 360 nm/Em 450 nm was monitored on an LS-3 fluorescence Spectrophotometer (Perkin-Elmer) to indicate LDH activity. Parallel dishes of cells were lysed with 0.1% Triton X-100 to determine total LDH activity. The LDH activity obtained from cell incubation medium was divided by the total LDH activity to calculate the percentage of LDH release.

Chemical Analyses. To measure ATP, cells were extracted with trichloroacetic acid. ATP in cell extracts was measured by luminometry of the luciferin firefly luciferase reaction (17,25). ATP values were expressed as nanomole per milligram of cell protein. Protein was quantitated with the bicinchoninic acid (BCA) reagent purchased from Pierce Chemical Company (Rockford, IL, USA).

RESULTS

Plasma Membrane Damage in PC-12 Cells Induced by ATP Depletion. In mammalian cells, there are two major pathways for ATP generation, oxidative phosphorylation via electron transport in the mitochondrion and anaerobic glycolysis in the cytosol. To induce ATP depletion, we incubated PC-12 cells with a physiological buffer that was free of glucose, the glycolytic substrate, to eliminate ATP generation through glycolysis. Moreover, CCCP, a mitochondrial uncoupler was included in the incubation medium to block ATP production through oxidative phosphorylation. Under these conditions, rapid declines of cellular ATP were observed. As shown in Fig. 1A, within

