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Effect of glycine on prelethal and postlethal increases in calpain activity in rat renal proximal tubules

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Effect of glycine on prelethal and postlethal increases in calpain activity in rat renal proximal tubules. The effect of glycine on hypoxia- and ionomycin-induced increases in calpain activity in rat proximal tubules was determined. Calpain activity was determined both *in vitro* and in the intact cell using the fluorescent substrate N-succinyl-Leu-Leu-Val-Tyr-7- amido-4-methyl coumarin (N-succinyl-Leu-Leu-Val-Tyr-AMC) and Western blotting for calpain-specific spectrin breakdown products (BDP), respectively. At 7.5 minutes of hypoxia (prelethal injury model) there was a significant (10-fold) increase in *in vitro* calpain activity that was not inhibited by glycine. At 15 minutes of hypoxia (postlethal injury model) there was a further increase in calpain activity that was inhibited by glycine. Normoxic tubules incubated with the calcium ionophore ionomycin (5 μM) for two minutes and 10 minutes had a significant increase in calpain activity that was not inhibited by glycine. After 15 minutes of hypoxia in the presence of glycine, there was an increase in calpain-specific spectrin breakdown products (BDP) in both Triton X-100 soluble and cytosolic extracts from proximal tubules. Glycine in concentrations up to 10 mM had no direct effect on the *in vitro* calpain activity of purified calpains. The present study demonstrates that: (*1*) prelethal increases in calpain activity stimulated by hypoxia and ionomycin treatment are not affected by glycine; (*2*) calpain-mediated spectrin breakdown during hypoxia occurs in the presence of glycine; (*3*) glycine does prevent the additional postlethal increase in calpain activity probably by maintaining membrane integrity to calcium influx.

Glycine is a well-known cytoprotective agent against proximal tubular injury [1, 2]. However, many of the pathophysiological events that occur in *in vitro* models of proximal tubule injury, such as ATP depletion [3–5], cytoskeletal changes [6, 7], prelethal influx of Ca^{2+} [5, 8], activation of phospholipase A_2 (PLA_2) [9] and nitric oxide synthase (NOS) [10] are not affected by glycine. These events therefore are more likely to be potential mediators of *in vivo* proximal tubule injury where glycine is present at physiological concentrations [1, 11].

Prelethal increases in free cytosolic calcium ($[Ca^{2+}]_i$) during hypoxia and ionomycin treatment have been shown to activate the Ca^{2+}-dependent protease, calpain, in proximal tubules [12, 13]. These prelethal increases in $[Ca^{2+}]_i$ are not inhibited by glycine [5, 8]. However, as membrane integrity becomes impaired in the absence of glycine in the media, there is a massive influx of Ca^{2+} from the extracellular medium [14, 15]. Glycine prevents this secondary Ca^{2+} influx by maintaining structural membrane integrity of the cell by a mechanism that is as yet incompletely understood.

It has been suggested that glycine may exert its cytoprotective effect by inhibition of protease activity [16]. In pre-lethal anoxic hepatocyte injury, glycine cytoprotection was demonstrated to be in part due to inhibition of Ca^{2+}-dependent degradative, nonlysosomal enzymes, including calpains [17]. There have also been preliminary reports that glycine cytoprotection against hypoxic and antimycin A-induced injury in rat proximal tubules may be mediated by calpain inhibition [18, 19]. However, whether this effect of glycine to inhibit calpain activity is a direct effect or the result of cytoprotection is controversial. In hepatocytes glycine has a direct effect on m-calpain more than μ-calpain [17]. However, in proximal tubules glycine did not have a direct effect on calpain activity [18]. Specifically, if glycine inhibits calpain directly, then the early prelethal increase in calpain activity would be blocked by glycine regardless of the glycine-independent increase in $[Ca^{2+}]_i$. On the other hand, if glycine does not directly inhibit calpain activity, it would be expected that the prelethal increase in Ca^{2+}-mediated calpain activity would persist. In either case, further increases in calpain activity during postlethal Ca^{2+} influx should be prevented by glycine.

The present study was designed to (*1*) dissociate prelethal versus postlethal effects of hypoxia or ionomycin treatment on calpain activity, and (*2*) determine whether the cytoprotective effect of glycine is mediated by calpain inhibition.

METHODS

Reagents

Glycine, digitonin, hyaluronidase type III from sheep testes, N-succinyl-Leu-Tyr-7-amido-4-methyl coumarin (N-succinyl-Leu-Tyr-AMC) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N-succinyl-Leu-Leu-Val-Tyr-AMC and AMC were made by the Peptide Institute (Osaka, Japan), and was obtained from Peptides International (Louisville, KY, USA). Ionomycin and purified μ-calpain (from porcine erythrocytes) was purchased from Calbiochem (San Diego, CA, USA); collagenase type B (lot number 0FAA133) from Boehringer Mannheim (Indianapolis, IN, USA); fura 2 pentapotassium from Molecular Probes (Eugene, OR, USA); fatty

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acid-free bovine albumin from ICN (Cleveland, OH, USA); polivinylidene diflouride (PVDF) membrane for immunoblotting from Millipore Corp. (Bedford, MA, USA). Rabbit polyclonal antibodies developed against the amino-terminal of the calpain cleavage site in alpha spectrin [breakdown product specific antibodies (BDPn)] were obtained from Dr. Ben Bahr, University of California (Irvine, CA, USA) [20]. Horseradish peroxidase conjugated sheep anti-mouse and donkey anti-rabbit immunoglobulin G from Amersham Corp. (Arlington Heights, IL, USA); chemiluminescence (ECL) kit from Amersham Corp.; and prestained protein markers were from BIORAD (Temecula, CA, USA).

