Kidney International, Vol. 49 (1996), pp. 449–460

Cytoprotection of kidney epithelial cells by compounds that target amino acid gated chloride channels

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Cytoprotection of kidney epithelial cells by compounds that target amino acid gated chloride channels. Glycine, strychnine and certain chloride channel blockers were reported to protect cells against lethal cell injury. These effects have been attributed to interactions with membrane proteins related to CNS glycine gated chloride channel receptors. We have investigated the pharmacology of these actions. Madin-Darby canine kidney (MDCK) epithelial cells were depleted of adenosine triphosphate (ATP) by incubation in glucose free medium containing a mitochondrial uncoupler. Medium Ca^{2+} was adjusted to 100 nM in the presence of an ionophore such that intracellular Ca^{2+} did not increase, and Ca^{2+}-related injury mechanisms were inhibited. This permitted more sensitive quantitation of protection against cell injury attributable to glycine or other agents whose actions might be related to those of the amino acid. Two classes of compounds showed cytoprotective activity in this system: (1) ligands at chloride channel receptors, such as glycine, strychnine and avermectin B1a; (2) chloride channel blockers, including cyanotriphenylboron and niflumic acid, both of which are known to bind to channel domains of CNS glycine receptors. Morphological and functional studies showed that the compounds preserved plasma membrane integrity, but permitted cell swelling. Substitution of medium chloride by gluconate, or chloride salts by sucrose, did not substantially modify lethal damage or its prevention by glycine or other drugs. The compounds did not modify ATP declines. At least for some compounds, cytoprotection appeared to be specific to structural features on the molecules. These observations are consistent with the hypothesis that a plasma membrane protein related to glycine-gated chloride channel receptors plays a significant role in cell injury, but indicate that the mechanisms of injury and protection by compounds active in this system are not related to chloride fluxes.

Glycine and related amino acids protect cells from the lethal effects of ATP depletion without modifying metabolism [1–10]. Strychnine has similar actions [11, 12]. Overlaps between structure-activity relationships of amino acids as agonists at ligand-gated chloride channel receptors [13], and as cytoprotective agents [2–4], have suggested that amino acids may protect cells by low affinity interactions with a protein that is related to CNS glycine receptors [3, 4]. It has been suggested that strychnine protects cells by a similar mechanism [14, 15]. According to this view, ATP depletion causes lethal chloride influx through the receptors, which is prevented by glycine and strychnine [14]. Reports that chloride channel blockers are also cytoprotective [14, 16] would seem to support this view. However, this hypothesis is inconsistent with actions of glycine and strychnine on glycine receptors; glycine stimulates chloride currents, whereas strychnine is an antagonist [13].

We investigated possible cytoprotective effects of two broad classes of compounds: agents with activities at amino acid gated chloride channels, and several species of chloride channel blockers. A premise for these experiments was that the protective actions of these compounds, if any, might be directed at mechanisms targeted by glycine. Consequently, we studied their effects in the context of membrane damage that is stringently dependent on glycine deficiency, is completely retarded by exogenous amino acid, and is uncomplicated by glycine-insensitive structural disruption caused by increased intracellular ionized (or "free") calcium (Caf). This was accomplished by using a model of injury to MDCK cells described recently. Cells are incubated with a mitochondrial uncoupler to deplete them of ATP in medium with Ca^{2+} adjusted to 100 nM using EGTA, with a calcium ionophore, so that Caf is clamped at 100 nM [17]. Because Caf cannot increase, injury caused by excessive Ca^{2+} is avoided, but damage that is prevented by glycine is fully expressed [17].

In the present experiments, the calcium clamping technique was particularly important for detecting pharmacological effects of compounds whose actions may be related to those of glycine, but weaker than those of the amino acid. Results using this model show that several compounds that bind to strychnine-sensitive glycine receptors, or block chloride channels, also prevent membrane damage in ATP depleted cells. However, isosmotic replacement of extracellular chloride by gluconate, or chloride salts by sucrose, did not significantly modify the parameters of injury or protection, suggesting that the underlying mechanisms are unrelated to membrane fluxes of chloride.

Methods

Experimental design

MDCK cells were grown as described [17], plated at 400,000 per 35 mm dish and used after overnight culture. Cells were incubated in glucose free Krebs-Ringer bicarbonate solution (KRB) containing (in mM) 115 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1 $KH2PO_4$, 1.25 $CaCl_2$, and 1 $MgSO_4$, gassed with 95% air, 5% CO_2. Carbonyl cyanide-m-chlorophenyl hydrazone (CCCP), an uncoupler of

Received for publication January 24, 1995
and in revised form September 18, 1995
Accepted for publication September 18, 1995