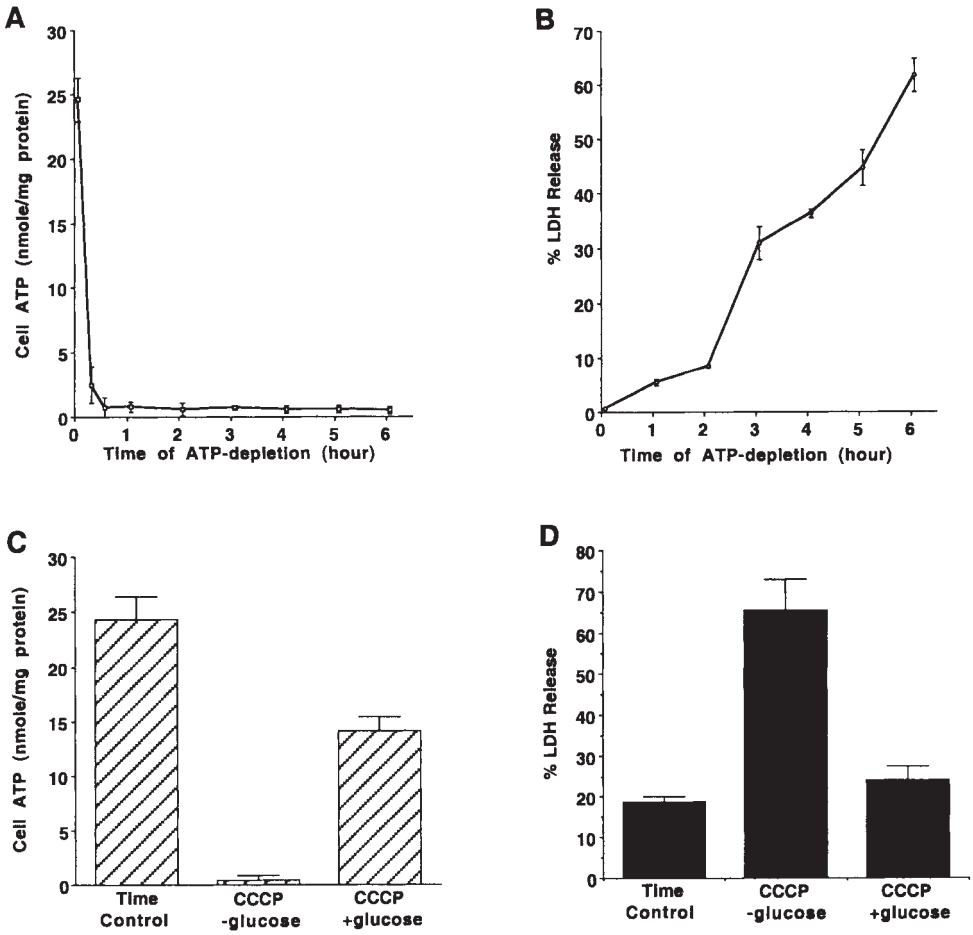

Fig. 1. ATP depletion–induced plasma membrane breakdown in PC-12 cells. **A,** Time course of ATP depletion in PC-12 cells induced by CCCP in glucose-free medium. Cell ATP declined rapidly after CCCP exposure. **B,** Release of cellular LDH into incubation medium during ATP depletion. ATP depletion led to plasma membrane damage, shown here as the release of cytosol LDH. **C,** ATP levels in PC-12 cells after 6 h of CCCP incubation in the absence or presence of 1 mg/ml glucose. Glucose facilitated ATP production through glycolysis in CCCP-treated cells and maintained cellular ATP to ~60% of control. **D,** LDH release from CCCP-treated PC-12 cells in the absence or presence of glucose. Glucose, while maintaining cell ATP, prevented cell injury or LDH release during CCCP incubation. Values shown in this figure are mean ± SD (n = 3).

15 min, ATP levels in treated cells decreased to less than 10% of control. At the end of 1 h of incubation, only a trace amount of cellular ATP was detected. ATP-depleted cells developed plasma membrane damage, as indicated by the leakage of LDH, a cytosolic protein of 136,000 D (Fig. 1B). After 3 h of ATP depletion, over 30% of cells lost the integrity of their plasma membranes, leaking LDH. As the incubation was prolonged, more cells were damaged and became permeable to macromolecules. By the end of 6 h of ATP depletion, over 60% of cells leaked LDH (Fig. 1B). Cell injury under these conditions was dependent on ATP depletion. As shown in Fig. 1C, glucose provided during CCCP treatment maintained cellular ATP at substantial levels. This was accompanied by the prevention of plasma membrane damage or LDH release (Fig. 1D).

Protection of ATP-Depleted PC-12 Cells by Glycine. To examine the effects of glycine on cell injury in PC-12 cells, we initially compared LDH release induced by ATP-depletion, in the presence or absence of 5 mM glycine. The results are shown in Fig. 2A. The time control group that was incubated in glucose-free medium without CCCP exposure had 13% LDH release. After 6 h of ATP depletion, almost 70% of cells lost their LDH into the incubation medium (Fig. 2A, CCCP-glycine). When 5 mM glycine was provided during ATP depletion, LDH release was drastically reduced to 25% (Fig. 2A, CCCP + glycine), indicating cytoprotection offered by this