Preparation of tubules

Proximal tubules were isolated from kidney cortex of male Sprague-Dawley rats (200 to 300 g body wt) using collagenase digestion and percoll centrifugation [21, 22]. Six milliliter aliquots of tubule suspension (~1 to 2 mg/ml) were placed in siliconized 25 ml Erlenmeyer flasks for a "recovery" period consisting of the following: (*1*) gassing with 95% O_2-5% CO_2 for five minutes on ice; (*2*) flasks were then capped with rubber stoppers and kept at room temperature for five minutes; and (*3*) flasks were then placed in a shaking water bath at 37°C for 10 minutes. To create hypoxia, the tubule suspension was regassed for five minutes with 95% N_2-5% CO_2 at a rate of 3 liter/min [23]. After regassing, the flasks were closed and kept in the shaking water bath for the period of hypoxia studied. At the end of the hypoxic period, 1 ml of tubule suspension was sampled for measurement of LDH and 2 ml for calpain activity.

The calcium ionophore, ionomycin, was used to induce an increase in $[Ca^{2+}]_i$. A stock solution of 1 mM ionomycin in DMSO was used. The increase in $[Ca^{2+}]_i$ in freshly isolated rat proximal tubules occurs after 30 seconds of exposure to ionomycin [8]. Normoxic tubules were exposed to ionomycin (5 μM) for 2 and 10 minutes. Control tubules were exposed to the vehicle, DMSO.

Glycine studies

A 200 mM stock solution of glycine in distilled deionized water was prepared fresh each day. After the recovery period, glycine 2 mM was added to the tubules before the induction of hypoxia and ionomycin treatment.

Measurement of lactate dehydrogenase release and protein

Lactate dehydrogenase (LDH) release was measured to evaluate cell damage, as previously described [24, 25]. The percentage of LDH released from tubules was calculated by determining the ratio of LDH in the supernatant compared to that in the lysed tubule pellet plus the supernatant. Tubule protein was measured by the Lowry method using bovine serum albumin as standard [26].

In vitro calpain assay

The calpain assay used in this study is based on that described by Sasaki et al for purified porcine kidney calpain [27]. N-succinyl-Leu-Leu-Val-Tyr-AMC was used as a susceptible substrate for calpain [27]. A stock solution of 10 mM was prepared in DMSO and stored at −20°C between use. The preparation of cytosolic extracts from proximal tubules and the calpain assay in freshly isolated proximal tubules has recently been described in detail by us [12, 13].

Briefly, the calpain assay was performed as follows. After exposure to normoxia, to ionomycin or to hypoxia, the tubule pellet was separated from 2 ml tubule suspension by centrifugation. The imidazole-HCl buffer used contained imidazole 63.2 mM—mercaptoethanol 10 mM, pH 7.3. The tubule pellet was immediately resuspended in a calcium free imidazole—HCl buffer containing in addition 1 mM EDTA and 10 mM EGTA (pH 7.3). The suspension was then incubated with digitonin (10 μM) at 37°C in a shaking water bath for five minutes. Ten micrometers of digitonin selectively permeabilized the plasma membrane but did not destroy lysosomal or mitochondrial membranes of hepatocytes [28] or mitochondrial membranes of rat proximal tubules [21]. In separate experiments, using the lysosomal dye Lucifer Yellow and a video imaging microscope that has been previously described [29], our laboratory confirmed that 10 μM digitonin selectively permeabilizes the plasma membrane of isolated rat proximal tubules without affecting the lysosomal membrane. Thus, 10 μM digitonin released cytosolic enzymes including calpain, while keeping the lysosomal membrane intact. After incubation with digitonin, the tubule pellet and supernatant containing released calpain were again separated by centrifugation. Calpain activity in the tubule pellet after digitonin incubation was zero, confirming that all the calpain had been released into the supernatant. The calpain assay was then performed on this supernatant as follows: 0.5 ml supernatant (0.5 to 1 mg tubule protein) and 10 μl of the substrate N-succinyl-leu-leu-val-tyr-AMC were added to the imidazole buffer with or without 5 mM $CaCl_2$. The total volume of the assay solution was made up to 2 ml with the imidazole buffer. The solution was incubated for 30 minutes at 37°C in a shaking water bath. In the assay performed without $CaCl_2$, the imidazole—HCl buffer containing 1 mM EDTA and 10 mM EGTA was used. After the a 30 minute incubation, fluorescence at 380 nm excitation and 460 nm emission was determined using a Hitachi F2000 spectrophotometer. Calpain activity was determined as the difference between the calcium dependent fluorescence and the non-calcium dependent fluorescence. An AMC standard curve was determined for each experiment. Calpain activity was expressed in pmol AMC released per minute of incubation time per mg of tubule protein.

