Neurosteroid inhibition of cell death

SHAYLA L. WATERS, GARY W. MILLER, MICHAEL D. ALEO, AND RICK G. SCHNELLMANN
Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Waters, Shayla L., Gary W. Miller, Michael D. Aleo, and Rick G. Schnellmann. Neurosteroid inhibition of cell death. *Am. J. Physiol.* 273 (*Renal Physiol.* 42): F869–F876, 1997.—Diverse γ-aminobutyric acid ($GABA_A$) receptor modulators exhibited novel cytoprotective effects and mechanisms of action in rabbit renal proximal tubules subjected to mitochondrial inhibition (antimycin A) or hypoxia. Cytoprotective potencies (50% effective concentration, EC_{50}) were 0.3 nM allopregnanolone (AP) > 0.4 nM 17α-OH-allopregnanolone (17α-OH-AP) > 30 nM dehydroepiandrosterone sulfate (DHEAS) = 30 nM pregnenolone sulfate (PS) > 500 nM pregnenolone (PREG) > 30 μM muscimol > 10 mM GABA following antimycin A exposure. Maximal protection with AP and 17α-OH-AP was 70%, whereas DHEAS, PS, PREG, and muscimol produced 100% cytoprotection. Experiments with AP, PS, and muscimol revealed the return of mitochondrial function and active Na^+ transport following hypoxia/reoxygenation. Muscimol inhibited the antimycin A-induced influx of both extracellular Ca^{2+} and Cl^- that occurs during the late phase of cell injury, whereas the neurosteroids only inhibited influx of Cl^-. Radioligand binding studies with AP and PS did not reveal a specific binding site; however, structural requirements were observed for cytoprotective potency and efficacy. In conclusion, we suggest that the $GABA_A$ receptor modulators muscimol and neurosteroids are cytoprotective at different cellular sites in the late phase of cell injury; muscimol inhibits Ca^{2+} and subsequent Cl^- influx, whereas the neurosteroids inhibit Cl^- influx.

renal proximal tubules; calcium influx; chloride influx; anoxia; γ-aminobutyric acid receptor

EXAMINATION OF THE TEMPORAL sequence of cell injury reveals that the onset of anoxia or the inhibition of mitochondrial function by a toxicant results in respiratory arrest, the loss of ATP, and decreased Na^+-K^+-adenosinetriphosphatase (Na^+-K^+-ATPase) activity (6). Na^+ influx and K^+ efflux occur concomitantly with the loss of Na^+-K^+-ATPase activity, and the normally negative membrane potential is diminished. During the late phase of cell injury in rabbit renal proximal tubules (RPT), influx of extracellular Ca^{2+} and Cl^- occurs (10, 17, 20, 29, 38, 39). Leaf (11) originally demonstrated that Cl^- influx is critical for the cell swelling produced by mitochondrial inhibition. Miller and Schnellmann (17, 20) subsequently showed that Cl^- influx occurs significantly after the loss of ATP and cation movement during the late phase of cell injury produced by a variety of toxicants. Decreasing the extracellular Cl^- concentration decreased Cl^- entry, cell swelling, and lysis, whereas increasing extracellular osmolality did not decrease Cl^- entry but did decrease cell swelling and lysis. These data suggest that Cl^- influx occurs during the late phase of cell injury and triggers the cell swelling that leads to cell lysis.

The cytoprotective effects of the neuronal glycine receptor agonist glycine and the antagonist strychnine have been reported in a number of in vitro and in vivo models against anoxia/hypoxia and a diverse group of toxicants. For example, glycine is cytoprotective in vivo against maleate-, ifosfamide-, and cisplatin-induced nephrotoxicity in the rat (8, 12, 25), against hypoxic injury in the isolated perfused rat kidney (2, 7, 33), reperfusion injury in the rat liver (40), cold ischemic injury in the isolated perfused rabbit and dog kidney (14, 30), and in vitro in rat hepatocytes (15), pulmonary artery endothelial cells (35), and human umbilical vein endothelial cells (40). The neuronal glycine receptor antagonist strychnine exhibits cytoprotective properties similar to glycine (16, 17, 42). Therapeutic realities of these compounds are limited due to the high concentrations (mM) necessary for cytoprotection and the additional neurotoxic effects of strychnine. Investigators have screened numerous agents in the hope of identifying more potent and efficacious cytoprotective compounds, including dipeptides and tripeptides with different amino acid constituents, glycine receptor antagonists, *N*-methyl-D-aspartate (NMDA) receptor antagonists, and other amino acid analogs (3, 9, 18, 41). However, none of the compounds thus far tested have been shown to meet or exceed the cytoprotective characteristics of glycine.

The γ-aminobutyric acid ($GABA_A$) receptor antagonist, bicuculline, and the benzodiazepine inverse agonist, norharmane, are partially protective to rabbit RPT exposed to the mitochondrial inhibitor antimycin A (1). Recently, Venkatachalam et al. (36) demonstrated that avermectin B_{1a} and its analogs, agonists at both the neuronal $GABA_A$ and glycine receptors, prevent hypoxic cell death in Madin-Darby canine kidney cells at concentrations as low as 100 μM. Thus avermectin B_{1a} is 10 times more potent than either glycine or strychnine. They also demonstrated the cytoprotective effects of the neuronal $GABA_A$ and glycine receptor antagonist cyanotriphenylboron (36).

These data prompted us to investigate the cytoprotective profile of additional $GABA_A$ receptor modulators in rabbit RPT including potency, efficacy, structural, and temporal aspects. We determined whether the $GABA_A$ receptor agonists GABA, muscimol, 4,5,6,7-tetrahydroisoxazolo[5.4-c]oyridin-3-ol hydrochloride (THIP), the $GABA_A$ receptor antagonist β-hydrastine, and the neurosteroid $GABA_A$ receptor modulators allopregnanolone (AP), 17α-OH-allopregnanolone (17α-OH-AP), pregnenolone sulfate (PS), pregnenolone (PREG), and dehydroepiandrosterone sulfate (DHEAS) (Fig. 1) *1*) prevent cell death produced by anoxia and mitochondrial inhibition, *2*) provide cytoprotection through the return of cellular functions, *3*) are cytoprotective during the late phase of cell injury, *4*) modulate the influx of extracellular Ca^{2+} that occurs during the late phase of cell injury, and *5*) modulate the Cl^- influx that occurs during the

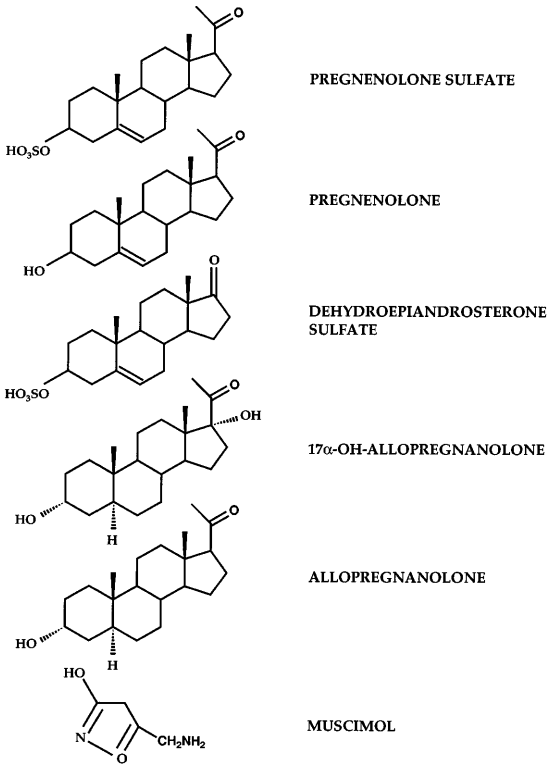

Fig. 1. Representative structures of five neurosteroids and muscimol.

late phase of cell injury. This study also examined the structural requirements necessary for neurosteroid cytoprotective potency or efficacy and whether the neurosteroids bind specifically to RPT.