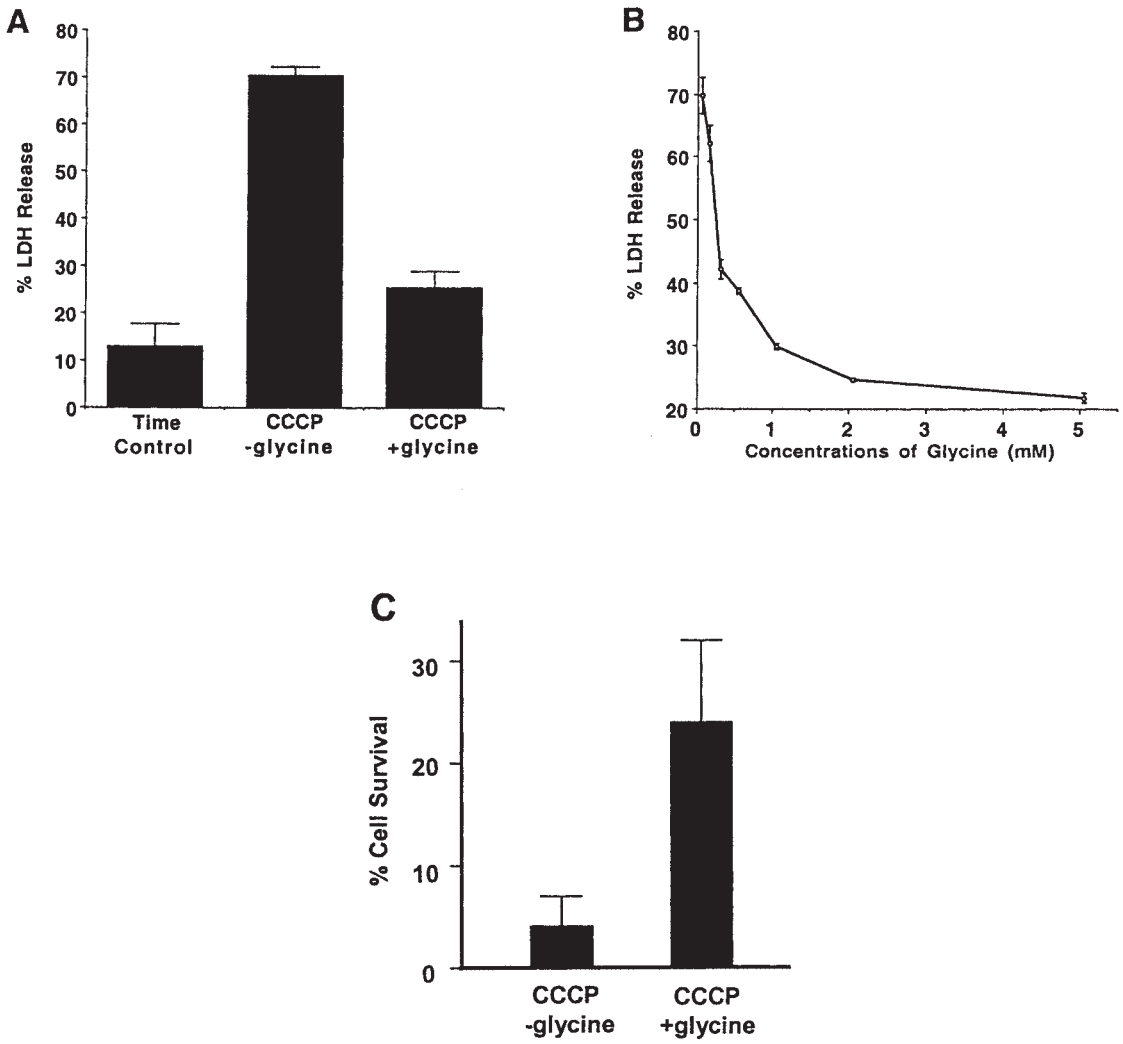

Fig. 2. Cytoprotective effects of glycine during ATP depletion of PC-12 cells. **A,** LDH release from ATP-depleted cells in the absence or presence glycine. Glycine at 5 mM reduced LDH release during 6 h of ATP depletion from 70% to 25%. **B,** Dose dependence of glycine cytoprotective effects. **C,** Cell survival. PC-12 cells were depleted of ATP for 6 h in the presence of 0–5 mM glycine, and LDH release from these groups of cells were measured to indicate cell injury. To determine cell survival, cells were collected after ATP depletion and washed twice with culture medium. The cells were replated into culture dishes and viable cells were counted after 24 h of culture. Survival rate was expressed as percent of cells that were subjected to ATP depletion. Values, mean ± SD, were obtained from three separate experiments. Significant protection was shown for glycine at concentrations of 0.25 mM or above.

amino acid. Glycine protection was also detected after longer periods of ATP depletion (not shown). Dose dependence of glycine protection is shown in Fig. 2B. Glycine at 0.1 mM already had slight protective effects, while evident protection was shown at concentrations of 0.25 mM or above. Maximal protection was achieved at glycine concentrations of 1–5 mM.