Calpain activity measured in the media used for incubation of the tubules was negligible in normoxia, hypoxia and ionomycin treatment, confirming that calpain does not leak out of the tubules into the media [12].

The calpain substrate used in our assay, N-succinyl-Leu-Leu-Val-Tyr-AMC, has been shown to be proteolyzed *in vitro* by the cathepsin, papain [27]. Therefore, to ensure the calpain selectivity of our assay and exclude any possible effect of lysosomal leakage of cathepsins in our calpain assay, the following measures were taken: (*1*) digitonin, which releases cytoplasmic contents while keeping lysosomes intact, thus limiting release of lysosomal cathepsins, was used to lyse the cells [8]; (*2*) the assay was performed at a pH of 7.3 at which cathepsins are known to be inactivated [30]; and (*3*) calpain activity was defined and calculated as calcium-dependent activity, thus excluding cathepsin activity, which is strictly calcium-independent [30].

Rat lung extract

Partially purified m-calpain controls were obtained from normal nonperfused rat lung tissue by a technique modified from Waxman and Krebs [31].

Calpain assay in intact cells by detection of spectrin breakdown products

Proteolysis of spectrin to its calpain specific breakdown products is an established measurement of calpain activation in intact cells [20, 32, 33]. Both Triton X-100 soluble and cytosolic fractions from proximal tubules were immunoblotted with antibodies developed against the amino-terminal of the calpain cleavage site in alpha spectrin (BDPn).

Preparation of triton soluble fractions

The association of spectrin with the cytoskeleton was determined by its solubility in Triton X-100. The cytoskeletal proteins that dissociate from the cytoskeleton become Triton X-100 soluble [7]. Freshly isolated rat proximal tubules were exposed to normoxic or hypoxic conditions for 15 minutes in the presence and absence of glycine. One milliliter of tubule suspension was mixed with an equal volume of ice-cold lysis buffer [0.5% Triton X-100, 300 mM sucrose, 5 mM tris(hydroxymethyl]aminomethane (Tris)-HCl, pH 7.4] containing the protease inhibitors pepstatin 20 μM, leupeptin 20 μM, aprotonin 1000 U/ml and phenylmethyl-sulfonyl fluoride (PMSF) 1 mM for 10 minutes on ice. The extracted suspension was then centrifuged at 5000 g for 10 minutes. The Triton soluble proteins were precipitated from the supernatant with 3.5 ml of 100% methanol and pelleted at 4,000 g for 10 minutes. The Triton soluble proteins were resuspended in polyacrylamide gel electrophoresis (PAGE) buffer [5% sodium dodecyl sulfate (SDS), 25% sucrose, 5 mM Tris-HCl and 5 mM EDTA] and mixed with 5× sample buffer (containing 0.35 M Tris base, 10% glycerol, 0.01% bromophenol blue, 15% SDS, 3.6 mM beta-mercaptoethanol, pH 6.5) and stored at −20°C. Samples were boiled at 100°C for three minutes before loading onto the gel. For spectrin, 100 μg samples were loaded onto the gel. The total protein in aliqouts precipitated from the Triton soluble fraction was resuspended in SDS-PAGE buffer and measured by the Lowry method using bovine serum albumin as the standard [26].

Preparation of cytosolic (soluble) fractions

Subcellular fractionation was performed as described by Michel, Li and Busconi [34]. Normoxic and hypoxic tubules (approximately 6 to 8 mg protein) in the presence of glycine (2 mM) were pelleted by gentle centrifugation. Pellets were resuspended in 2 ml homogenizing buffer (50 mM Tris-HCl, 1 mM EDTA, 2 mM *b*-mercaptoethanol, 1 mM EDTA, pH 7.4) with the protease inhibitors pepstatin 20 μM, leupeptin 20 μM, phenylmethyl-sulfonyl fluoride (PMSF) 200 μM. Tubules were then disrupted by freeze-thawing three times followed by homogenization in a teflon-glass homogenizer. Homogenate was then centrifuged at 3000 g for five minutes. The supernatant was centrifuged at 100,000 g at 4°C for one hour. The high-speed supernatant was kept as the cytosolic fraction. Aliquots of the cytosolic fraction were mixed with 5× sample buffer and frozen at −20°C. Aliquots were kept for protein determination using the Bradford method as described in the Bio-rad protein assay kit with bovine serum albumin as standards. Samples were boiled at 100°C for three minutes before loading onto the gel. For spectrin, 100 μg samples were loaded onto the gel.