MATERIALS AND METHODS

Materials. Antimycin A, GABA, THIP, β-hydrastine, and the neurosteroids AP (5α-pregnan-3α-ol-20-one), 17α-OH-AP (5α-pregnane-3α,17α-diol-20-one), PREG, PS, and DHEAS were purchased from Sigma Chemical (St. Louis, MO). Muscimol was obtained from Research Biochemicals International (Natick, MA). $Na^{36}Cl$, [^{14}C]dextran (85,000 mol wt), [^{3}H]AP, [^{3}H]PREG, and $^{45}CaCl_2$ were purchased from Dupont-NEN (Boston, MA). All other chemicals and agents were of reagent grade and obtained from either Sigma Chemical or Aldrich Chemical (Milwaukee, WI).

Isolation and incubation of rabbit RPT. RPT were isolated and purified by the method of Rodeheaver et al. (28) from female 1.5- to 2.0-kg New Zealand White rabbits (Myrtle's Rabbitry, Thompson Station, TN). RPT were suspended at a concentration of 1 mg/ml in an incubation buffer containing (in mM) 1 alanine, 5 dextrose, 2 heptanoate, 4 lactate, 5 malate, 115 NaCl, 15 $NaHCO_3$, 5 KCl, 2 NaH_2PO_4, 1 $MgSO_4$, 1 $CaCl_2$, and 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.4, 295 mosmol/kgH_2O). RPT suspensions were incubated under 95% air-5% CO_2 at 37°C in a gyrating water bath (180 revolutions/min). Following a 15-min preincubation period, 1 μM antimycin A and/or various concentrations of AP, 17α-OH-AP, PREG, PS, DHEAS, muscimol, THIP, GABA, or diluent (dimethyl sulfoxide, ≤0.5% total volume) was added to the RPT suspensions, and lactate dehydrogenase (LDH) release was determined 30 min later. In some experiments, muscimol or the neurosteroids were added 15 min after antimycin A, and LDH release was determined 15 min later. Tracer concentrations of $^{36}Cl^-$ or $^{45}Ca^{2+}$ were added 15 min after antimycin A, and aliquots were taken for analysis 15 min later.

Hypoxia/reoxygenation studies. Following a 15-min exposure to oxygenated conditions, RPT were subjected to hypoxia by incubating the tubules under 95% N_2-5% CO_2 for 1 h. Cytoprotectants were added immediately prior to the initiation of hypoxia. After the hypoxic exposure, RPT were reoxygenated with 95% air-5% CO_2 for 1 h, and oxygen consumption was measured. LDH samples were taken following both hypoxia and reoxygenation.

Biochemical analysis. Aliquots of RPT suspensions were taken at various times, and RPT were separated from the surrounding buffer by rapid centrifugation through a layer of dibutylphthalate-dioctylphthalate (2:1). The pellets were resuspended in Triton solubilization buffer [100 mM tris(hydroxymethyl)aminomethane, 150 mM NaCl, and 0.05% Triton X-100 at pH 7.5], and aliquots were taken for liquid scintillation spectrometry and protein content. Extracellular $^{36}Cl^-$ and $^{45}Ca^{2+}$ were corrected for by using the extracellular water marker [^{14}C]dextran. The release of LDH into the incubation buffer was used as a marker of cell death/lysis as described previously by Moran and Schnellmann (24). RPT ATP and protein contents were determined using high-performance liquid chromatography analysis (31) and the biuret method (5), respectively.

Oxygen consumption analysis. Oxygen consumption in RPT was measured using a Clark-type electrode as previously described (31). Following the measurement of basal oxygen consumption, the Na^+-K^+-ATPase inhibitor ouabain (0.1 mM) was added to obtain ouabain-insensitive oxygen consumption. Ouabain-sensitive oxygen consumption was calculated as the difference between basal and ouabain-insensitive oxygen consumption.

Radioligand binding. Concentration-dependent binding of [^{3}H]AP and [^{3}H]PS was determined by incubating RPT with radiolabeled compound (10 nM to 10 μM) in the absence and presence of 1 mM unlabeled AP and PS, respectively, at 4°C for 30 min. Time-dependent neurosteroid binding was conducted by incubating RPT with 10 nM [^{3}H]AP or 100 nM [^{3}H]PS in the absence and presence of 1 mM unlabeled AP or PS, respectively. Samples were taken at 1, 5, 10, 15, 30, and 60 min at 4°C or at 1, 2, 5, 10, and 15 min at 37°C. RPT were separated from the surrounding media by centrifugation through a layer of dibutylphthalate-dioctylphthalate (2:1), resuspended in Triton X-100 solubilization buffer as above, and radioactivity was determined using liquid scintillation spectrophotometry. Protein concentration was determined as above.

Statistics. Data are expressed as means ± SE. Each tubule preparation represented a separate experiment ($n = 1$). Percent protection was calculated using the following formula

$$100 - \left[\frac{(\%LDH_{sample} - \%LDH_{control})}{(\%LDH_{antimycin\,A} - \%LDH_{control})} \times 100\right]$$

$$= \text{percent protection}$$

where $\%LDH_{sample}$, $\%LDH_{antimycin\ A}$, and $\%LDH_{control}$ are the percent of LDH released in the sample, that treated with antimycin A, and in the control. Data were analyzed by analysis of variance. Multiple means were tested for significance using Student-Newman-Keuls post hoc examination of multiple groups ($P < 0.05$).

RESULTS

The cytoprotective properties of GABA, THIP, muscimol, β-hydrastine, and the neurosteroids in RPT exposed to antimycin A are illustrated in Fig. 2. The

neurosteroids were the most potent compounds examined, providing 50% cytoprotection at ~0.3 nM AP, 0.4 nM 17α-OH-AP, 30 nM DHEAS, 30 nM PS, and 500 nM PREG. Maximal cytoprotection of ~70% was observed at 10 nM with AP and 17α-OH-AP. In contrast, complete cytoprotection was observed with DHEAS, PS, and PREG at ~3, 3, and 30 μM, respectively. Muscimol and β-hydrastine were less potent cytoprotectants, providing 50% protection at ~30 and 20 μM, respectively. GABA and THIP were only partially protective at millimolar concentrations. GABA provided ~55% protection at 10 mM, whereas THIP provided ~45% protection at 1 mM.