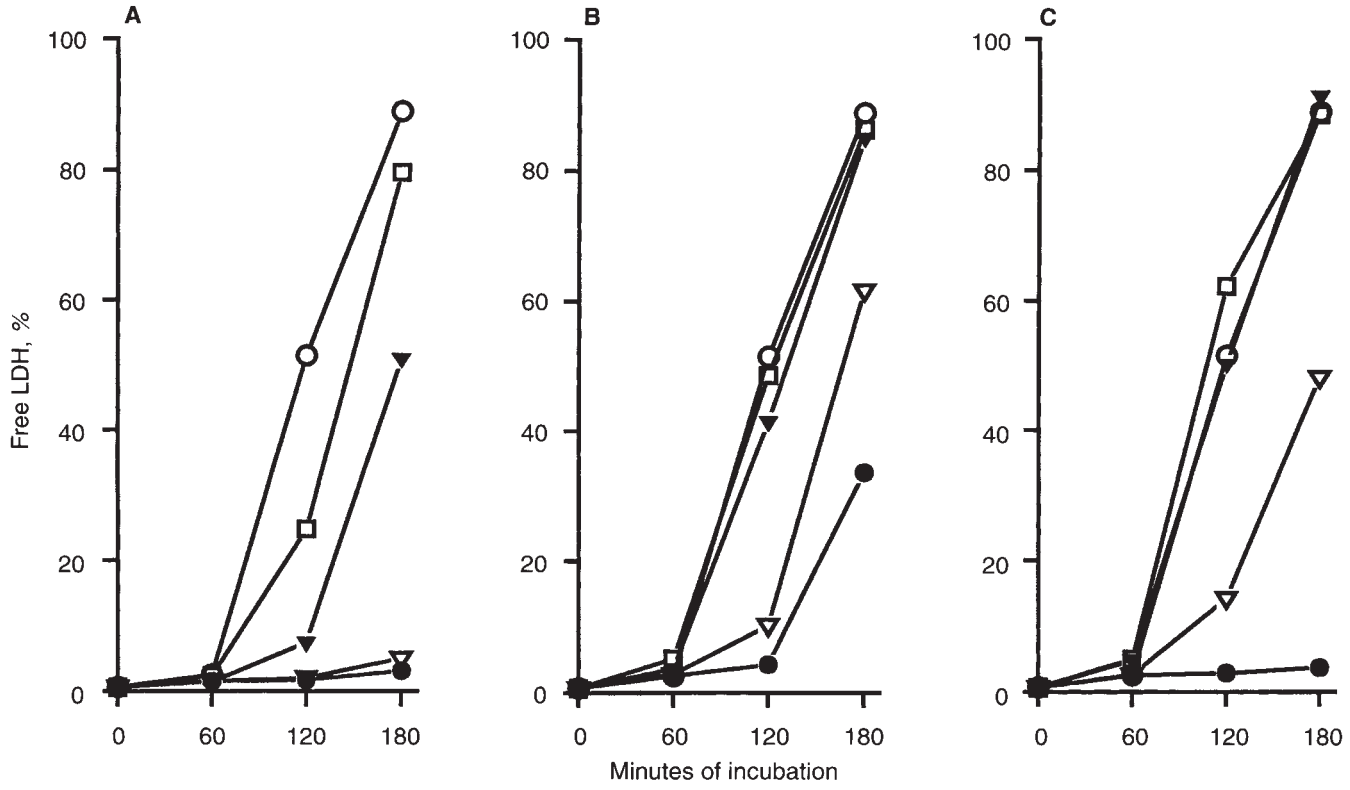

Fig. 1. *MDCK cells were incubated in KRB containing 100 nM Ca^{2+} with 5 μM ionomycin and 15 μM CCCP and no further additions (NFA) or various concentrations of* (**A**) *glycine,* (**B**) *avermectin B_1a, or* (**C**) *strychnine (drug concentrations are indicated in millimolar).* Symbols in (A) are: (○) NFA; (□) 0.10; (▼) 0.25; (▽) 1.0; (●) 5.0. Symbols in (B) are: (○) NFA; (□) 0.0001; (▼) 0.001; (▽) 0.01; (●) 0.10. Symbols in (C) are: (○) NFA; (□) 0.1; (▼) 0.25; (▽) 0.5; (●) 1.0. Free LDH in the medium was measured at 60, 120 and 180 minutes ($N = 5$). At two hours, glycine was significantly protective in concentrations down to 0.1 mM (100 μM), but protection at three hours required concentrations of 0.25 mM or higher. Avermectin was protective in concentrations of 0.01 and 0.1 mM (10 and 100 μM) at both time periods. With strychnine, 0.5 or 1 mM drug was required to achieve significant decrease of LDH release relative to the NFA group. On a molar basis, avermectin was most protective at all time periods. Error bars are not shown for clarity.

oxidative phosphorylation, and ionomycin, a Ca^{2+} ionophore, were added as 1000× solutions in ethanol at final concentrations of 15 μM and 5 μM. The medium contained 2.25 mM EGTA so that Ca^{2+} was buffered to 100 nM [8]. Incubations were at 37°C under 6% CO_2 in air; pH of the incubation medium was between 7.43 to 7.45. Drugs were added to the media as 100× stock solutions in DMSO, ethyl alcohol or water. Controls received vehicle alone. In some experiments, chloride ions or chloride salts in the incubation medium were isosmotically replaced by gluconate or sucrose, respectively. To replace chloride ions, Na gluconate and K gluconate were substituted for NaCl and KCl, and $CaSO_4$ was used instead of $CaCl_2$. Medium bicarbonate content was increased to 26 mM to keep pH at 7.43-7.45. To replace chloride salts, NaCl and KCl were replaced isosmotically by sucrose, and $CaCl_2$ was replaced by Ca acetate.

Methods

Lactate dehydrogenase (LDH), protein and ATP were measured as described [8, 9, 18]. Cells were fixed in glutaraldehyde and osmium tetroxide, and processed for electron microscopy by standard techniques. Plasma membrane permeability to fluorescein (molecular wt 332) and propidium iodide (PI, molecular wt 668) was assessed [19]. Experimental medium was replaced with 0.02 M phosphate buffered saline (PBS) containing 18.75 μg/ml fluorescein diacetate (FDA) and 2 μg/ml PI. After three minutes, cells were transferred to PBS, and photographed using epifluorescence optics. Fluorescein and PI were viewed simultaneously using a 420 to 490 nm excitation filter, 505 nm beam splitter and 520 nm long pass barrier filter.

Reagents

Avermectin B1a and derivatives were gifts from Drs. Sam Cifelli and Michael Fisher, Merck Research Laboratories (Rahway, NJ, USA). Cyanotriphenylboron sodium salt was a gift from Dr. Heinrich Betz, Max Planck Institute for Brain Research (Frankfurt, Germany). Indanyloxyacetic acid 94 (IAA), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and N-phenylanthranilic acid (diphenylamine-2-carboxylic acid, DPC) were from Research Biochemicals International (Natick, MA, USA). All other chemicals were from Sigma Chemicals Co. (St. Louis, MO, USA).

Statistics

Values are means ± SE. Data were analyzed by analysis of variance for repeated measure designs and the Neuman-Keuls test for multiple comparisons. $P < 0.05$ or lower in a two tailed comparison was considered significant.

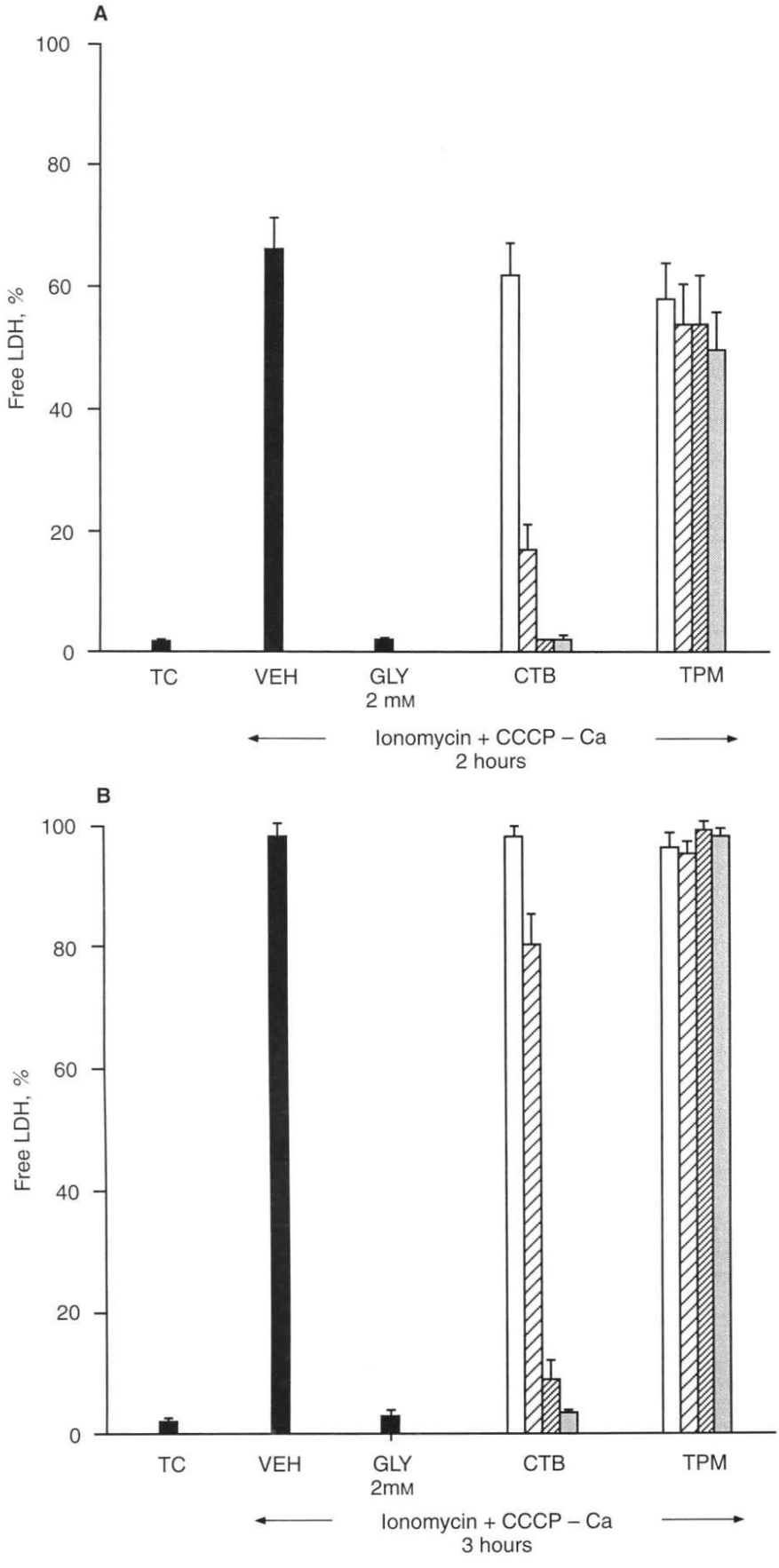

Fig. 2. *MDCK cells were incubated for two hours* (**A**) *or three hours* (**B**) *in 100 nM Ca^{2+} medium with ionomycin and CCCP, with vehicle alone (VEH), 2 mM glycine (GLY), cyanotriphenylboron sodium salt (CTB, 0.01, 0.025, 0.05 or 0.1 mM), or triphenylmethylphosphonium bromide (TPM, 0.01, 0.025, 0.05 or 0.1 mM).* Symbols are: (□) 0.01 mM, (▨) 0.025 mM, (▨) 0.05 mM, (▣) 0.1 mM. Time controls (TC) were incubated without ionomycin or CCCP in the presence or absence of 0.1 mM CTB or TPM. There was dose dependent inhibition of LDH release by CTB, significant for all concentrations 0.025 mM and higher. TPM was ineffective. ($N = 4$).