Glycine protection was associated with increased survival of the cells. As shown in Fig. 2C, without glycine, less than 5% of cells were viable 24 h after ATP depletion. On the contrary, over 20% of cells recovered after ATP depletion, when protected by glycine. Of note, there was a significant difference between viability measurements using LDH release or cell survival as parameters. For example, 25% LDH release was shown for glycine protected cells (Fig. 2A), indicating cell viability of 75%. However, the survival rate for this group of cells was only 23% (Fig. 2C). There were at least two possibilities to account for the differences. First, some of the cells that did not leak LDH might have been injured not at the plasma membrane level but at other critical sites, including DNA breakdown. Second, cells detached during ATP

depletion regardless of the presence or absence of glycine. To determine survival rates, the detached cells were replated and cultured overnight. During replating, some cells did not attach to the dish and ultimately lost their viability.

Glycine also protected ATP depletion–induced injury in differentiated PC-12 cells. For differentiation, PC-12 cells were cultured in the presence of nerve growth factor for 8–10 days. In a typical experiment, control differentiated cells showed 1.9% LDH release and ATP depletion increased LDH release to 66%. When 1 mM glycine was provided, only 8.4% LDH release was detected.

Glycine Does Not Prevent ATP Declines in CCCP-Treated PC-12 Cells. Our results have shown impressive cytoprotective effects of glycine against ATP depletion–induced injury in PC-12 cells. An obvious question was: does glycine protect the cells by maintaining cellular ATP? To answer this question, we compared ATP levels in glycine-protected and unprotected cells during CCCP incubation. The results are shown in Fig. 3. After 15 min of CCCP incubation, ATP levels declined to 12% and 9% of control in glycine-protected and unprotected cells, respectively. By the end of 1 h CCCP incubation, cellular ATP declined to less than 2% of control, regardless of the presence or absence of glycine. Only traces of ATP were detected in these cells after longer CCCP treatment (Fig. 3). These data indicate clearly that glycine protected PC-12 cells without preserving cellular ATP.

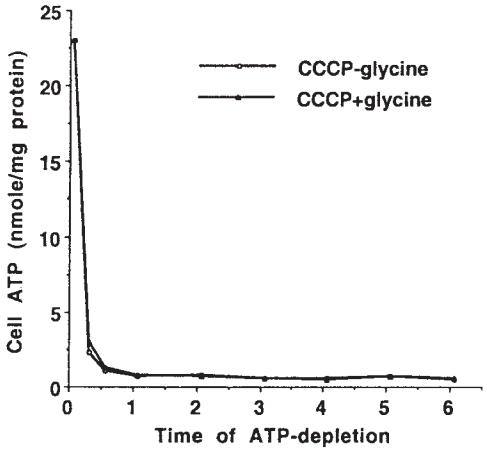

Fig. 3. Glycine does not preserve cell ATP during CCCP treatment in glucose free medium. PC-12 cells were incubated for 0–6 h with CCCP in glucose-free medium in the absence or presence of 5 mM glycine. ATP in these two groups of cells was determined by luminometry of the luciferin firefly luciferase reaction. Values were averages of three separate experiments; error bars (SD) were not shown for clarity. ATP depletion was induced by CCCP, regardless the presence or absence of glycine.

Cytoprotective Action of Glycine Depends on Structure of the Amino Acid. Glycine protection of ATP-depleted cells appeared to depend on the molecular structure of this amino acid. As shown in Fig. 4, alanine, which has an additional methyl group to the glycine moiety, was cytoprotective during ATP depletion, but it was not as effective as glycine. Whereas 1 mM glycine lowered the LDH release from ATP-depleted cells from 64% to 27%, 40% LDH release was detected in the group with 1 mM alanine. Further modification of the amino acid structure led to complete loss of cytoprotective effects. For example, no significant protection was shown for glutamate (not shown) or γ-aminobutyric acid (GABA) (Fig. 4). These results suggest that protective action of glycine might be determined by its structure.