To determine whether the cytoprotective properties of the neurosteroids and muscimol applied only to the prevention of cell lysis or also would allow RPT to regain mitochondrial function and active ion transport following the removal of the injurious insult, a model of RPT hypoxia/reoxygenation was utilized. The antimycin A model cannot be used for these studies, because antimycin A cannot be removed from the RPT (unpublished observations). Studies were conducted to determine the effects of AP, PS, and muscimol on LDH release, mitochondrial function, and active Na^+ transport following hypoxia/reoxygenation. Following a 15-min oxygenation period, 10 nM AP, 3 μM PS, or 100 μM muscimol was added to RPT immediately prior to 1 h of hypoxia, followed by 1 h of reoxygenation. LDH release increased to 70% following the hypoxic period and did not further increase after the reoxygenation period (Fig. 3). AP, PS, and muscimol completely ameliorated hypoxia-induced LDH release both after the hypoxic exposure and following reoxygenation. Associated with hypoxia/reoxygenation was a marked inhibition of basal oxygen consumption; however, RPT incubated with muscimol, PS, or AP had basal oxygen consumption

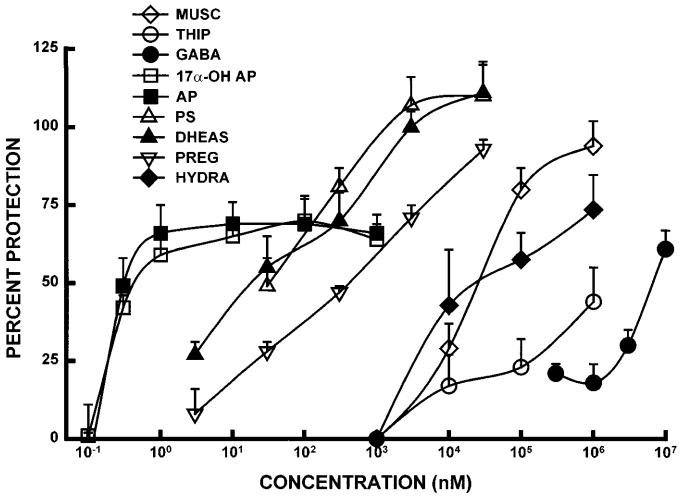

Fig. 2. Concentration-response curves of cytoprotective γ-aminobutyric acid ($GABA_A$) receptor modulators. Allopregnanolone (AP), 17α-OH-allopregnanolone (17α-OH-AP), pregnenolone sulfate (PS), pregnenolone (PREG), dehydroepiandrosterone sulfate (DHEAS), muscimol (MUSC), β-hydrastine (HYDRA), 4,5,6,7-tetrahydroisoxazolo[5.4-c]oyridin-3-ol hydrochloride (THIP), or GABA was added immediately prior to addition of 1 μM antimycin A. Lactate dehydrogenase (LDH) release was determined 30 min later and was 33 ± 1%. Control LDH release was 8 ± 2%. Symbols are means ± SE; n = 3–8.

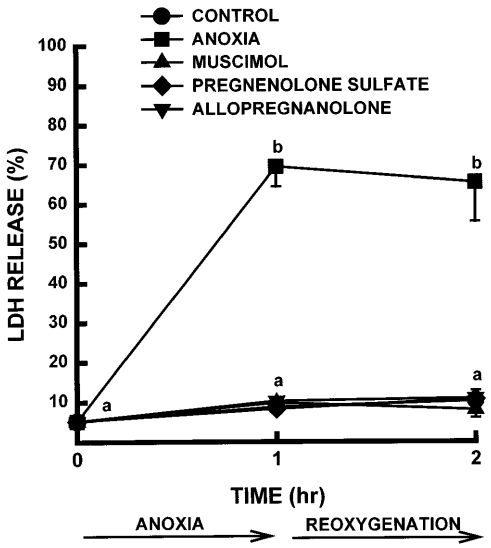

Fig. 3. Effect of muscimol, PS, and AP on LDH release following anoxia and reoxygenation. Muscimol (100 μM), AP (10 nM), or PS (3,000 nM) was added immediately prior to a 1-h anoxia exposure followed by an additional 1-h incubation under oxygenated conditions. Data points are means ± SE; n = 3–5. Data points with different superscripts are significantly different from each other ($P < 0.05$).

levels ~1.6 times greater than RPT subjected to hypoxia/reoxygenation (Fig. 4). Dissection of the basal respiration into its ouabain-sensitive and ouabain-insensitive components, representing respiration associated with active Na^+ transport and other ATP-consuming pathways, respectively, revealed that both components were inhibited following hypoxia/reoxygenation. Ouabain-insensitive respiration decreased ~28% and was not increased by muscimol, PS, or AP (data not shown). In contrast, ouabain-sensitive respiration was decreased 90% by hypoxia/reoxygenation, and muscimol, PS, and AP increased ouabain-sensitive respiration approximately eightfold following hypoxia/reoxygenation. These data suggest that not only do muscimol, PS, and AP prevent cell lysis as indicated by the inhibition of LDH release, but that these compounds also allow RPT cells to regain mitochondrial function and active Na^+ transport following hypoxia/reoxygenation.

To determine the temporal relationship for neurosteroid and muscimol cytoprotection, maximally cytoprotective concentrations of muscimol or the neurosteroids were added 15 min after antimycin A addition, during the late phase of cell injury. We have previously determined that ATP depletion occurs during the first 10 min following antimycin A addition and that ATP depletion results in Na^+ influx and K^+ efflux (unpublished observations). Muscimol and the neurosteroids produced similar cytoprotective effects when added 15 min after antimycin A (Fig. 5). Furthermore, 17α-OH-AP and AP were equally efficacious, and PS, PREG, DHEAS, and muscimol were equally efficacious. Additional evidence that the neurosteroids and muscimol act subsequent to ATP depletion was provided by the observation that these compounds do not reverse the ATP depletion produced by antimycin A. ATP content under control conditions was 6.8 ± 0.9 nmol/mg pro-