Western blots

Western blotting was performed using a standard protocol [35, 36]. Extracted proteins were separated by electrophoresis on a denaturing 7.5% SDS polyacrilamide gel (constant current of 150 mA for 2 hr). Proteins were then transferred to a polivinylidene diflouride (PVDF) membrane by wet electroblotting (100 V for 105 min). Blots were blocked for one hour with 5% non fat dry milk in TTBS (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20, pH 7.5). Western blot analysis was performed with rabbit polyclonal antibodies developed to the amino-terminal of the calpain cleavage site in alpha spectrin [breakdown product specific antibody (BDPn)] [20]. The blotted proteins were then probed with BDPn antibodies (1:50) overnight at 4°C. Secondary antibody used was horseradish peroxidase conjugated donkey anti-rabbit immunoglobulin G 1:1000. BDPn protein (147 kDa) were detected by enhanced chemiluminescence (ECL) according to manufacturer's instructions. Prestained proteins markers were used for molecular mass determinations. Positive controls for BDPs were run with calcium-treated hippocampal slices [20].

Statistical analysis

Multiple group comparisons were done using the analysis of variance (ANOVA) with post-test according to Newman-Keuls. A P value of less than 0.05 was considered statistically significant. Values are expressed as means ± standard error (SEM).

RESULTS

Effect of glycine on hypoxia-induced calpain activity

At 7.5 minutes there was no increase of LDH release in hypoxic and glycine treated tubules compared to controls (Fig. 1). There was a prelethal increase in calpain activity that was not significantly decreased by glycine. Calpain activity was 17 ± 7 pmol/min/mg protein in controls, 166 ± 22 pmol/min/mg protein after 7.5 minutes hypoxia ($P < 0.001$ vs. control) and 147 ± 22 pmol/min/mg protein in hypoxic tubules treated with glycine ($P < 0.001$ vs. control, NS vs. hypoxia alone; $N = 12$; Fig. 1).

At 15 minutes of hypoxia there was a fourfold increase of LDH release in hypoxic tubules that was completely prevented by glycine (Fig. 2). Calpain activity increased to a higher level than seen at 7.5 minutes of hypoxia and glycine significantly reduced the calpain activity to a level seen at 7.5 minutes of hypoxia. Calpain activity was 7 ± 7 pmol/min/mg protein in controls, 308 ± 42 pmol/min/mg protein after 15 minutes of hypoxia ($P < 0.001$ vs. control, $P < 0.05$ vs. 7.5 min hypoxia), and 138 ± 23 pmol/min/mg protein in hypoxic tubules treated with glycine ($P < 0.01$ vs. control, $P < 0.01$ vs. hypoxia alone; $N = 5$; Fig. 2).

Effect of glycine on ionomycin-induced calpain activity

Normoxic tubules were incubated with 5 μM ionomycin for 2 and 10 minutes, in the presence and absence of glycine. Control tubules were incubated with DMSO, the vehicle for the ionomycin.

At two minutes there was no increase of LDH release in ionomycin and glycine treated tubules compared to controls (Fig. 3). There was a prelethal increase in calpain activity that was not decreased by glycine. Calpain activity was 80 ± 40 pmol/min/mg protein in normoxic controls, 358 ± 64 pmol/min/mg protein after two minutes of ionomycin ($P < 0.01$ vs. control) and 297 ± 73 pmol/min/mg protein in ionomycin treated tubules preincubated

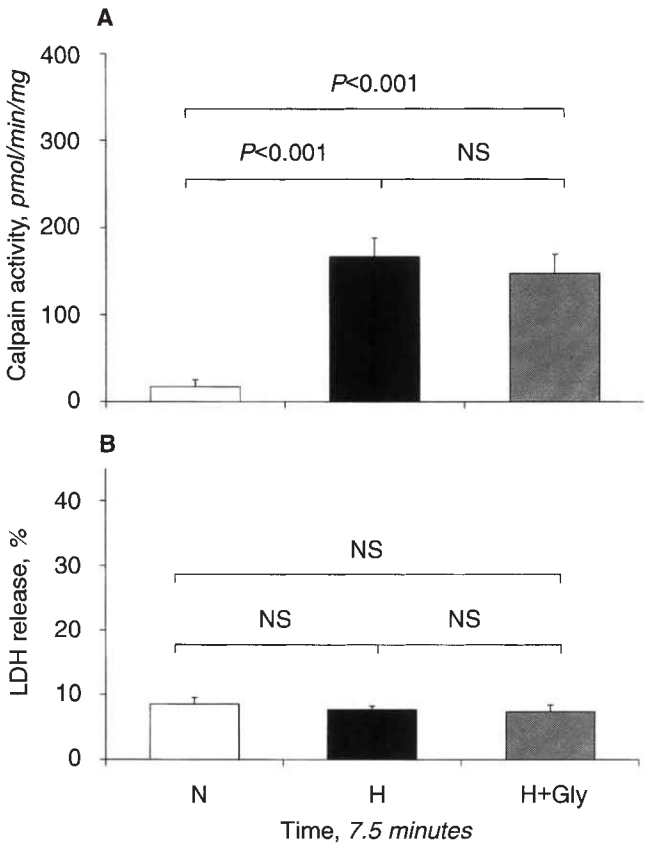

Fig. 1. Effect of 2 mM glycine (Gly) on (*A*) calpain activity and (*B*) LDH release at 7.5 minutes of hypoxia (H) (prelethal injury model). There was a prelethal increase in calpain activity compared to normoxic controls (N). Addition of 2 mM glycine did not inhibit the prelethal increase in calpain activity ($N = 12$).