Protection of ATP-Depleted PC-12 Cells by Strychnine. Our results suggest that glycine protection might be specific for the structure of small amino acids (Fig. 4). This observation points to interactions of the amino acids with molecular targets in the cells. In nervous system, receptors for glycine have been identified (27). These receptors, also known as glycine-gated chloride channels, are pentameric proteins in the plasma membranes, which upon glycine binding transform into channels that are specifically permeable to chloride (27). To test the involvement of these receptors in glycine cytoprotection, we examined the effects of strychnine, a well-known antagonist of glycine receptors in the nervous system (27). The results are shown in Fig. 5. ATP depletion for 6 h

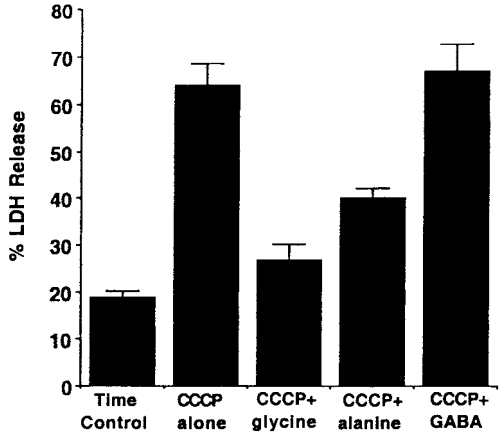

Fig. 4. Alanine but not larger amino acids share the cytoprotective action of glycine. PC-12 cells were depleted of ATP for 6 h in the absence or presence of 1 mM glycine, alanine, or GABA. Values, mean ± SD, were obtained from three separate experiments. Significant cytoprotective effects were shown for glycine and alanine but not for GABA. Glutamate did not protect ATP-depleted PC-12 cells either (data not shown).

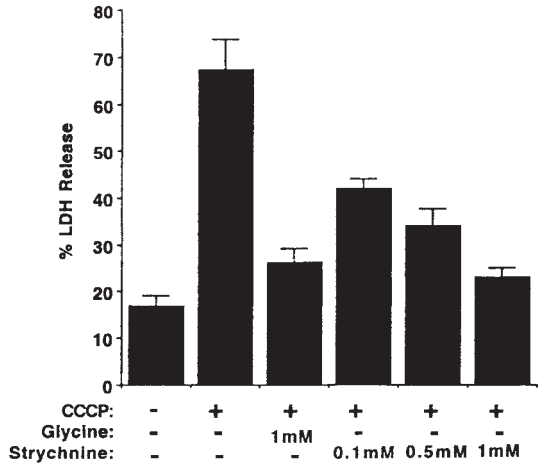

Fig. 5. Cytoprotective effects of strychnine during ATP depletion of PC-12 cells. PC-12 cells were depleted of ATP for 6 h in the absence or presence of 1 mM glycine or 0.1–1 mM strychnine. LDH release from these cells was monitored to indicate cell injury. Values, mean ± SD, were obtained from three separate experiments. Strychnine was shown to be cytoprotective at concentrations as low as 0.1 mM.

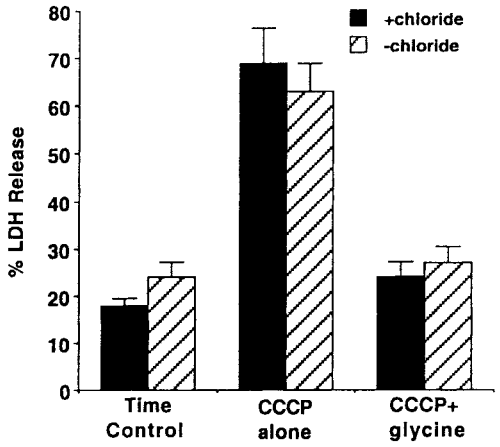

Fig. 6. Effects of Cl^- omission on ATP depletion–induced cell injury and its prevention by glycine. PC-12 cells were depleted of ATP for 6 h in medium containing regular (119.75 mM) Cl^- or in Cl^--free medium. In Cl^--free medium, Cl^- was substituted with gluconate. Glycine at 5 mM was added during ATP depletion to examine it protective effects in the presence or absence of Cl^-. Cell injury (LDH release) was monitored as described in the Experimental Procedure section. Values, mean ± SD, were obtained from three separate experiments. Similar extents of cell injury and glycine protection were shown in ATP-depleted cells for media with or without Cl^-.