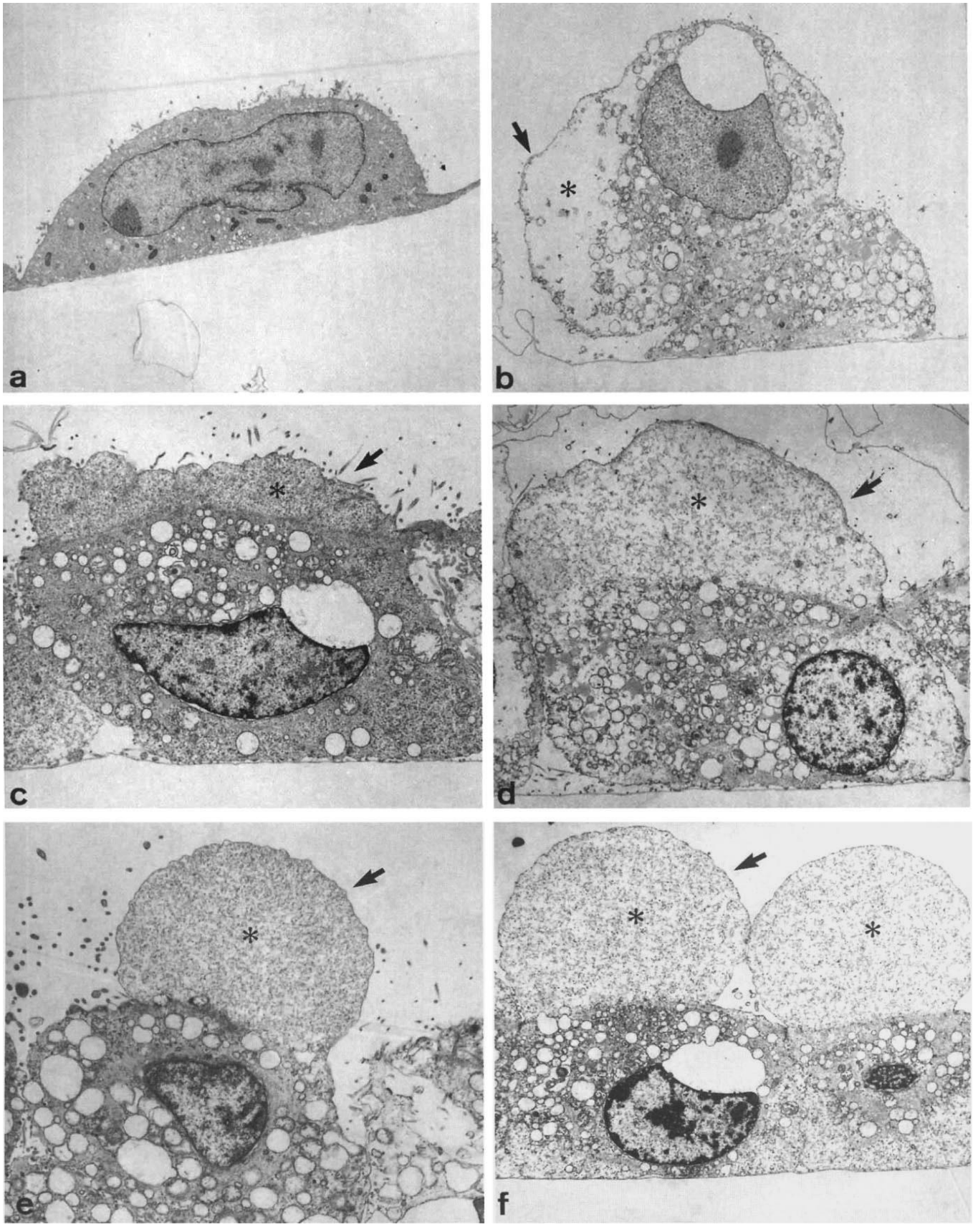
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Fig. 3. *Electron microscopy of injured and protected MDCK cells incubated for three hours in 100 nM Ca^{2+} medium without further additions (a), with ionomycin and CCCP alone (b) and with ionomycin and CCCP in the presence of 5 mM glycine (c), 1 mM strychnine (d), 0.1 mM avermectin B1a (e) or 0.1 mM cyanotriphenylboron sodium salt (f).* Compared to the time control in (a), cells with ionomycin and CCCP and no further additions (b) show swelling of cell bodies and all organelles including the nuclear envelope. The cytoplasm is vacant, with noticeable lack of electron dense material (asterisk), particularly in large, swollen areas underlying the plasma membrane (arrow). Cells with ionomycin and CCCP, and either glycine, strychnine, avermectin or cyanotriphenylboron (c–f) also exhibit severe swelling of all organelles and cytoplasmic swelling forming blebs, but the cytoplasmic density is greater, and they retain cytosolic protein, visible as electron dense granular material shown strikingly well within swollen blebs (asterisks), limited by the plasma membranes (arrows). LDH release assessed in the medium prior to fixation for EM, expressed as percentage of total LDH in parallel dishes were as follows: time control (a), 7%; ionomycin and CCCP (I+C) with no further additions (b), 100%; I+C with glycine (c), 5%; I+C with strychnine (d), 7%; I+C with avermectin (e), 23%; I+C with cyanotriphenylboron (f), 5%. Magnifications are ×3200 for a, b, d and f, and ×5000 for c and e.

Results

Incubation of MDCK cells with ionomycin and CCCP in 100 nM Ca^{2+} medium with no further additions led to progressive loss of plasma membrane integrity measured as LDH release (Fig. 1).

Compounds with activity at strychnine-sensitive CNS glycine receptors prevent leakage of LDH from ATP depleted MDCK cells

Avermectin B1a is an agonist at several types of ligand gated chloride channels in lower animals but also binds to $GABA_A$ and glycine receptor chloride channels in mammalian CNS [13, 20–23]. Strychnine is an antagonist of inhibitory glycine receptors [13]. Avermectin B1a and strychnine hydrochloride were included in the incubation medium and their effects on cell injury compared to that of glycine (Fig. 1). Concentrations of 0.5 and 1.0 mM strychnine strongly inhibited LDH release. Avermectin B1a had the same effect in much lower concentrations of 0.01 and 0.1 mM. In comparison, glycine was only moderately effective at 0.1 mM, with much stronger protection being observed in the range of 0.25 to 5 mM (Fig. 1). Cyanotriphenylboron, which blocks glycine and GABA gated chloride channels [24], potently inhibited the release of LDH (Fig. 2 A, B). Strong inhibitory effects were observed using concentrations as low as 0.025 mM, although higher concentrations were required to maintain protection during longer durations of incubation. As noted earlier, much higher concentrations of glycine were required to obtain equivalent protective effects (Fig. 1).

Structural correlates of LDH release or its prevention

By electron microscopy, there was marked swelling of cells and organelles at three hours, with large empty spaces in the cytoplasm (Fig. 3B). Cells with glycine, strychnine, avermectin or cyanotriphenyboron were similarly swollen, but maintained denser cytoplasmic texture, and contained granular electron dense material even in the most swollen areas, consistent with retention of cytosolic protein by plasma membranes (Fig. 3 C-F). Plasma membranes were separated from the cytoplasm, forming blebs containing granular electron dense material (Fig. 3 C-F). These changes were absent in vehicle controls (Fig. 3A), and untreated cells (not shown).