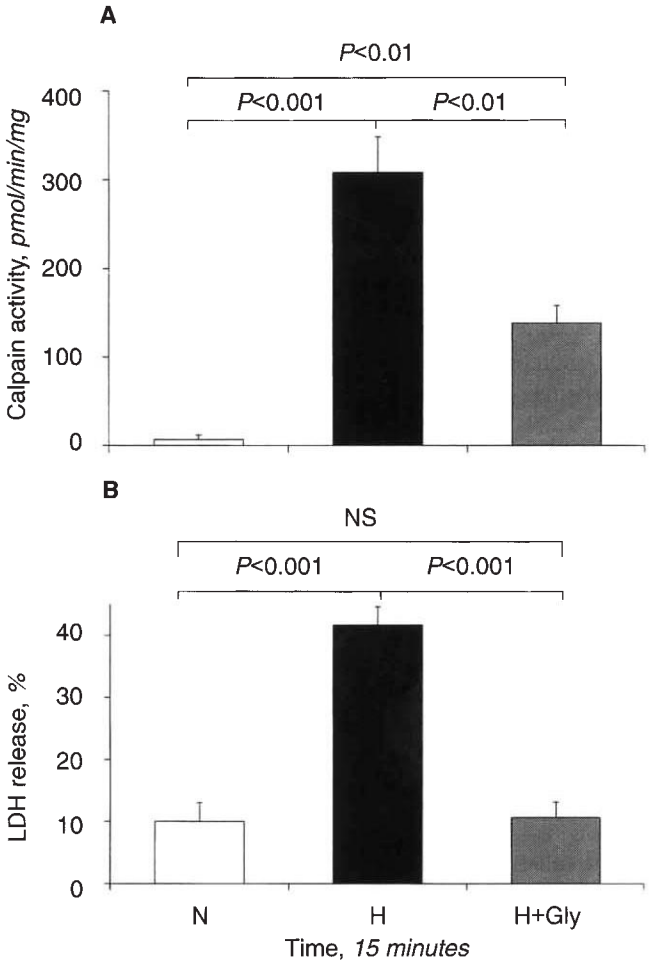

Fig. 2. Effect of 2 mM glycine (Gly) on (*A*) calpain activity and (*B*) LDH release at 15 minutes hypoxia (H) (postlethal injury model). At 15 minutes of hypoxia there was a fourfold increase of LDH release in hypoxic tubules that was completely prevented by glycine. Calpain activity increased to a higher level than seen at 7.5 minutes of hypoxia, and glycine significantly reduced the calpain activity to a level seen at 7.5 minutes of hypoxia ($N = 5$).

with glycine ($P < 0.05$ vs. control, NS vs. ionomycin treatment alone; $N = 4$; Fig. 3).

At 10 minutes there was an increase of LDH release in ionomycin treated tubules that was prevented by glycine (Fig. 4). There was an increase in calpain activity compared to normoxic controls that was not decreased by glycine. Calpain activity at 10 minutes was similar to that at two minutes. Calpain activity was 80 ± 40 pmol/min/mg protein in controls, 301 ± 33 pmol/min/mg protein after 10 minutes ionomycin ($P < 0.01$ vs. control) and 272 ± 5 pmol/min/mg protein in ionomycin treated tubules preincubated with glycine ($P < 0.05$ vs. control, NS vs. ionomycin treatment alone; $N = 4$; Fig. 4).

Calpain-specific spectrin breakdown products (intact cell assay)

Triton X-100 soluble and cytosolic extracts from normoxic and hypoxic (15 min) tubules were immunoblotted for spectrin breakdown products (BDPn). In Triton X-100 soluble extracts there was an increase in BDPn even in the presence of glycine (Fig. 5). In cytosolic extracts there was also an increase in BDPn in the presence of glycine (Fig. 6).

Direct effect of glycine on calpain activity

To ascertain whether glycine directly inhibits calpain activity, using the substrate N-succinyl-Leu-Tyr-AMC that we have found to be more sensitive than N-succinyl-Leu-Leu-Val-Tyr-AMC for detecting activity of purified calpains, we measured the *in vitro* activity of purified calpains in the absence and presence of increasing doses of glycine. The calpain activity of purified calpains was not affected by 2 mM and 10 mM glycine. The calpain activity (pmol/min/mg) of purified μ-calpain (20 μg) was 495 ± 33 with no added glycine, 561 ± 20 after the addition of 2 mM glycine and 522 ± 40 after the addition of 10 mM glycine ($N = 3$). The calpain activity (pmol/min/mg) of partially purified m-calpain (280 μg of lung extract) was 18 ± 1 with no added glycine, 18 ± 1 after addition of 2 mM glycine and 17 ± 2 after the addition of 10 mM glycine ($N = 3$). The calpain activity (pmol/min/mg) of extracts (500 μg of tubule protein) from hypoxic tubules was 39 ± 8 with no added glycine, 36 ± 8 after addition of 2 mM glycine and 38 ± 8 after addition of 10 mM glycine ($N = 5$). The calpain activity (pmol/min/mg) of extracts (500 μg of tubule protein) from ionomycin (5 μM) treated tubules was 26 ± 8 with no added glycine, 30 ± 10 after addition of 2 mM glycine and 30 ± 10 after addition of 10 mM glycine ($N = 5$).