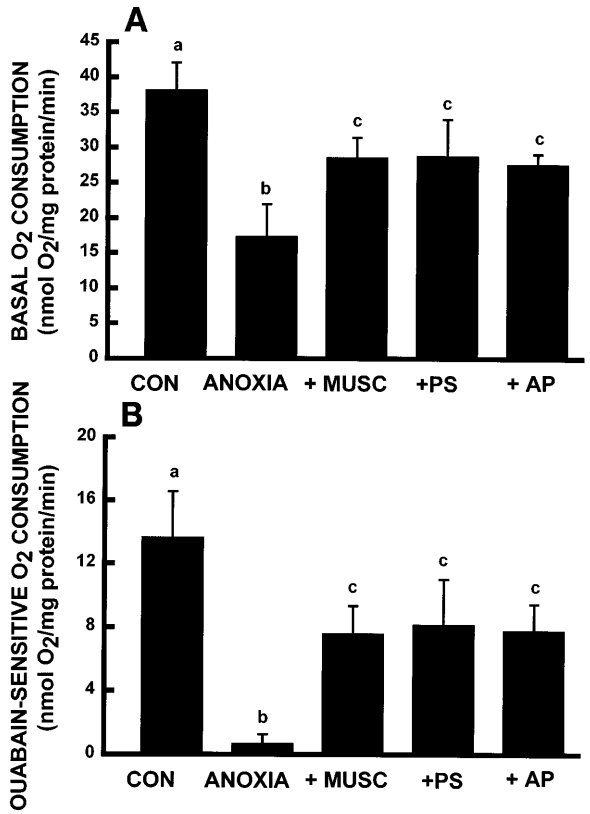

Fig. 4. Effect of muscimol, PS, and AP on basal (*A*) and ouabain-sensitive (*B*) oxygen consumption following anoxia (1 h) and reoxygenation (1 h). Muscimol (100 μM), AP (10 nM), or PS (3,000 nM) was added immediately prior to anoxia (Con, control). Bars are means ± SE; $n = 3–4$. Bars with different superscripts are significantly different from each other ($P < 0.05$).

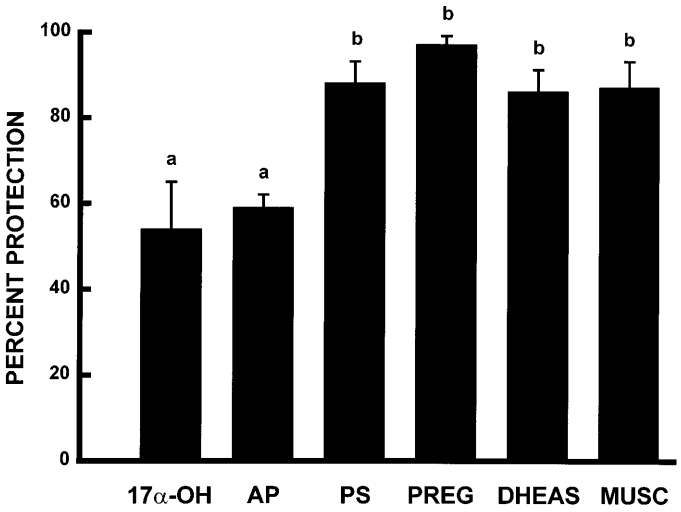

Fig. 5. Effect of late addition of the neurosteroids and muscimol on antimycin A-induced LDH release. AP (10 nM), 17α-OH-AP (10 nM), PS (3,000 nM), PREG (3,000 nM), DHEAS (3,000 nM), or muscimol (100 μM) was added 15 min after 1 μM antimycin A, and LDH release was determined 15 min later. LDH release in rabbit RPT incubated with antimycin A alone was 31 ± 2%. Control LDH release was 9 ± 1%. Bars are means ± SE; $n = 3–5$. Bars with different superscripts are significantly different from each other ($P < 0.05$).

tein. After a 30-min incubation in the presence of antimycin A, ATP levels decreased to 1.4 ± 0.2 nmol/mg protein and remained decreased in the presence of 100 μM muscimol (1.3 ± 0.3 nmol/mg protein), 10 nM AP (1.6 ± 0.3 nmol/mg protein), or 3 μM PS (1.7 ± 0.2 nmol/mg protein).

Influx of extracellular Ca^{2+} occurs in the late phase of mitochondrial inhibitor-induced cell injury (10, 29, 39). To determine the effect of neurosteroids and muscimol on antimycin A-induced Ca^{2+} influx, a tracer amount of $^{45}Ca^{2+}$ was added to RPT suspensions 15 min after the simultaneous addition of antimycin A and muscimol or a neurosteroid. Antimycin A increased Ca^{2+} influx 3.2-fold over vehicle control values (Fig. 6). Cytoprotective concentrations of muscimol, but not the neurosteroids, inhibited extracellular Ca^{2+} influx.

Cl^- influx also occurs during the late phase of cell injury and directly contributes to the cell swelling and death/lysis produced by antimycin A as well as a variety of other toxicants (17, 20, 27, 38). Therefore, we determined whether the neurosteroids and muscimol also inhibited Cl^- influx. A tracer amount of $^{36}Cl^-$ was added to RPT suspensions 15 min after the simultaneous addition of antimycin A and muscimol or a neurosteroid. Antimycin A increased RPT $^{36}Cl^-$ content 2.5-fold over controls (Fig. 7). Muscimol and the neurosteroids all blocked antimycin A-induced Cl^- influx. These results suggest that muscimol and the neurosteroids act in the late phase of cell injury at two distinct sites. Muscimol acts by blocking the influx of extracellular Ca^{2+}, whereas the neurosteroids act subsequent to Ca^{2+} influx but prior to the influx of Cl^-.

Radioligand binding studies were conducted to determine whether a specific binding site for the neurosteroids could be identified. No difference in binding was observed with increasing concentrations of [^{3}H]PS (10 nM to 10 μM) in the absence and presence of 1 mM unlabeled PS (Fig. 8*A*). No difference in binding with

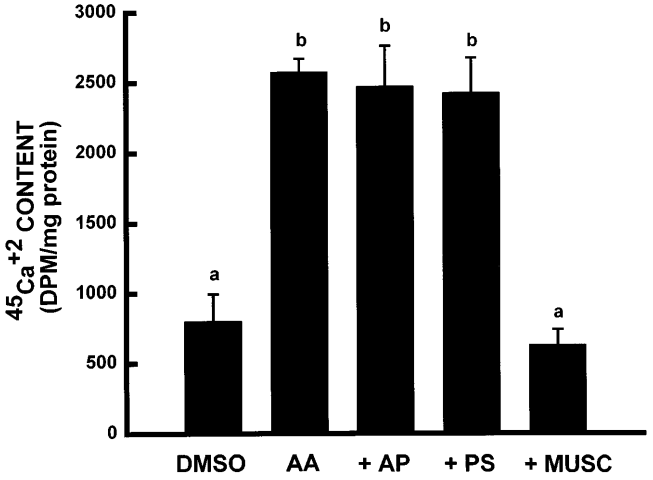

Fig. 6. Effect of muscimol, PS, and AP on antimycin A-induced extracellular Ca^{2+} influx. Muscimol (100 μM), AP (10 nM), or PS (3,000 nM) was added immediately prior to 1 μM antimycin A. A tracer quantity of $^{45}Ca^{2+}$ was added 15 min after antimycin A, and RPT $^{45}Ca^{2+}$ content was determined 15 min later. Bars are means ± SE; $n = 3$. DMSO, dimethyl sulfoxide. Bars with different superscripts are significantly different from each other ($P < 0.05$).

respect to time (from 1–60 min) was observed in RPT incubated with 100 nM [^{3}H]PS in the absence and presence of 1 mM unlabeled PS (Fig. 8*B*). Incubating RPT with 3,000 nM [^{3}H]PS in the presence of 1 μM antimycin A also did not result in specific neurosteroid binding (data not shown). Similar results were obtained using [^{3}H]AP. These data suggest that the cytoprotective effects of the neurosteroids may not be at the extracellular surface of the plasma membrane.