Following exposure to FDA and PI, control cells showed green, but no red fluorescence, indicating that plasma membranes were impermeable to fluorescein and PI (Fig. 4 A, B). The large majority of cells incubated with CCCP and ionomycin alone for three hours showed red, but no green fluorescence; the rare exceptions fluoresced green (Fig. 4C). In contrast, the vast majority of cells incubated with optimal concentrations of glycine, strychnine or cyanotriphenylboron were green (Fig. 4 D, E, H).

Table 1. Effects of Avermectin analogs on cell injury

	Free LDH, %
Time Control	3 ± 1
I+C, Vehicle control	88 ± 2
I+C, glycine 5 mM	2 ± 1[a]
I+C, Avermectin B_1a (AVM)	55 ± 7[d]
I+C, 22,23-dihydro AVM (Ivermectin) (A)	65 ± 2[e]
I+C, 22,23-dihydro AVM-4″-O-phosphate (B)	90 ± 4
I+C, Avermectin B_2a (C)	88 ± 2
I+C, AVM-4″-O-phosphate (D)	87 ± 3
I+C, 22,23-dihydro AVM aglycone (E)	78 ± 3
I+C, 22,23-dihydro AVM monosaccharide (F)	70 ± 2
I+C, AVM monosaccharide (G)	88 ± 5
I+C, AVM-5-ketone (H)	81 ± 5
I+C, 2,3,8,9,10,11,22,23-octahydro-AVM (I)	85 ± 3
I+C, 13-deoxy-AVM aglycone (J)	36 ± 6[c]
I+C, 13-deoxy-22,23-dihydro AVM aglycone (K)	36 ± 8[c]
I+C, Emamectin (MK 244) (L)	36 ± 6[c]
I+C, 13-O-methoxymethylene AVM (M)	15 ± 7[b]
I+C, 13-Epi-O-methoxy ethoxy methoxy-H_2 AVM (N)	43 ± 15[c]

MDCK cells were incubated in KRB with 100 nM Ca^{++} containing ionomycin and CCCP (I+C), and either vehicle alone (DMSO), Avermectin B_1a, or an Avermectin analog (compounds A–N) for 3 hours. A concentration of drug that was submaximally protective for Avermectin B_1a (10 μM) was used, so that increased as well as decreased protection by analogs could be detected ($N = 4$).

[a] less than all other groups except the time control

[b] less than all other groups except the time control, and I & C with glycine

[c] less than all other groups except time control, I+C with glycine, I+C with compound M, and I+C with AVM

[d] less than I+C vehicle control, and I+C with compounds B through I

[e] less than I+C vehicle control, and I+C with compounds B,C,D and G

Higher fractions of cells exposed to avermectin B1a showed red fluorescence (Fig. 4F). The incidence of red and green cells faithfully reflected the release of LDH. Thus, plasma membranes of protected cells were impermeable not only to the protein LDH (molecular wt 136,000), but also to the small molecules fluorescein (molecular wt 332) and propidium iodide (molecular wt 668).

Effects of compounds related to avermectin and cyanotriphenylboron

Availability of avermectin analogs permitted investigation of the structural specificity of cytoprotection by avermectin B1a. Modifications of avermectin structure result in alterations of binding affinity at C. elegans muscle glutamate-gated chloride channels and increase or decrease of vermicidal, insecticidal or miticidal activities of avermectins [20, 21]. Cells were injured in the presence of vehicle alone, or an avermectin analog for three hours. A concentration of drug that was submaximally protective

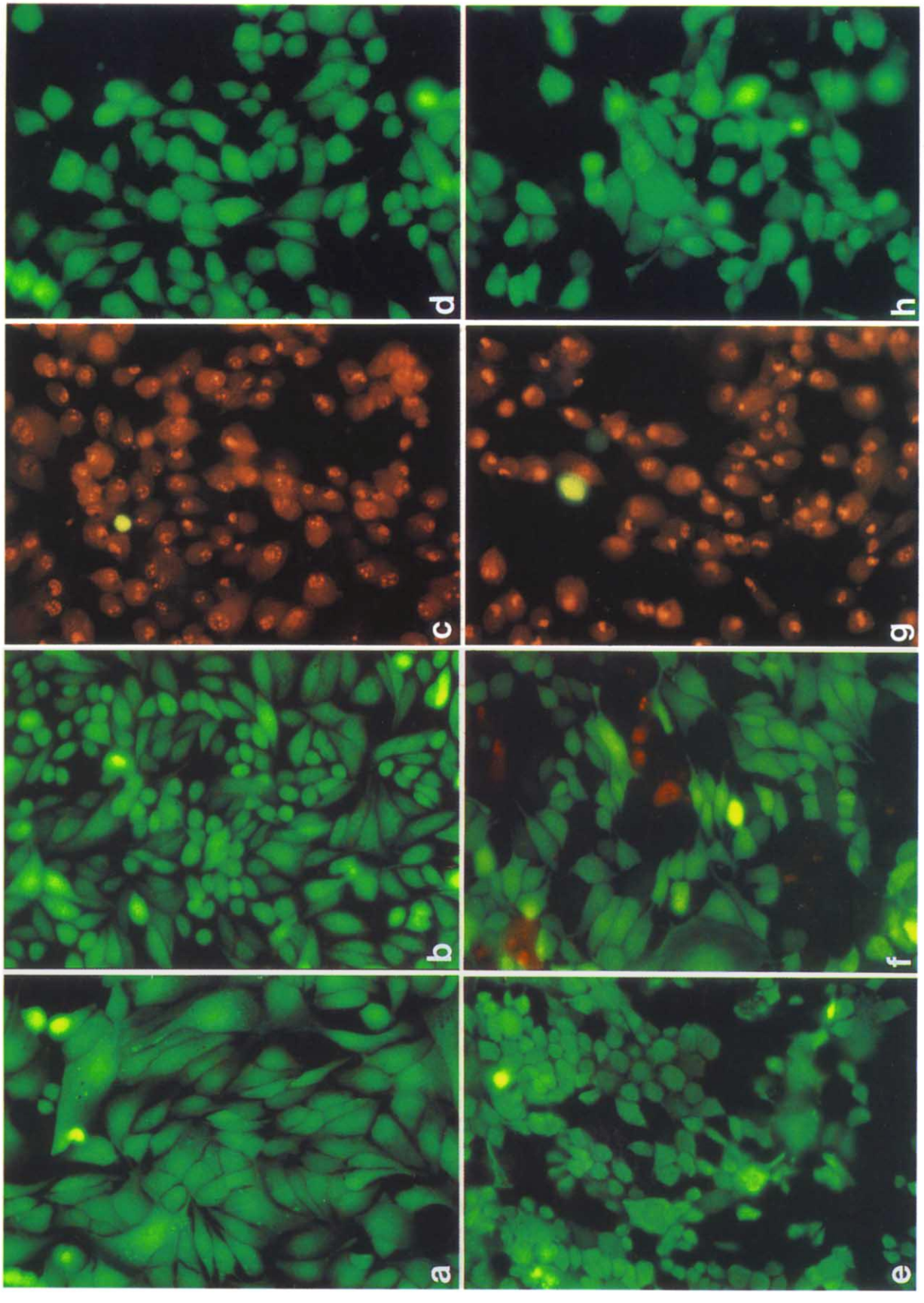

Fig. 4. *MDCK cells were stained intravitally with fluorescein diacetate and propidium iodide simultaneously as described under Methods, without any further treatment* (**a**)*, following incubation for three hours in 100 nM* Ca^{2+} *medium alone* (**b**)*, after incubation for three hours in 100 nM* Ca^{2+} *medium containing ionomycin and CCCP and no further additions* (**c**)*, or with 5 mM glycine* (**d**)*, 1 mM strychnine* (**e**)*, 0.1 mM avermectin B1a* (**f**)*, 0.1 mM triphenylmethylphosphonium bromide* (**g**) *or 0.1 mM cyanotriphenylboron sodium salt* (**h**). Cells with ionomycin and CCCP alone or with triphenylmethylphosphonium fluoresce red due to staining of DNA and RNA by propidium iodide and have lost free fluorescein from their cytoplasm with rare exceptions which are green or yellow (c and g). All cells in both controls (a and b) and groups incubated with ionomycin and CCCP with glycine, strychnine and cyanotriphenylboron retain free fluorescein within the cytoplasm fluorescing green, and do not have cells with red signal. All magnifications are ×200.