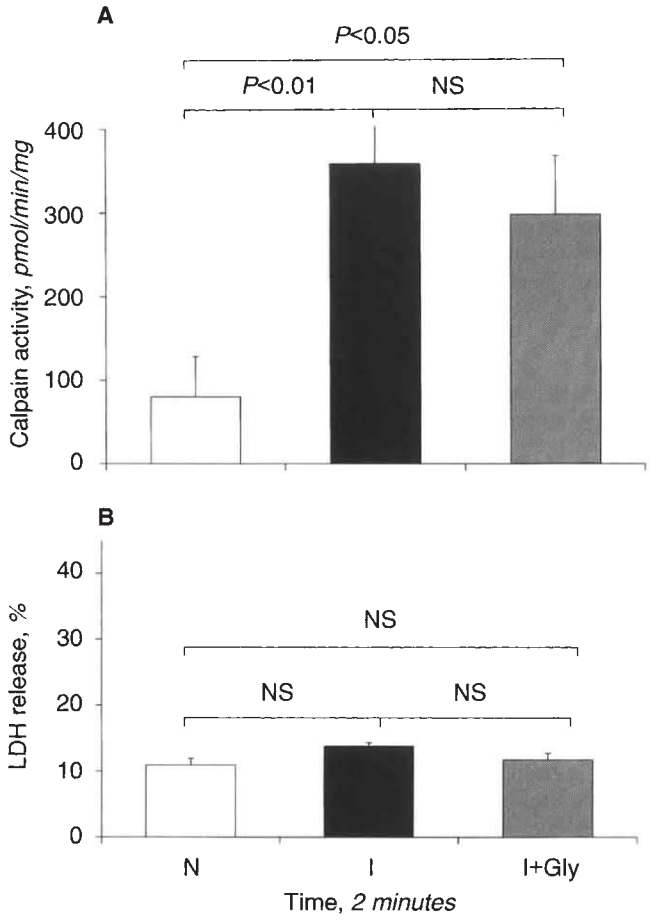

Fig. 3. Effect of two minutes of incubation of 5 μM ionomycin (I) and 2 mM glycine (Gly) on (*A*) calpain activity and (*B*) LDH release in normoxic (N) tubules. There was a prelethal increase in calpain activity in ionomycin treated tubules compared to normoxic controls. Addition of 2 mM glycine did not inhibit the prelethal increase in calpain activity ($N = 4$).

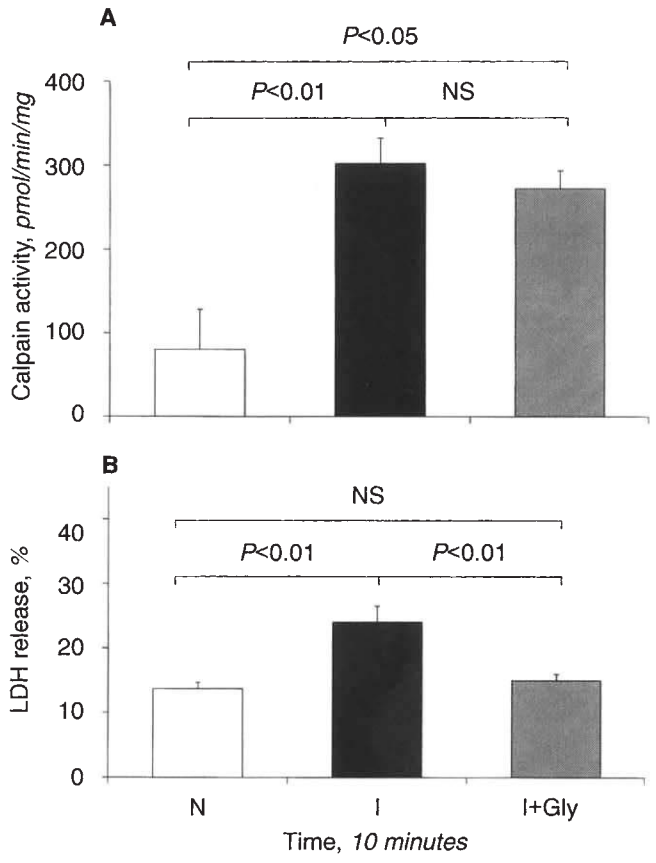

Fig. 4. Effect of 10 minutes of incubation of 5 μM ionomycin (I) and 2 mM glycine (Gly) on (*A*) calpain activity and (*B*) LDH release in normoxic (N) tubules. There was an increase in LDH release compared to normoxic controls. Addition of 2 mM glycine gave complete cytoprotection against cell membrane damage. There was an increase in calpain activity similar to that seen at two minutes ionomycin incubation. Addition of 2 mM glycine had no effect on ionomycin-induced calpain activity at 10 minutes ($N = 4$).