DISCUSSION

We have examined a variety of compounds that interact with the neuronal $GABA_A$ receptor for cytoprotective effects against mitochondrial inhibition and anoxia in rabbit RPT (Table 1). The neurosteroids AP and 17α-OH-AP [50% effective concentration (EC_{50}) of ~0.3 nM] were the most potent neurosteroids tested followed by PS, DHEAS, and PREG (EC_{50} values of ~30–500 nM). The $GABA_A$ receptor agonists THIP and GABA were the least potent cytoprotectants ($EC_{50} > 1$ mM). Thus the neurosteroids are ~10^3- to 10^5-fold more potent than muscimol, β-hydrastine, and avermectin B_{1a} and 10^4- to 10^6-fold more potent than bicuculline, norharmane, THIP, GABA, glycine, and strychnine in preventing antimycin A-induced RPT cell death (1, 36). The rank order of cytoprotective potency of $GABA_A$ receptor agonists/antagonists/modulators is AP = 17α-OH-AP > DHEAS = PS > PREG >>> β-hydrastine = muscimol = avermectin B_{1a} >>> THIP = bicuculline = norharmane > GABA.

Although AP and 17α-OH-AP were the most potent cytoprotectants, their efficacy was only 70%. In contrast, the neurosteroids PS, DHEAS, and PREG were completely efficacious. These studies demonstrate that neurosteroid structure is an important determinant of cytoprotective efficacy and potency. Structure activity analysis revealed that the absence of the double bond between C-5 and C-6 (AP and 17α-OH-AP) and/or the hydroxyl group in the α-position increases cytoprotective potency and decreases efficacy. The difference in the degree of potency and efficacy between DHEAS and PS and between AP and 17α-OH-AP suggests that substitution at the C-17 position is not as critical. Although Majewska (13) has suggested that PREG is not likely to be an active $GABA_A$ receptor modulator without a sulfate group, it is not clear whether the decreased cytoprotective potency of PREG is due to the absence of the sulfate group per se or decreased ability of RPT to sulfate PREG. Considering the cytoprotective potency of the neurosteroids and the structural requirements for cytoprotection, it is likely that the neurosteroids are acting in a specific manner.

To test whether the neurosteroids were acting at a receptor on the RPT plasma membrane, ligand binding studies were conducted. Radioligand binding assays did not reveal any specific binding of cytoprotective concentrations of the neurosteroids to the extracellular surface of the RPT plasma membrane. In contrast, this assay method did identify a specific strychnine binding site on the basolateral membrane of RPT (19), indicat-

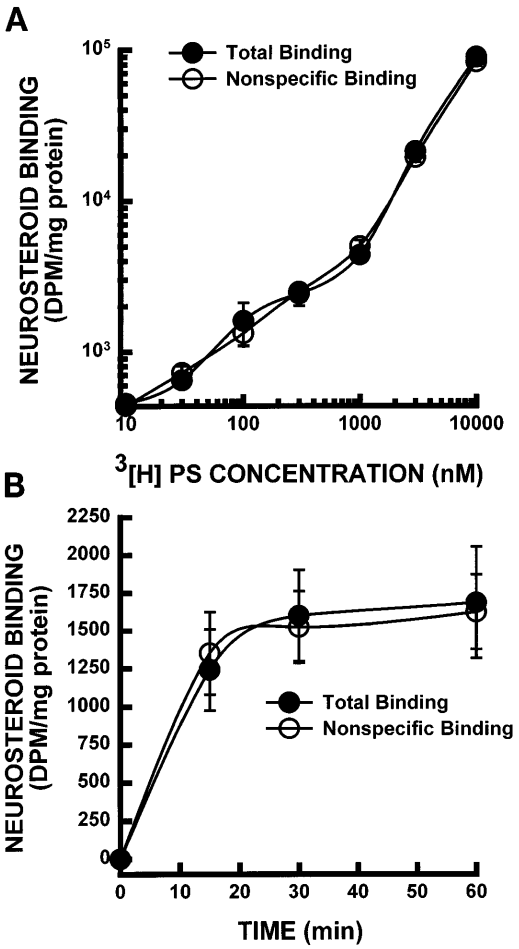

Fig. 8. Concentration-dependent (*A*) and time-dependent (*B*) binding of [^{3}H]PS to RPT. Nonspecific binding was determined in presence of 1 mM unlabeled PS. Time-dependent binding was conducted with 100 nM [^{3}H]PS. Data points are means ± SE; n = 4–5.

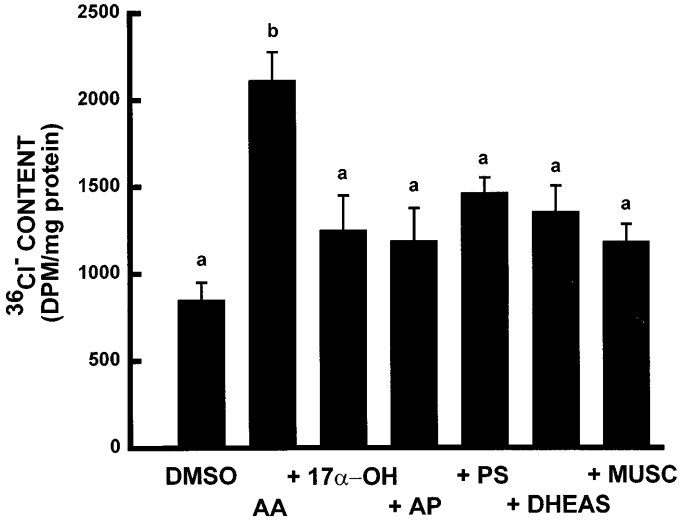

Fig. 7. Effect of neurosteroids and muscimol on antimycin A-induced Cl^- influx. AP (10 nM), 17α-OH-AP (10 nM), PS (3,000 nM), DHEAS (3,000 nM), or muscimol (100 μM) was added immediately prior to 1 μM antimycin A. A tracer quantity of $^{36}Cl^-$ was added 15 min after antimycin A, and RPT $^{36}Cl^-$ content was determined 15 min later. Bars are means ± SE; n = 4–5. Bars with different superscripts are significantly different from each other ($P < 0.05$).