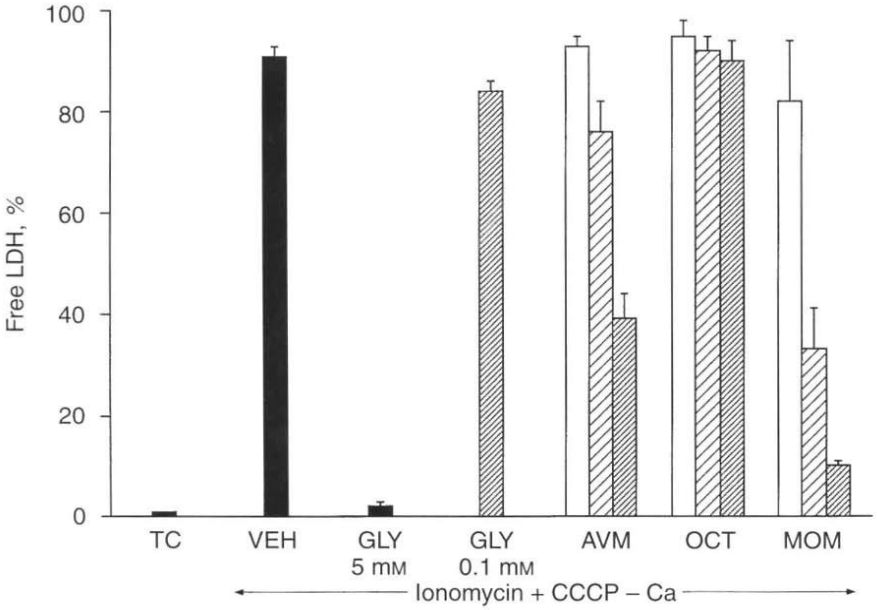

Fig. 5. *MDCK cells were incubated for three hours in 100 nM Ca^{2+} medium with ionomycin and CCCP, with vehicle alone (VEH), glycine (GLY, 5 mM or 0.1 mM), or different concentrations (0.001 mM, 0.01 mM, 0.1 mM) of avermectin B_1a (AVM), 2,3,8,9,10,11,22,23-octahydro-AVM (OCT) or 13-0-methoxy-methylene AVM (MOM).* Whereas OCT was inactive, both AVM and MOM protected significantly at 10 μM and 100 μM, and MOM was more effective than AVM at both concentrations. TC-Time Control without ionomycin or CCCP ($N = 4$). Symbols are: (□) 0.001 mM, (▨) 0.01 mM, (▨) 0.1 mM.

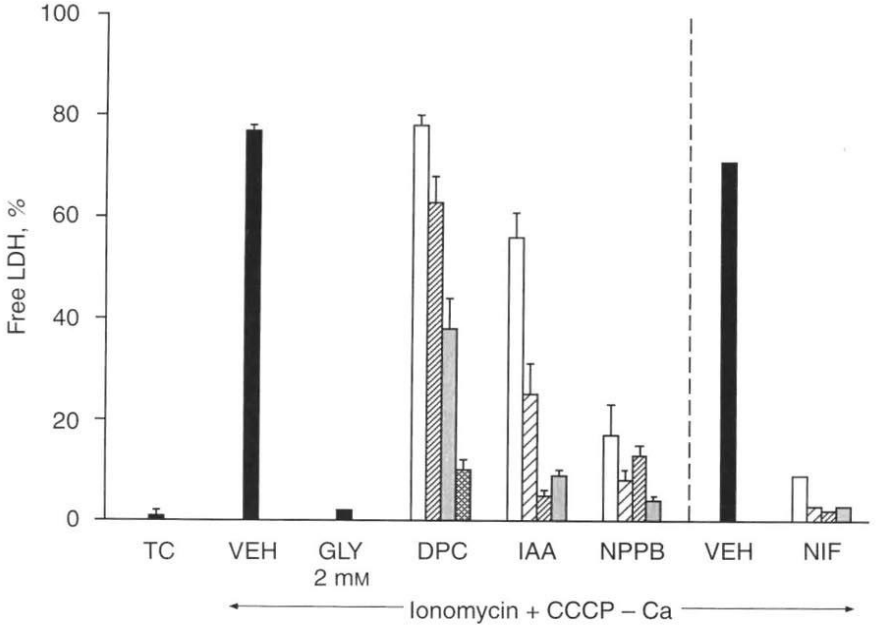

Fig. 6. *MDCK cells were incubated in 100 nM Ca^{2+} medium with no further additions (time control, TC), or containing ionomycin and CCCP with vehicle alone (VEH), 2 mM glycine (GLY), or different concentrations of chloride channel blockers.* Free LDH in the medium (y-axis) was measured at two hours ($N = 5$). DPC-diphenylamine-2-carboxylic acid. IAA-indanyloxyacetic acid 94. NPPB-5-nitro-2-(3-phenylpropylamino) benzoic acid. NIF-niflumic acid. Protection against LDH release by DPC, IAA and NPPB was significant and dose dependent. There were significant differences between the drugs, NPPB being the most potent. The average of two experiments done in quadruplicate with NIF, are shown to the right of the dotted line. They were done separately with their own vehicle controls (VEH). There was little variation between the quadruplicates and the two experiments. LDH release by time controls and injured cells protected by glycine was similar to that shown in the left panel (not shown). Symbols are: (□) 0.1 mM, (▨) 0.25 mM, (▨) 0.5 mM, (▨) 1.0 mM, (▩) 2.0 mM.

for avermectin B1a (0.01 mM) was used, so that increased as well as decreased protection by analogs could be detected. Avermectin B1a and its 22,23-dihydro derivative Ivermectin were moderately protective, whereas others were ineffective (Table 1). However, five compounds, four of which lack the 2-deoxy sugar glycoside substitutive and are modified at the 13 position, were also effective, among which one, 13-O-methoxymethylene avermectin B1a, was strongly cytoprotective, even better than the parent compound (Table 1). Dose response effects for avermectin B1a, an inactive octahydro derivative and the 13-O-methoxymethylene analog are shown in Figure 5.

To investigate the specificity of cytoprotection by the anionic cyanotriphenylboron, we used triphenylmethylphosphonium bromide (TPMP), which has the same triphenyl structure and equivalent hydrophobicity, but is positively charged [24]. TPMP blocks cation channels of nicotinic acetylcholine receptors, but not the anion channels of glycine receptors [24], although the two channels have structural similarities [25]. Conversely, cyanotriphenylboron is

Table 2. ATP levels with chloride or gluconate

	With chloride medium		With gluconate medium	
	ATP	LDH	ATP	LDH
TC	14.7 ± 1.4	2 ± 0	13.8 ± 1.5	3 ± 1
VEH	0.7 ± 0.1	5 ± 2	0.4 ± 0.1	9 ± 4
+GLY	0.6 ± 0.1	3 ± 1	0.4 ± 0.1	4 ± 1
+CTB	0.4 ± 0.1	3 ± 1	0.3 ± 0.1	3 ± 1
+TPM	0.9 ± 0.3	6 ± 2	0.4 ± 0.1	12 ± 5

ATP content and release of LDH from cells incubated for 1 hour in 100 nM Ca^{++} KRB containing ionomycin and CCCP in the presence of 5 mM glycine (+GLY), 100 μM cyanotriphenylboron sodium salt (+CTB), 100 μM triphenylmethylphosphonium bromide (+TPM), or vehicle alone without drugs (VEH) and in time controls without ionomycin and CCCP (TC). Decreases of ATP relative to controls were of equal magnitude with no significant differences between groups in chloride or gluconate media. ATP, nmol/mg protein. LDH, percent free LDH of total (N = 4).