led to ~70% LDH release. In the presence of 1 mM glycine, LDH release was reduced to 26%, confirming our previous observations of cytoprotection by this amino acid. Of interest, protection was also shown for strychnine, the glycine receptor antagonist (Fig. 5). As a matter of fact, strychnine showed significant protective effects at the concentration of 0.1 mM, a dose at which glycine had only marginal effects (Fig. 2B). Glycine is an agonist and strychnine an antagonist of chloride-channel receptors; thus these results, while suggesting the involvement of the receptor protein in cytoprotection, indicate clearly that the cytoprotective effects are not associated with channel activity of the receptors.

Dissociation of Glycine Protection from Chloride Flux. To further examine whether glycine protects ATP-depleted cells through activating the chloride channels, we monitored cell injury and glycine protection in chloride-free medium. In this medium, chloride was completely replaced with gluconate, an anion that is not permeable to chloride channels. The results are shown in Fig. 6. Omission of chloride did not have significant effects on cell injury induced by ATP depletion. In regular chloride medium, ATP depletion for 6 h led to 70% LDH release and to 64% for cells in chloride-free medium. Of significance, glycine protected ATP-depleted cells, regardless of the presence or absence of chloride. With 1 mM glycine, LDH release during 6 h ATP depletion was reduced to less than 30%, in chloride-containing as well as chloride-free media. These results suggest that cytoprotective effect of glycine can be dissociated from chloride flux through glycine-gated channels.

DISCUSSION

The current study has demonstrated cytoprotective effects of glycine against injury by ATP depletion in PC-12 cells. To our knowledge, this is also the first report on glycine protection of cells with neuronal property or of neuronal origin. The protective activity appears to be rather specific for the structure of small amino acids and is lost in larger amino acids such as glutamate or GABA. Of interest, similar protective activity was shown for strychnine, an antagonist of glycine receptors in the nervous system. Both glycine and strychnine interact with neural chloride channel-receptors; thus these results may suggest the involvement of the receptor protein in cytoprotective activity of these chemicals. On the other hand, glycine and strychnine regulate channel activity of the receptors in opposite directions and yet display similar cytoprotection. Thus the cytoprotection seems to be dissociated from the channel activity of the receptor. Further experiments showed clearly that removal of chloride did not affect cell injury during ATP depletion and, of sig-

nificance, it did not interfere with glycine cytoprotection either. The results indicate that cytoprotective effects of glycine are dissociated from chloride influx, providing additional support for the notion that the glycine receptor may participate in cytoprotection in a way that is independent of its channel activity.

Since the first demonstration of the protective effects of glycine, several hypotheses on glycine protection have been proposed and tested. It has been shown that glycine does not help the cells generate or conserve ATP. Moreover, glycine does not protect ATP-depleted cells by preservation of ion homeostasis, modification of intracellular pH, stabilization of the cytoskeleton, quenching of reactive oxygen species, or inhibition of phospholipid hydrolysis (32,34–36). In addition, glycine cytoprotection does not rely on metabolism of the amino acid (37). On the other hand, studies by us and others have shown that the protective activity is shared by a family of closely related amino acids and several neural chloride channel modulators, including the antagonist strychnine (24–26,38). With these observations, we proposed that glycine and the related compounds may protect ATP-depleted cells by low-affinity interactions with a multimeric channel protein, destabilization of which may otherwise lead to formation of pathological pores (25). Such porous defects in plasma membranes of ATP-depleted cells have been characterized by our recent studies (16). The membrane defects were shown to evolve from small pores permeable only to propidium iodide (668 D), before enlarging to become permeable to larger molecules such as LDH. Glycine provided during ATP depletion blocked the development of membranous pores completely. Of importance, the glycine effect could be mimicked by a cell-impermeant cross-linker that was expected to tether the subunits of multimeric proteins in the plasma membrane and prevent their breakdown (16). Further studies showed that-cell impermeable strychnine derivatives were able to protect kidney epithelial cells and hepatocytes (17). These observations are consistent with the hypothesis that glycine cytoprotection may depend on its interaction with a multimeric protein target in the plasma membranes that are related to neuronal glycine receptors. Despite these studies, classical glycine receptors have thus far been identified only in neuronal cells. The demonstration of glycine protection of PC-12 cells in this study may therefore lay a background for further investigation of glycine receptors in cytoprotective effects of the amino acid.