Table 1. *Cytoprotective effects of agents that act on $GABA_A$ receptors*

Compound	EC_{50}	Reference
	Antagonists	
Competitive		
Bicuculline	~1 mM	1
Norharmane	~1 mM	1
β-Hydrastine	~20 μM	PS*
RU-5135	No effect (10 μM to 1 mM)	UO
Noncompetitive		
Picrotoxin	No effect (100 nM to 1 mM)	UO*
TBPS	No effect (0.03 nM to 3 μM)	UO*
Cyanotriphenylborate	~1 μM	35*
	Agonists	
Endogenous		
GABA	~10 mM	PS
Exogenous		
Muscimol	~30 μM	PS
THIP	~1 mM	PS
	Positive allosteric modulators	
Endogenous		
Allopregnanolone	~0.3 nM	PS
17α-OH-allopregnanolone	~0.4 nM	PS
Exogenous		
Flurazepam	No effect (1 nM to 1 mM)	UO
	Negative allosteric modulators	
Exogenous		
β-Carbolines	No effect (1 μM to 1 mM)	UO
	Bidirectional allosteric modulators	
Endogenous		
Pregnenolone	~500 nM	PS
Pregnenolone sulfate	~30 nM	PS
Dehydroepiandrosterone sulfate	~30 nM	PS
Exogenous		
Avermectin B_{1a}	~50 μM	35*
	Chloride channel inhibitor	
Niflumic acid	~30 μM	36

*Studies that used MDCK cells treated with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) in the absence of medium glucose; all other studies used rabbit renal proximal tubule suspensions treated with antimycin A. EC_{50}, 50% effective concentration; TBPS, *t*-butylbicyclophosphorothionate; THIP, 4,5,6,7-tetrahydroisoxazolo[5.4-c]oydrin-3-ol hydrochloride; PS, present study; UO, unpublished observations.

ing the validity of the binding assay. These results suggest that, although the neurosteroids are very potent and require strict structural requirements for cytoprotection, they do not bind specifically to the extracellular site of a plasma membrane receptor in a classic ligand/receptor fashion. Since the neurosteroids are very lipophilic, it is possible that they act within the lipid environment of the membrane or intracellularly.

Investigations have revealed temporal markers of initial and late phases in rabbit RPT cell injury. Gullans et al. (6) have shown that respiration is inhibited in RPT within 1 min of addition of antimycin A. During the next 10 min, ATP levels decrease, Na^+-K^+-ATPase activity is inhibited, intracellular K^+ levels decrease, and intracellular Na^+ levels increase. During the late phase of cell injury in rabbit RPT, Cl^- influx occurs followed by cell swelling and lysis (17, 20). The observations that the neurosteroids and muscimol prevented cell death/lysis when added 15 min after antimycin A, did not preserve ATP levels, and blocked antimycin A-induced Cl^- influx indicate that these compounds are acting subsequent to mitochondrial dysfunction, ATP depletion, and Na^+/K^+ imbalances and prior to Cl^- influx in the late phase of cell injury. The inability of the neurosteroids and muscimol to preserve ATP concentrations is consistent with studies showing that cytoprotective concentrations of glycine do not reverse the ATP depletion caused by antimycin A (36).

Ca^{2+} influx also occurs in the late phase of cell injury in rabbit RPT (10, 29, 39). Furthermore, we have recently shown that *1*) Ca^{2+} influx occurs in the late phase of cell injury produced by antimycin A, *2*) the Ca^{2+} channel blocker nifedipine blocks antimycin A-induced Ca^{2+} influx, *3*) nifedipine blocks cell death/lysis, and *4*) nifedipine blocks antimycin A-induced Cl^- influx (37). These results are consistent with the previous observation that Cl^- channel inhibitors reported to inhibit Ca^{2+}-activated Cl^- channels (34), 5-nitro-2-(3-phenylpropylamino)benzoate, indanyloxyacetic acid (i.e., IAA-94), and niflumic acid, inhibit Cl^- influx and are cytoprotective against antimycin A-induced RPT cell death/lysis (27, 38). Experiments designed to determine whether the neurosteroids and muscimol inhibit antimycin A-induced Ca^{2+} influx revealed that muscimol inhibited Ca^{2+} influx, whereas the neurosteroids did not. Therefore, the cytoprotective actions of muscimol and the neurosteroids are at two different sites; muscimol acts prior to and blocks Ca^{2+} influx, whereas the neurosteroids act subsequent to Ca^{2+} influx and prior to Cl^- influx. Figure 9 illustrates our current hypothesis for the mechanism of cytoprotection produced by the neurosteroids and muscimol in the late phase of cell injury. Further research is needed to thoroughly dissect the cytoprotective mechanisms of action of the neurosteroids and muscimol and correlate cytosolic free Ca^{2+} levels with cell injury and cytoprotection.

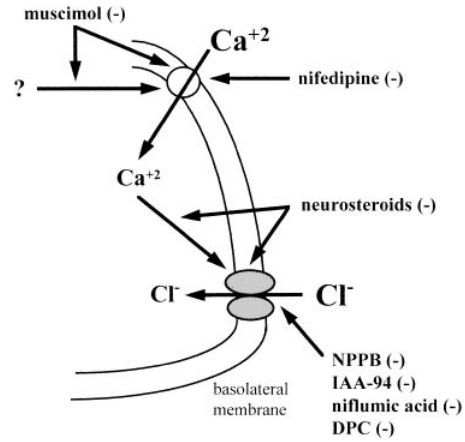

Fig. 9. Current hypothesis for the mechanism of cytoprotection produced by the neurosteroids and muscimol during the late phase of cell injury following mitochondrial inhibition. NPPB, 5-nitro-2-(3-phenylpropylamino)benzoate; IAA-94, indanyloxyacetic acid; DPC, diphenylamine-2-carboxylate.

Our laboratory has observed that a variety of compounds including glycine receptor agonists and antagonists, $GABA_A$ receptor agonists, antagonists, and modulators (1, 16–18; also, above data), and Cl^- channel inhibitors (38) inhibit toxicant- and anoxia-induced extracellular Cl^- influx and cell death/lysis in rabbit RPT suspensions. These results support Leaf's (11) original demonstration that Cl^- influx is critical for the cell swelling produced by mitochondrial inhibition. In contrast, Venkatachalam et al. (36) have suggested that the cytoprotective effects of glycine, strychnine, cyanotriphenylboron, and avermectin B_{1a} and its analogs are not the result of the inhibition of Cl^- influx. However, in their experiments, Cl^- influx was not examined. In addition, their Cl^- substitution experiments were conducted under conditions of complete Cl^- substitution with either gluconate or sucrose, a condition which is inherently damaging to the cells (Miller and Schnellmann, unpublished observations). Although it is not clear whether glycine and $GABA_A$ receptor modulators act directly on a Cl^- channel to inhibit Cl^- influx or indirectly immediately prior to Cl^- influx, the data strongly suggest that the inhibition of Cl^- influx is involved in the cytoprotection.

Characterization of a large variety of $GABA_A$ receptor modulators demonstrated that agonists, antagonists, and modulators were cytoprotective in RPT (Table 1). The original characterizations of the actions of these $GABA_A$ receptor compounds and their subsequent categorization into pharmacological groups were conducted in neuronal tissue. Their action as cytoprotectants in nonneuronal tissue does not appear to follow the same pattern as that observed in neuronal tissue. In some cases, millimolar concentrations of antagonists and agonists exhibit cytoprotection (bicuculline, norharmane, GABA, THIP), whereas other antagonists and modulators exhibit no cytoprotection (RU-5135, picrotoxin, *t*-butylbicyclophosphorothionate, flurazepam, β-carboline-3-carboxylic acid *N*-methyl). In addition, differences were observed in the mechanism of cytoprotection between the agonist muscimol and the neurosteroids. Finally, the lack of a specific binding site for the most potent cytoprotectants with specific structural determinants implies that these compounds may act at a site other than the extracellular surface of the plasma membrane. These observations question whether a $GABA_A$-like receptor is present in RPT and plays a role in cell injury/death.