Table 3. ATP levels with chloride or gluconate

	With chloride medium		With gluconate medium	
	ATP	LDH	ATP	LDH
TC	13.0 ± 0.5	3 ± 0	12.3 ± 0.5	3 ± 0
VEH	0.5 ± 0.1	6 ± 3	0.2 ± 0.1	14 ± 6
+GLY	0.5 ± 0.1	3 ± 0	0.4 ± 0.0	3 ± 1
+STR	0.4 ± 0.1	3 ± 0	0.3 ± 0.0	3 ± 0
+AVM	0.9 ± 0.1	3 ± 1	0.6 ± 0.0	4 ± 1
+DPC	0.4 ± 0.1	6 ± 1	0.2 ± 0.0	9 ± 2
+IAA	0.1 ± 0.0	6 ± 1	0.2 ± 0.0	4 ± 1
+NPP	0.2 ± 0.1	1 ± 0	0.2 ± 0.1	2 ± 0
+NIF	0.5 ± 0.1	4 ± 1	0.3 ± 0.1	5 ± 1

ATP content and release of LDH from cells incubated for 1 hour in 100 nM Ca^{++} KRB containing ionomycin and CCCP in the presence of 5 mM glycine (+GLY), 1 mM strychnine (+STR), 100 μM Avermectin B1a (+AVM), 2 mM diphenylamine-2-carboxylic acid (+DPC), 1 mM indanyloxyacetic acid (+IAA), 1 mM 5-nitro-2-(3-phenylpropylamino) benzoic acid (+NPP), 1 mM niflumic acid (+NIF) or vehicles without drugs (VEH), and in time controls without ionomycin and CCCP (TC). Decreases of ATP relative to controls were of equal magnitude with no significant differences between groups in chloride or gluconate media. ATP, nmol/mg protein. LDH, percent free LDH of total (N = 4).

anion channel selective [24]. TPMP did not have protective activity, measured as LDH release (Fig. 2), membrane permeability of fluorescein and PI (Fig. 4G) and structural disruption by electron microscopy (not shown).

Protection by other chloride channel blockers

Several other chloride channel blockers were tested. These were: Indanyloxyacetic acid 94 (IAA), N-phenylanthranilic acid (diphenylamine-2-carboxylic acid or DPC), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and niflumic acid (NIF). All four compounds were cytoprotective (Fig. 6). With IAA, NPPB and NIF, LDH release was inhibited progressively between concentrations of 0.25 to 1.0 mM (Fig. 6). NPPB and NIF were the most effective, protection being seen at concentrations down to 0.025 mM in the case of NIF (not shown). On the other hand, larger amounts of DPC were required to obtain equivalent inhibition of LDH release (Fig. 6).

Cell ATP

Declines of ATP were equivalent and virtually complete by one hour of incubation in all groups of protected and unprotected cells (Tables 2 and 3).

Membrane damage is not related to medium chloride

It was suggested that chloride influx mediates cell damage by ATP depletion [14], and chloride substitution by impermeant anions was reported to enhance hypothermic tissue preservation [26]. Chloride ions may modulate ligand binding at chloride channel receptors [13, 22, 23] and compete with channel blockers for functional sites [27]. For these reasons we examined whether substitution of chloride ions by gluconate or chloride salts by sucrose had any effects on injury or protection parameters. Equivalent declines of ATP occurred during injury in the presence or absence of test compounds, in both chloride or gluconate media (Tables 2 and 3). LDH release from cells injured without test compounds or in the presence of TPMP was not affected by gluconate substitution (Fig. 7). Glycine, strychnine, cyanotriphenylboron and other chloride channel blockers were equally protective in media with gluconate or chloride, but, in the case of avermectin B1a, there was partial loss of activity (Fig. 7). The ultrastructure of cells which had leaked LDH during incubation in gluconate media was indistiguishable from that of equally injured cells in chloride medium (Fig. 8). On the other hand, protected cells in gluconate media were not swollen or blebbed (shown for glycine, Fig. 8). Cells protected by other agents had a similar appearance (not shown).

Substantial injury occured during incubations in media containing sucrose substituted for chloride salts, in spite of the potential benefits that the impermeant solute sucrose might be expected to have. Cell damage under these circumstances was significantly prevented by glycine and other test compounds. Values for percent LDH release after three hours of incubation in 100 nM Ca^{2+} sucrose media were: controls, 1 ± 0; ionomycin and CCCP, 74 ± 1; ionomycin and CCCP with 5 mM glycine, 2 ± 1, with 1 mM strychnine, 3 ± 1, with 0.1 mM avermectin B1a, 18 ± 3, with 0.1 mM cyanotriphenylboron, 2 ± 0, and with 1 mM niflumic acid, 3 ± 1 (N = 4).

Effects of medium Ca^{2+} on cytoprotective activity

Cells were exposed to ionomycin and CCCP also in a "high calcium" medium containing 1.25 mM Ca^{2+}, that is, without added EGTA. Such cells exhibit rapid and sustained increases of Caf to high levels [10]. High concentrations of glycine (5 mM) protect MDCK cells under these conditions also, but the effects are ultimately overcome by Ca^{2+} dependent, glycine insensitive damage processes [17]. In the context of current experiments using Ca^{2+} clamped cells, we wanted to compare relative potencies of cytoprotective compounds under high calcium conditions also. The results show that uncontrolled increases of Caf have a major adverse impact on total cell damage and make impossible precise analyses of Ca^{2+}-independent processes sensitive to, or related to glycine.

LDH release from cells injured or protected by test compounds (0.1, 0.5 and 1.0 mM) in 1.25 mM Ca^{2+} medium for two hours is shown in Table 4. With few exceptions at the 1 mM level, all concentrations of test compounds were less effective in 1.25 mM Ca^{2+} medium than in 100 nM Ca^{2+} media (Table 4; compare with data for cells in 100 nM Ca^{++} media, Figs. 1, 2A and 6). Avermectin could not be tested at concentrations higher than 0.1 mM because of insolubility. At 0.1 mM, protection was nearly

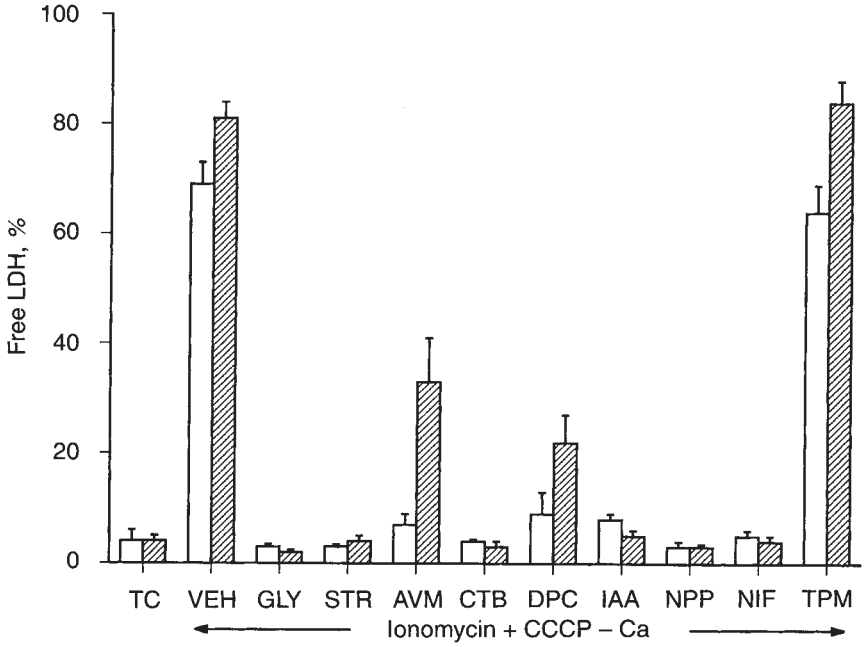

Fig. 7. *MDCK cells were incubated for two hours in 100 nM Ca^{2+} medium with no further additions (time control, TC), or containing ionomycin and CCCP with vehicle (VEH), 5 mM glycine (GLY), 1 mM strychnine (STR), 0.1 mM avermectin B1a (AVM), 0.1 mM cyanotriphenylboron sodium salt (CTB), 2 mM diphenylamine-2-carboxylic acid (DPC), 1 mM indanyloxyacetic acid 94 (IAA), 1 mM 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPP) 1 mM niflumic acid (NIF) or 0.1 mM triphenylmethylphosphonium bromide (TPM).* The incubations were done in the standard medium containing chloride (□) or medium in which chloride was substituted by gluconate (▨). Substitution by gluconate did not reduce the extent of injury. There was a trend for increased injury in gluconate groups with DPC or TPM or vehicle alone with no further additions, but this did not reach significance. In groups with AVM, there was significantly more injury in gluconate than in chloride medium. Nevertheless, cells with AVM in gluconate medium released significantly less LDH than corresponding cells in gluconate medium without added protective agents. In all other groups, the extent of inhibition of LDH release was equivalent in either chloride or gluconate media ($N = 4$).

absent in 1.25 mM Ca^{2+} medium for most compounds, including glycine. During incubations lasting three hours, the deleterious effects of high calcium conditions on cytoprotection was more strikingly shown, even for glycine. Values for LDH release from cells exposed for three hours to injury conditions in high calcium medium with 1 mM glycine and 1 mM strychnine were 72 ± 3 and 63 ± 2 ($N = 4$), whereas the two compounds were completely protective at the same concentrations in 100 nM Ca^{2+} medium (Fig. 1).