DISCUSSION

Calpain is the major Ca^{2+}-dependent cytosolic protease and is ubiquitously present in most cell types [37, 38]. Calpain exists in the cytosol as the inactive proenzyme, procalpain. Procalpain translocates from the cytosol to membrane in the presence of Ca^{2+}. Autocatalytic activation of procalpain occurs when it attaches to a phosphatidylinositol-binding site on the cell membrane (membrane activation theory of calpain activation) [38, 39]. Binding to the membrane-phospholipid is proposed to lower the Ca^{2+} concentration required for autolysis. The activated calpain is either released into the cytosol, where with its reduced Ca^{2+} requirement it is able to proteolyze substrate proteins, or it remains associated with the membrane and degrades cytoskeletal proteins involved in the interaction of the cell cytoskeleton with the plasma membrane. This activated calpain is measured in the *in vitro* assay. Since calpain is strictly a Ca^{2+}-dependent enzyme, the calpain activity measured *in vitro* is defined as only that which is Ca^{2+}-dependent. There is not much further autocatalytic conversion of procalpain to calpain in the *in vitro* assay as there is no cell membrane present.

Calpain is now known to be a mediator of hypoxic/ischemic injury to brain, liver, heart and kidneys [12, 13, 40–43]. In proximal tubules exposed acutely to cyclosporine, calpain activity increased, suggesting that calpain is involved in acute cyclosporin toxicity [44]. Also in proximal tubules, $HgCl_2$ toxicity correlated with increased ionized Ca^{2+} and the toxicity was diminished by calpain inhibitors [45]. Thus, the role of calpain as a mediator of cell injury is well established.

It is also well known that the availability of glycine in a cell is a major determinant of lethal cell membrane damage to anoxic, hypoxic, ischemic and toxin-induced insults in hepatocytes, proximal tubules and endothelial cells [1, 2, 17, 46, 47]. Since the physiological concentrations of glycine present *in vivo* [1, 11] are cytoprotective, the mechanism of calpain-induced structural and functional changes during cell injury is better understood and needs to be assessed in the presence of glycine. Studies were therefore undertaken to determine whether the cytoprotective effects of glycine involve calpain inhibition.

We have previously demonstrated the calcium-dependency of hypoxia-induced calpain activity [13]. Low $[Ca^{2+}]_i$ induced by both a low Ca^{2+} medium and the intracellular Ca^{2+} chelator 1,22-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), attenuated the increase in calpain activity seen at 15 minutes of

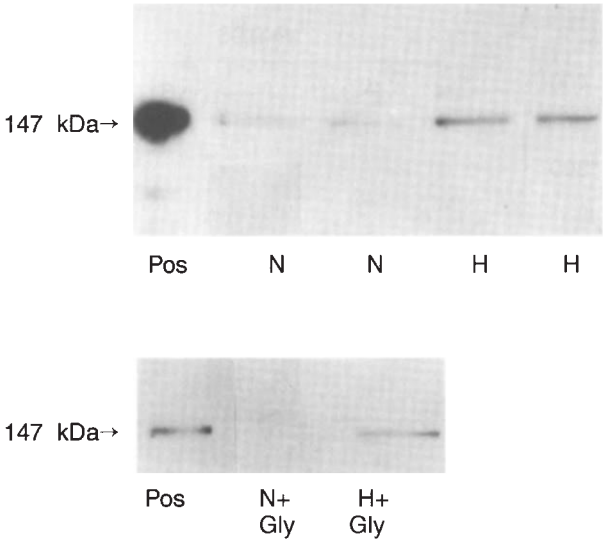

Fig. 5. Calpain specific spectrin breakdown products (BDP) in Triton X-100 soluble extracts from normoxic (N) and hypoxic (H; 15 min) tubules. BDP were increased in the presence and absence of 2 mM glycine (Gly). Pos represents positive controls (calcium-treated hippocampal slices; $N = 3$).

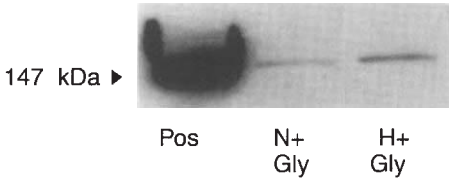

Fig. 6. Calpain-specific spectrin breakdown products (BDP) in cytosolic extracts from normoxic (N) and hypoxic (H; 15 min) tubules. BDP were increased even in the presence of glycine (Gly). Pos represents positive controls (calcium treated hippocampal slices; $N = 3$).

hypoxia. This attenuation of calpain activity by low $[Ca^{2+}]_i$ was also observed at 7.5 minutes before hypoxia-induced cell membrane damage (prelethal injury model). As further evidence of the calcium-dependency of calpain activity, we have demonstrated in normoxic proximal tubules that the calcium ionophore, ionomycin, which is known to induce a dose-dependent increase in $[Ca^{2+}]_i$, also induces a dose-dependent increase in calpain activity that preceeds cell membrane damage [12]. Using Fura-2/AM and Video imaging fluorescence microscopy (VIFM) [8], we have confirmed in PT that 3 μM and 5 μM ionomycin induce a dose-dependent increase in $[Ca^{2+}]_i$ within 30 seconds to levels of 1140 nM and 2160 nM, respectively.