Preliminary studies by Plotkin et al. (26) and Molony et al. (21, 22) identified the $GABA_A$ receptor β_3-subunit in rat kidney cortex and α_1-subunit mRNA in rat kidney medulla, respectively. Investigators have shown also that GABA-catabolizing and GABA-anabolizing enzymes, and two GABA uptake systems exist in the rat renal cortex (4, 32). Recent studies have shown that the $GABA_A$ receptor agonists GABA and muscimol and the antagonist bicuculline elicit a concentration-dependent increase in the fractional excretion of both water and sodium in the isolated perfused rat kidney (23). The differential cytoprotective effects and potencies of the $GABA_A$ receptor agonists/antagonists/modulators indicate that if a $GABA_A$-like receptor does exist in the kidney, then it is significantly different from the neuronal form of the receptor.

AP, PS, and muscimol completely ameliorated hypoxia/reoxygenation-induced cell lysis as determined by the prevention of LDH release. The cytoprotective effects of the neurosteroids and muscimol were not restricted to the prevention of cell lysis but also allowed the RPT to regain cellular functions. The addition of AP, PS, or muscimol to RPT subjected to hypoxia/reoxygenation improved basal oxygen consumption 1.6-fold. Furthermore, AP, PS, and muscimol increased active Na^+ transport from 10% of controls in hypoxia/reoxygenation-treated RPT to 80% of controls. These data strongly suggest that the neurosteroids and muscimol not only prevent RPT cell lysis but also enable the restoration of normal mitochondrial function and ion transport and therefore are true cytoprotectants.

Numerous investigators have discussed the cascade of events that lead to cell death and the "point of no return." The idea is that once the point of no return is reached, any intervention will not prevent the cell from dying. Although our data do not specifically identify the point of no return, the observation that the neurosteroids do not block extracellular Ca^{2+} influx but allow the cells to regain mitochondrial function and active Na^+ transport suggests that the point of no return is distal to Ca^{2+} influx.

In summary, this study has demonstrated that compounds that interact with the neuronal $GABA_A$ receptor are potent cytoprotectants. Nanomolar concentrations of the neurosteroids are cytoprotective in RPT subjected to mitochondrial inhibition and hypoxia with strict structural requirements for both efficacy and potency. The mechanism of action for neurosteroid cytoprotection does not appear to involve specific receptor binding to the extracellular surface of the RPT plasma membrane. The neurosteroids and muscimol are cytoprotective during the late phase of cell death and act differently: muscimol inhibits antimycin A-induced Ca^{2+} influx, whereas the neurosteroids inhibit Cl^- influx.

We thank Satinder S. Sarang and Jeffrey H. Moran for technical assistance.

S. L. Waters is a recipient of an Arkansas American Heart Association Predoctoral Fellowship.

Portions of this work have been published in abstract form and were presented at the 1995 American Society of Nephrology meeting in San Diego, CA (*J. Am. Soc. Nephrol.* 6: 1006, 1995), at the 1996 Society of Toxicology meeting in Anaheim, CA (*Fund. Appl. Toxicol.* 30: 304, 1996), and at the 1996 American Society of Nephrology meeting in New Orleans, LA (*J. Am. Soc. Nephrol.* 7: 1836, 1996).

Current addresses: G. W. Miller, Dept. of Cell Biology, Duke Univ., Durham, NC 27710; and Michael D. Aleo, Drug Safety Evaluation, Pfizer, Groton, CT 06340.

Address for reprint requests: R. G. Schnellmann, Division of Toxicology, Slot 638, 4301 W. Markham, Little Rock, AR 72205-7199.

Received 21 January 1997; accepted in final form 24 July 1997.

REFERENCES

1. **Aleo, M. D., and R. G. Schnellmann.** The neurotoxicants strychnine and bicuculline protect renal proximal tubules from mitochondrial inhibitor induced cell death. *Life Sci.* 51: 1783–1787, 1992.

2. **Baines, A. D., N. Shaikh, and P. Ho.** Mechanism of perfused kidney cytoprotection by alanine and glycine. *Am. J. Physiol.* 259 (*Renal Fluid Electrolyte Physiol.* 28): F80–F87, 1990.
3. **Cox, A. G., E. W. Taylor, G. W. Miller, and R. G. Schnellmann.** Comparative molecular field analysis of amino acids having a cytoprotective effect in renal proximal tubules. *Proc. Natl. Med. Chem. Symposium 1994.*
4. **Erdo, S. L., and J. R. Wolff.** γ-Aminobutyric acid outside the mammalian brain. *J. Neurochem.* 54: 363–372, 1990.
5. **Gornall, A. G., C. J. Bardawill, and M. M. David.** Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177: 751–766, 1949.

5a. **Groves, C. E., and R. G. Schnellmann.** Suspension of rabbit renal proximal tubules. In: *Methods in Renal Toxicology*, edited by R. K. Zalups and L. H. Lash. Boca Raton, FL: CRC, 1996, p. 147–162.