Discussion

Our studies show that several compounds which have activities at diverse chloride channels, including amino acid gated chloride channels, share cytoprotective actions in a well characterized model of ATP depletion in MDCK cells. Beneficial effects of the compounds were not mediated by improvement of energy status, as demonstrated previously for glycine and related amino acids.

Use of the Ca^{2+} clamping technique [9, 17] was required to clearly characterize and compare cytoprotective activity. Beneficial effects of glycine and other drugs were blunted, and in some cases lost, when incubations were done in 1.25 mM Ca^{2+} media. This can be attributed to uncontrolled Ca^{2+} influx through ionomycin permeabilized membranes and ensuing Ca^{2+} dependent damage [10, 17]. Increased Caf can ultimately overwhelm even the robust protection afforded by optimal concentrations (5 mM) of glycine [17]. For this reason, most studies reported here were done in Ca^{2+} clamped cells, where injury is completely glycine dependent, and effects of the amino acid are fully expressed. In that many test compounds were less potent than glycine, it became important to use Ca^{2+} clamped cells to study their specific actions also, free of non-specific damage caused by increased Caf. Taken in this context, the striking benefits afforded by glycine and other drugs with activity at chloride channels suggest that the cytoprotective mechanisms of this heterogeneous group of compounds may indeed be related. Our morphological results are consistent with this notion in that the ultrastructure of cells protected by diverse compounds was similar, with the common feature of preserved plasma membrane integrity despite considerable swelling of cells.

Cytoprotection appeared to be specific for chemical structure, at least for some classes of compounds, and the benefits were apparently not mediated by non-specific effects such as hydrophobic interactions. In proximal tubules, as well as in MDCK and endothelial cells, amino acid cytoprotection is stringently dependent on specific molecular features [3, 10, 28]. Structural specificity for the actions of amino acids could be shown even more rigorously for MDCK cells injured under low calcium conditions (unpublished observation). Comparison of chemical derivatives showed structural requirements for the actions of avermectins also (Table 2), arguing against non-specific stabilizing effects of the compound on proteins or lipids as the explanation for their cytoprotective activity. Similarly, TPMP, which has the same triphenyl structure as cyanotriphenylboron [24], but is positively charged, was inactive, whereas the negatively charged cyanotriphenylboron was protective. Brucine (2,3-dimethoxy-strychnine) has been reported to be cytoprotective similar to strychnine [15], but an examination of the structural requirements for the activity of these alkaloids has not been possible owing to the lack of availability of modified derivatives. Cytoprotection by other chloride channel blockers may also show structural specificity. Different classes of these aromatic compounds share functionally important structural features and the property of negative charge [27, 29]. DPC, NPPB and NIF are closely related arylaminobenzoates, but NPPB and NIF have higher binding affinities than DPC to anion channels [29, 30]. In this regard, it is notable that NPPB and NIF were more potent cytoprotectants than DPC. Being somewhat different structurally, it is difficult to relate the activity of IAA to the other three.

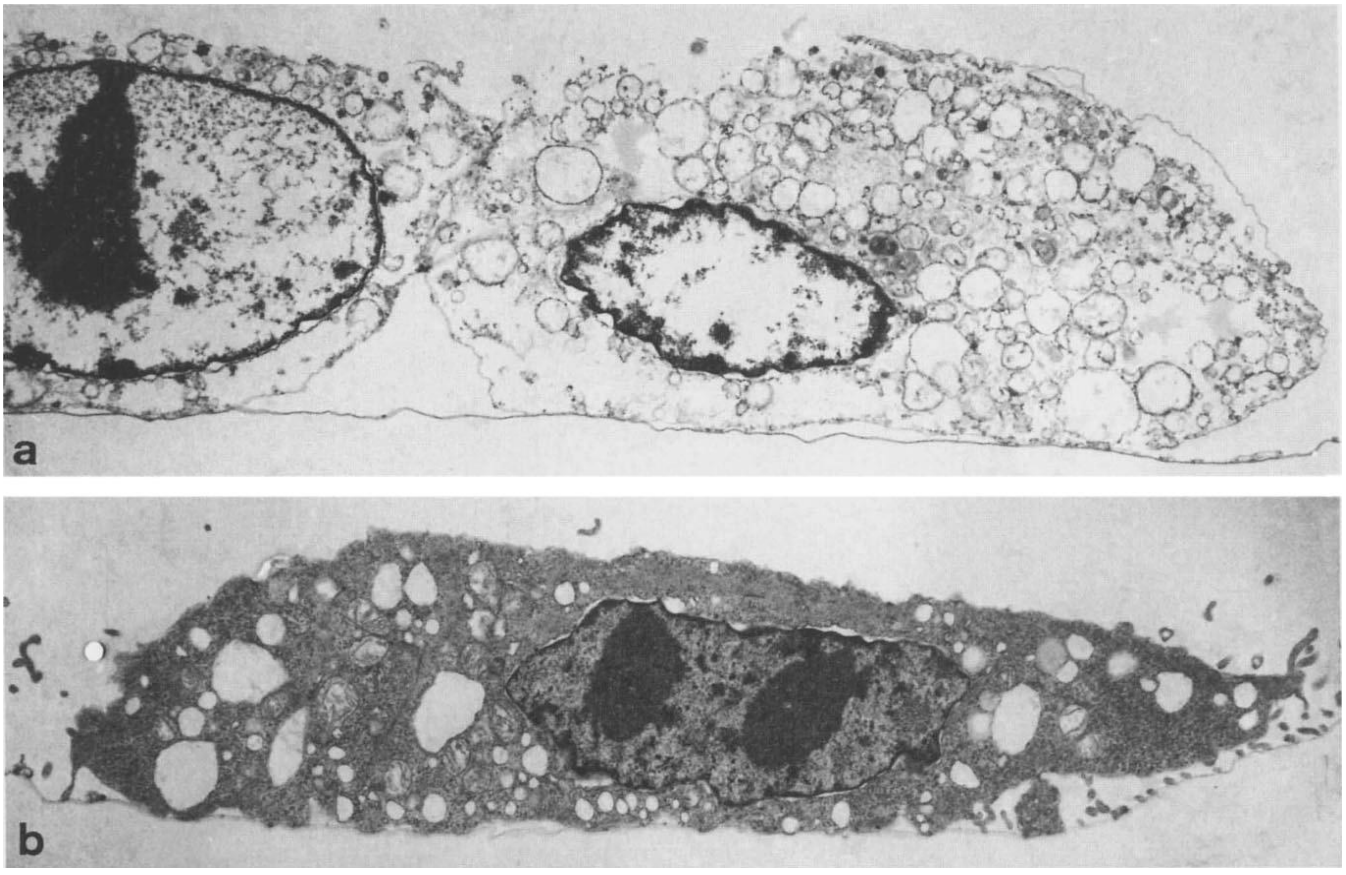

Fig. 8. *Electron microscopy of injured and protected MDCK cells incubated in 100 nM Ca^{2+} medium containing ionomycin and CCCP without further additions (a) or with 5 mM glycine (b) for three hours.* The medium was formulated to replace chloride by gluconate. Cells with no further additions (a) show an injury pattern that is indistinguishable from that seen in chloride medium (compare with Fig. 3b). On the other hand, there is notable absence of cytoplasmic swelling and blebbing in the cell protected by glycine (Fig. 8b), compared to the corresponding cell protected by glycine in chloride medium (Fig. 3c), although all organelles remain swollen nevertheless. Release of free LDH in the medium prior to fixation for EM was 95% from cells with ionomycin and CCCP (I+C) alone (Fig. 8a), and 6% from cells with I+C and glycine (Fig. 8b). Magnifications are ×5000 (a) and 6500 (b).