Results from this study suggest that, although glycine receptors may be involved in cytoprotection by glycine, channel activity of the receptor proteins can be dissociated. The involvement of glycine receptors is suggested by the observation that modulators of the receptors, including strychnine, share the cytoprotective action of glycine. On the other hand, strychnine opposite to glycine is a classical antagonist of neuronal glycine receptors and is a potent inhibitor of their channel activity. Cytoprotective effects of glycine and strychnine as well suggest that, while glycine receptors may be involved in cytoprotection, channel activity of the receptors is dispensable. The conclusion that chloride channel activity of the receptors can be dissociated is further suggested by the experiment of chloride removal. Omission of chloride from incubation medium did not significantly alter cell injury by ATP depletion or modify glycine protection (Fig. 6).

The loss of ion homeostasis, particularly intracellular Ca^{2+} increase, has been implicated in cell injury under in vitro as well as in vivo conditions of ATP depletion (7,8). However, the focus of this study is not Ca^{2+}-dependent cell injury or its modification by glycine. Instead, this study has employed a technique of Ca^{2+} "clamping" to tightly control intracellular Ca^{2+} to a level of 100 nM to minimize Ca^{2+}-dependent injury. This enables a full expression of glycine-sensitive injury that is Ca^{2+}-independent (16). The technique of intracellular Ca^{2+} clamping has been established in our previous studies (16,33). In this technique, the Ca^{2+} ionophore ionomycin is added to the incubation medium at a concentration of 5 μM. This leads to specific permeabilization of the plasma membrane to Ca^{2+} and an equilibration of Ca^{2+} between the intracellular and intercellular spaces. For normal cells with ATP, it may take some time to reach the equilibrium, because Ca^{2+} can be pumped out of the cell or sequestered into mitochondria and endoplasmic reticulum in an energy dependent manner. However, in this study, cells were depleted of ATP and lost their ability to actively maintain Ca^{2+} homeostasis. Under these conditions, rapid Ca^{2+} equilibrations across the plasma membrane have been demonstrated in all cell types examined including MDCK epithelial cells, BEC endothelial cells, and 3T3 fibroblasts (ref. [33]; unpublished data). Based on these data, the intracellular Ca^{2+} in this study is expected to be clamped at the extracellular level of 100 nM. This assumption is supported by the observation of impressive cytoprotective effects of glycine in the current study; as shown before, glycine alone cannot protect cells from damage by uncontrollable increases of cellular Ca^{2+} (25).

Finally, it should be pointed out that demonstration of cytoprotective effects of glycine in neuronal cells

in vitro does not necessarily suggest that similar beneficial effects of glycine can be achieved during tissue ischemia in the nervous system. First of all, nervous systems, including the brain, are far more complex than the liver, intestine, or kidneys, structurally as well as functionally. Second, neuronal cells, as excitable as they are, possess many unique properties, compared with hepatocytes or epithelial or endothelial cells. In particular, glycine acts as an agonist of glycine-gated chloride channels, but it is also a potent ligand for NMDA-sensitive glutamate receptors (for a review see [39]). At low micromolar concentrations, glycine binds to NMDA receptors and behaves as a coagonist. At millimolar concentrations, glycine reportedly can activate NMDA receptors by itself. Under these conditions, glycine may exhibit neurotoxicity, presumably through its activation of NMDA receptor channels followed by Ca^{2+} influx. These observations suggest that whether glycine and related compounds indeed hold a therapeutic promise for neuronal tissue damage in vivo remains to be investigated.

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