6. **Gullans, S. R., P. C. Brazy, B. C. Soltoff, V. W. Dennis, and L. J. Mandel.** Metabolic inhibitors: effects of metabolism and transport in the proximal tubule. *Am. J. Physiol.* 243 (*Renal Fluid Electrolyte Physiol.* 12): F133–F140, 1982.
7. **Heyman, S. N., M. Brezis, F. H. Epstein, K. Spokes, and S. Rosen.** Effect of glycine and hypertrophy on renal outer medullary hypoxic injury in ischemia reflow and contrast nephropathy. *Am. J. Kidney Dis.* 19: 578–568, 1992.
8. **Heyman, S. N., S. Rosen, P. Silva, K. Spokes, M. J. Egorin, and F. H. Epstein.** Protective action of glycine in cisplatin nephrotoxicity. *Kidney Int.* 40: 273–279, 1991.
9. **Heyman, S. N., K. Spokes, M. J. Egorin, and M. J. Epstein.** Mechanism of glycine protection in hypoxic injury: analogies with glycine receptor. *Kidney Int.* 42: 41–45, 1992.
10. **Jacobs, W. R., M. Sgambati, G. Gomez, P. Vilaro, M. Higdon, P. D. Bell, and L. J. Mandel.** Role of cytosolic Ca in renal tubule damage induced by anoxia. *Am. J. Physiol.* 260 (*Cell Physiol.* 29): C545–C554, 1991.
11. **Leaf, A.** On the mechanism of fluid exchange of tissues in vitro. *Biochemistry* 62: 241–248, 1956.
12. **Li, Q., C. J. Bowmer, and M. S. Yates.** Amelioration of cisplatin nephrotoxicity with glycine: dose dependency in rats. *J. Pharm. Pharmacol.* 47: 223–226, 1995.
13. **Majewska, M. D.** Neurosteroids: endogenous bimodal modulators of the $GABA_A$ receptor. Mechanism of action and physiological significance. *Prog. Neurobiol.* 38: 379–395, 1992.
14. **Mangino, M. J., M. K. Murphy, G. G. Grabau, and C. B. Anderson.** Protective effects of glycine during hypothermic renal ischemia-reperfusion injury. *Am. J. Physiol.* 261 (*Renal Fluid Electrolyte Physiol.* 30): F841–F848, 1991.
15. **Marsh, D. C., P. K. Hjelmhaug, P. K. Vreugdenhill, F. O. Belzer, and J. H. Southard.** Glycine prevention of cold ischemic injury in isolated hepatocytes. *Cryobiology* 28: 105–109, 1991.
16. **Miller, G. W., E. A. Lock, and R. G. Schnellmann.** Strychnine and glycine protect renal proximal tubules from various nephrotoxicants and act in the late phase of necrotic cell injury. *Toxicol. Appl. Pharmacol.* 125: 192–197, 1994.
17. **Miller, G. W., and R. G. Schnellmann.** Cytoprotection by inhibition of chloride channels: the mechanism of action of glycine and strychnine. *Life Sci.* 53: 1211–1215, 1993.
18. **Miller, G. W., and R. G. Schnellmann.** A putative cytoprotective receptor in the kidney: relation to the neuronal strychnine-sensitive glycine receptor. *Life Sci.* 55: 27–34, 1994.
19. **Miller, G. W., and R. G. Schnellmann.** A novel low-affinity strychnine binding site on renal proximal tubules: role in toxic cell death. *Life Sci.* 53: 1203–1209, 1993.
20. **Miller, G. W., and R. G. Schnellmann.** Inhibitors of renal chloride transport do not block toxicant-induced chloride influx in the proximal tubule. *Toxicol. Lett.* 76: 179–184, 1995.
21. **Molony, D., R. Hamilton, A. Holian, and P. S. Mehta.** Renal localization of mRNA for a murine $GABA_A$ chloride channel and increased expression after in vivo pesticide exposure (Abstract). *J. Am. Soc. Nephrol.* 5: 294, 1994.
22. **Molony, D., T. Pressley, T. DuBose, and P. S. Mehta.** Murine renal medulla expresses a $GABA_A$ α_1 Cl^- channel that differs in cDNA sequence from the CNS channel (Abstract). *J. Am. Soc. Nephrol.* 4: 875, 1993.
23. **Monasterolo, L. A., L. Trumper, and M. M. Elias.** Effects of γ-aminobutyric acid agonists on the isolated perfused rat kidney. *J. Pharmacol. Exp. Ther.* 279: 602–607, 1996.
24. **Moran, J. H., and R. G. Schnellmann.** A rapid β-NADH-linked fluorescence assay for lactate dehydrogenase in cellular death. *J. Pharmacol. Toxicol. Methods* 36: 41–44, 1996.
25. **Nissim, I., and J. M. Weinberg.** Glycine attenuates Fanconi syndrome induced by maleate or ifosfamide in rats. *Kidney Int.* 49: 684–695, 1996.
26. **Plotkin, M., R. Kojima, and S. R. Gullans.** Colocalization of GABA transporter and receptor in rat kidney (Abstract). *J. Am. Soc. Nephrol.* 5: 318, 1994.
27. **Reeves, W. B.** Effects of chloride channel blockers on hypoxic injury in rat proximal tubules. *Kidney Int.* 51: 1529–1534, 1997.
28. **Rodeheaver, D. P., M. D. Aleo, and R. G. Schnellmann.** Differences in enzymatic and mechanical isolated rabbit renal proximal tubules: comparison in long-term incubation. *In Vitro Cell. Dev. Biol.* 26: 898–904, 1990.
29. **Rose, U. M., R. J. M. Bindels, J. W. C. M. Jansen, and C. H. van Os** Effects of Ca^{2+} channel blockers, low Ca^{2+} medium and glycine on cell Ca^{2+} and injury in anoxic rabbit proximal tubules. *Kidney Int.* 46: 223–229, 1994.
30. **Schilling, M. K., G. den Butter, A. Saunder, S. Lindell, F. O. Belzer, and J. H. Southard.** Membrane stabilizing effects of glycine during kidney cold storage and reperfusion. *Transplant. Proc.* 23: 2387–2389, 1991.
32. **Sidhu, H. S., and J. D. Wood.** Gamma-aminobutyric acid uptake by rat kidney brush-border membrane vesicles. *Experientia* 45: 726–728, 1989.
33. **Silva, P., S. Rosen, K. Spokes, and F. H. Epstein.** Effect of glycine on medullary thick ascending limb injury in perfused kidneys. *Kidney Int.* 39: 653–658, 1991.
34. **Sorota, S.** Pharmacologic properties of the swelling-induced chloride current of dog atrial myocytes. *J. Cardiovasc. Electrophysiol.* 5: 1006–1016, 1994.
35. **Varani, J., I. Ginsburg, K. J. Johnson, D. F. Gibbs, J. M. Weinberg, and P. A. Ward.** Amino acids and metal ions protect endothelial cells from lethal injury (Abstract). *FASEB J.* 5: 887, 1991.
36. **Venkatachalam, M. A., J. M. Weinberg, Y. Patel, P. Saikumar, and Z. Dong.** Cytoprotection of kidney epithelial cells by compounds that target amino acid gated chloride channels. *Kidney Int.* 49: 449–460, 1996.
37. **Waters, S. L., S. S. Sarang, K. K. W. Wang, and R. G. Schnellmann.** Calpains mediate calcium and chloride influx during the late phase of cell injury. *J. Pharmacol. Exp. Ther.* In press.
38. **Waters, S. L., and R. G. Schnellmann.** Extracellular acidosis and chloride channel inhibitors act in the late phase of cellular injury to prevent death. *J. Pharmacol. Exp. Ther.* 278: 1012–1017, 1996.
39. **Weinberg, J. M., J. A. Davis, N. F. Roeser, and M. A. Venkatachalam.** Role of increased cytosolic free calcium in the pathogenesis of rabbit proximal tubule cell injury and protection by glycine or acidosis. *J. Clin. Invest.* 87: 581–590, 1991.
40. **Weinberg, J. M., J. Varani, K. J. Johnson, N. F. Roeser, M. K. Dame, J. A. Davis, and M. A. Venkatachalam.** Protection of human umbilical vein endothelial cells by glycine and structurally related amino acids against calcium and hydrogen peroxide-induced lethal cell injury. *Am. J. Pathol.* 140: 457–471, 1992.
41. **Weinberg, J. M., M. A. Venkatachalam, R. Garza-Quintero, N. F. Roeser, and J. A. Davis.** Structural requirements for protection by small amino acids against hypoxic injury in kidney proximal tubules. *FASEB J.* 4: 3347–3354, 1990.
42. **Zhong, A., S. Jones, and R. G. Thurman.** Glycine minimizes reperfusion injury in a low-flow reflow liver perfusion model in the rat. *Am. J. Physiol.* 270 (*Gastrointest. Liver Physiol.* 33): G332–G338, 1996.