It was suggested that cytoprotection by glycine, strychnine and certain chloride channel blockers is due to prevention of large chloride fluxes through glycine receptor like proteins during terminal phases of cell injury [14]. However, this hypothesis is inconsistent with dissimilar effects of these drugs on chloride transport. In our studies, replacement of medium chloride by gluconate did not prevent membrane damage. Substantial glycine preventable injury occured when chloride salts in media were replaced by sucrose. These data show that lethal damage of MDCK cells that was specifically preventable by glycine and other drugs under our experimental conditions cannot be attributed to chloride influx. Rather, they suggest that pathological membrane pores must develop first in compromised cells, followed by abnormal and irreversible transmembrane fluxes of solutes.

A notable feature of protected cells in gluconate media was prevention of swelling and blebbing. This is consistent with the concept that influx of chloride, accompanying that of sodium, and followed by water, causes cell swelling when sodium pump function is compromised [31]. Gluconate substitution may have prevented cell swelling in the presence of glycine and other agents because of osmotic effects across protected plasma membranes impermeant to this large anion. On the other hand, failure by gluconate to prevent swelling of cells in the absence of glycine or other drugs is consistent with abnormal porosity of unprotected plasma membranes. In this situation, large fluxes of gluconate (and water) would be expected to occur after the membranes have developed porous defects. Prevention of cell swelling is expected, *a priori*, to decrease lethal cell damage and improve cell viability [31–33]. However, such benefits were insubstantial under our experimental conditions, and plasma membranes became abnormally porous in spite of the presence of osmotically active agents if glycine or other protective drugs were not also present. Viewed in this context, our findings underscore the uniqueness of the preservation of plasma membrane integrity by glycine, strychnine, avermectin B1a, cyanotriphenylboron and other chloride channel blockers. Their effects were not only of large magnitude, but appeared to be similar to the extent that they were directed towards at least one common structural locus in the cell (the plasma membrane), and occurred under circumstances where the deleterious effects of other factors contributing to cell injury such as increases of Ca^{2+} and cell swelling had been scrupulously avoided.

Given that modification of energy metabolism or chloride

Table 4. Cytoprotective effects in 1.25 mM Ca^{2+} medium

	Drug concentration *mM*				Time control
	0	0.1	0.5	1.0	
GLY	92 ± 2	87 ± 1	54 ± 6	22 ± 2	2 ± 1
STR	92 ± 2	92 ± 2	66 ± 7	16 ± 1	2 ± 1
AVM	92 ± 2	97 ± 2	NT	NT	2 ± 1
CTB	91 ± 4	58 ± 5	16 ± 2	3 ± 0	2 ± 0
IAA	95 ± 2	89 ± 3	27 ± 4	17 ± 2	1 ± 0
NPP	95 ± 2	74 ± 6	17 ± 5	4 ± 2	1 ± 0
DPC	95 ± 2	94 ± 3	90 ± 5	77 ± 6	1 ± 0
NIF	95 ± 2	88 ± 4	69 ± 6	68 ± 12	1 ± 0

Percent release of LDH from cells exposed to ionomycin and CCCP for 2 hours in KRB containing 1.25 mM Ca^{++} (No added EGTA) without or with 0.1 mM, 0.5 mM or 1.0 mM glycine (GLY), strychnine (STR), avermectin B1a (AVM), cyanotriphenylboron (CTB), indanyloxyacetic acid (IAA), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPP), niflumic acid (NIF) and from time controls without ionomycin and CCCP ($N = 4$). NT, not tested because of drug insolubility.

transport cannot explain the protection afforded by diverse compounds with known activities at chloride channels and chloride channel receptors, hypotheses for their actions have to be speculative at best. Although this cannot be proven at the present time, the circumstances of injury and patterns of protection suggest to us that the mechanisms of action may be related. The possibility that these effects are due to non-specific "membrane stabilizing" actions [34] appears unlikely, although rigorous structure activity studies are needed to resolve this question. However, based on known information regarding the interactions of glycine and other agents with ion channels, we would like to offer a highly speculative explanation for their cytoprotective effects. This is an extension of the hypothesis that the protective effects of glycine may be mediated by interaction with proteins related in structure to glycine receptors in the CNS.

Glycine receptors are hetero-oligomeric transmembrane proteins, whose functions are regulated by protein conformation and subunit interactions. Chloride channels, including those of glycine receptors, are known to alternate between multiple conductance states indicating frequent variations of channel size [35, 36]. Such physiological regulation of channel structure may break down during ATP depletion, and pathological pores develop, because of proteolysis or altered phosphorylation of channel protein subunits. Receptor occupancy by the appropriate ligand could perhaps preserve channel structure by maintaining protein conformation and subunit interactions. It is of interest that mammalian glycine and $GABA_A$ receptors and invertebrate glutamate gated chloride channels (which are the targets of the avermectins) are similar overall in their subunit and molecular structure, particularly in the channel forming M2 domains [25, 37], and avermectin B1a binds to, and is an agonist, not only at invertebrate glutamate receptors, but also at mammalian chloride channel receptors [20–23, 38, 39]. Bicuculline, which has activity at $GABA_A$ and glycine receptors [13], was shown to protect ATP depleted proximal tubules [12]. The binding of avermectin to the mammalian brain receptor site is sensitive to medium chloride [23], and if the hypothesis offered here for its cytoprotective activity is correct, this may explain the weakening of the benefits of the compound in gluconate medium.

Reservations regarding these interpretations are that the concentrations required for cytoprotection by some of the putative ligands are excessive, relative to known affinities at chloride channel receptors, and that at least one of the effects is aberrant. For example, the Kd for strychnine at the adult-spinal cord glycine receptor is < 10 nM and strychnine is an antagonist of glycine, whereas in the cytoprotective system, the actions of the alkaloid are like that of glycine and require high concentrations. That the analogy is not farfetched is suggested by the fact that there are glycine receptor variants with much lower affinities for glycine and strychnine within the CNS itself [40]. Subtle mutations of the glycine receptor can drastically alter affinity constants and binding properties [41].

The protective actions of chloride channel blockers cannot be reconciled with a hypothesis based on ligand binding. However, these agents bind to channel domains through hydrophobic and ionic interactions, and it is theoretically possible that subunit interactions in a putative multimeric channel protein may be stabilized by such binding. In this context, cyanotriphenylboron and niflumic acid, which were the most protective chloride channel blockers, are known to bind glycine receptor M2 channel domains [24, 42].

It is not farfetched to consider that proteins related to neurotransmitter receptors may be found in non-neuronal, parenchymal cells. Low affinity avermectin binding was detected in rat liver and kidney [23], and recently, β subunits of $GABA_A$ receptors were reported to be present in rat renal nephron segments [43]. The strength of the hypothesis being presented here rests not on the actions of one individual agent or a single family of drugs, but on the remarkable cytoprotection offered by several families of compounds, all of which have activity at a specific group of ligand-gated chloride channel receptors. The involvement of specific proteins of this type in cell injury will have to be investigated in future work. The present studies provide substantially expanded pharmacological tools and an improved model system that will be important for this purpose.

Acknowledgments

This work was supported by the National Institutes of Health grants DK37139 and DK48417 (M.A.V.), DK34275 and DK39255 (J.M.W.) and the Veterans Administration (J.M.W.). We thank Sue Clayton for secretarial assistance.

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