We have previously shown in rat proximal tubules that the $[Ca^{2+}]_i$ increases from 100 nM to 400 nM during 7.5 minutes of hypoxia, and that this rise in $[Ca^{2+}]_i$ preceeds cell membrane injury as measured by propidium iodide (PI) uptake by the nuclei of necrotic cells [8]. Glycine was also shown to have no effect on this early prelethal increase in $[Ca^{2+}]_i$ [8]. In the present study, calpain activity was therefore measured at 7.5 minutes of hypoxia. There was a large increase in calpain activity at 7.5 minutes of hypoxia that was not significantly inhibited by glycine (Fig. 1).

However, the postlethal increase in $[Ca^{2+}]_i$ during hypoxic injury is inhibited by glycine [5, 8]. At 15 minutes, hypoxia-induced LDH release was 40%. In these lethally damaged cells, the cell membranes become Ca^{2+}-permeable, $[Ca^{2+}]_i$ increases and eventually equilibrates with that of the extracellular medium [15]. As a consequence of this postlethal increase in $[Ca^{2+}]_i$, there was a further increase in calpain activity at 15 minutes of hypoxia as compared to 7.5 minutes of hypoxia. Glycine, by preventing the postlethal increase in $[Ca^{2+}]_i$, also prevented the further increase in calpain activity. Specifically, calpain activity in the presence of glycine was the same at 7.5 and 15 minutes of hypoxia (Figs. 1 and 2).

By inducing an increase in $[Ca^{2+}]_i$, ionomycin (5 μM) stimulated an increase in calpain activity to levels seen at 15 minutes of hypoxia and higher than those seen at 7.5 minutes of hypoxia. This increase in calpain activity was maximal after two minutes of 5 μM ionomycin, before the LDH release occurred. The increase in calpain activity induced by ionomycin was not inhibited by glycine. This finding is compatible with earlier results that ionomycin-induced increases in $[Ca^{2+}]_i$ are not affected by glycine [5]. Thus, the cytoprotective effect of glycine seen at 10 minutes of ionomycin treatment is not mediated by inhibition of calpain.

Proteolysis of spectrin to its calpain-specific breakdown products is established as a measurement of calpain activation in intact cells [20, 32, 33]. This technique was therefore used to complement the direct *in vitro* measurement of calpain activity. A prelethal increase in calpain-specific spectrin breakdown products in rat proximal tubules was observed in the presence of glycine, thus confirming the prelethal calpain activation that was detected in the *in vitro* assay. A spectrin breakdown pattern similar to that induced by μ-calpain proteolysis of spectrin has been demonstrated in whole kidney extracts after both early [48] and late [14] ischemia. Molitoris, Dahl and Hosford [49] have recently demonstrated that intact spectrin in cultured proximal tubule cells dissociates from the cytoskeleton and translocates to the cytoplasm in a prelethal model of antimycin A-induced ATP depletion. In this previous study, spectrin was not degraded during prolonged ATP depletion. However, it was acknowledged in the paper that it was possible that the antibody used did not recognize spectrin breakdown products.

In hepatocytes, glycine has been found to more potently inhibit the high calcium-sensitive isoform, m-calpain, than the low calcium-sensitive isoform, μ-calpain [17]. The activity of m-calpain in hepatocytes is fivefold greater than the activity of μ-calpain [50]. It is also known that the increase in $[Ca^{2+}]_i$ during anoxia in rat hepatocyte suspensions is biphasic, consisting of an early small increase in $[Ca^{2+}]_i$ from 127 nM to 389 nM and a later larger increase reaching 1450 nM [51]. Thus, in hepatocytes, glycine's inhibition of later larger increases in $[Ca^{2+}]_i$ may inhibit activation of the high calcium-sensitive m-calpain. In rat proximal tubules, the low calcium-sensitive isoform, μ-calpain, is the active calpain isoform during hypoxia and ionomycin treatment [13].

In summary, calpain activity was measured both *in vitro* and in the intact cell. The present results demonstrate that (*1*) prelethal increases in calpain activity induced by hypoxia and the calcium ionophore, ionomycin, are not inhibited by glycine; and (*2*) the additional calpain activation observed after postlethal acceleration of calcium entry into the cell was inhibited by glycine. This study is also the first demonstration of prelethal hypoxia-induced spectrin breakdown in freshly isolated proximal tubules.

Thus, the cytoprotective effects of glycine in proximal tubules are not directly mediated by calpain inhibition. Rather, glycine

prevents hypoxia-induced membrane damage and thus further postlethal increases in $[Ca^{2+}]_i$ and calpain activity. The study of prelethal changes in Ca^{2+}-dependent enzymes therefore needs to be performed in the presence of glycine.

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