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Cyclic GMP-dependent cytoprotection against ethanol-induced damage in rabbit isolated gastric parietal cells

Hideki Sakai [1], Akira Ikari [1], Takahiro Shimizu, Takahiro Sato, Noriaki Takeguchi *

Department of Pharmaceutical Physiology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

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Abstract

Prostaglandin E_2 stimulates a nitric oxide/cyclic GMP (NO/cGMP) pathway which activates basolateral Cl^- channels in rabbit gastric parietal cells. We examined whether the NO/cGMP pathway protects parietal cells from ethanol (EtOH)-induced cytotoxicity, using a parietal cell-rich suspension purified from rabbit gastric mucosa. Cytotoxicity was assayed by measuring the release of a fluorescent dye from the cells. N^2,O^2-dibutyryl guanosine 3′,5′-cyclic monophosphate (DBcGMP) showed a concentration-dependent protective effect against EtOH-induced cytotoxicity. The half-maximal effect of DBcGMP was observed at 24 μM. DBcGMP in a concentration-dependent manner opened the basolateral Cl^- channels of parietal cells, the EC_{50} value being 44 μM. The EtOH-induced cytotoxicity decreased as the Cl^- concentration of medium decreased. A 30-s treatment with 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), an inhibitor of the Cl^- channel, had a cytotoxic effect which was not prevented by pre-incubation with DBcGMP. The cytotoxic effects of EtOH and NPPB were additive and the NPPB effects did not depend on the medium Cl^- concentration. The present study showed that cGMP protects the gastric parietal cell from EtOH-induced cytotoxicity, and this cytoprotection is related to basolateral Cl^- channel activity in the plasma membrane via an unknown mechanism(s). © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Parietal cell; Cytoprotection; cGMP; Cl^- channel

1. Introduction

Ethanol (EtOH) is known as a necrotizing substance that causes gastric mucosal injury (for review see the works of Guslandi (1987), Glavin and Szabo (1992) and Konturek (1997)). In rat gastric mucosa, prostaglandin E_2 has a protective action against EtOH. The in vitro effects of exogenous prostaglandin E_2 on EtOH-induced damage were assessed on isolated surface epithelial cells (Arakawa et al., 1988), mucosal cells (Glavin et al., 1996) and gastric glands (Tarnawski et al., 1988; Ko and Cho, 1995). Histological investigations showed that the prostaglandin E_2-induced cytoprotective effects against EtOH were more evident in cells located in deep regions of gastric glands, especially in parietal cells (Lacy and Ito, 1982; Schmidt and Miller, 1988; Robert et al., 1992). Prostaglandin E_2 was shown to have cytoprotective effects against EtOH in isolated gastric parietal cells (Barr et al., 1988). The mechanism of prostaglandin E_2-induced cytoprotection in gastric parietal cells is not known.

We have recently found that exogenous prostaglandin E_2 increases the intracellular cyclic GMP (cGMP) content, an effect which is mediated via increases in the intracellular free Ca^{2+} concentration and the production of nitric oxide (NO) in rabbit isolated gastric parietal cells (Sakai et al., 1995). This prostaglandin E_2-elicited NO/cGMP pathway leads to opening of the basolateral sub-picosiemens Cl^- channel (Sakai et al., 1995). Recently, NO/cGMP pathways were reported to protect against cellular damage in cultured bovine pulmonary artery endothelial cells (Polte et al., 1997) and tissue damage in rat lung (Pinsky et al., 1994) and guinea pig gastric mucosa in vitro (Yanaka et al., 1995). In the present study using isolated rabbit gastric parietal cells, we investigated whether (1) cGMP and NO protect parietal cells from EtOH-induced cytotoxicity and whether (2) the cGMP-dependent cytoprotection is related to the NO/cGMP-activated opening of the basolateral Cl^- channel.

* Corresponding author. Tel.: +81-764-34-2281 Ext. 2665; Fax: +81-764-34-5051; E-mail: ntakeguc@ms.toyama-mpu.ac.jp

[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Chemicals

5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB; Funakoshi, Tokyo, Japan), diphenylamine-2-carboxylate (DPC; Wako Pure Chemical Industries, Osaka, Japan), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS; Sigma, St. Louis, MO, USA), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; Sigma) and niflumic acid (Sigma) were dissolved in dimethyl sulfoxide, and diluted to final concentrations just before use. The dimethyl sulfoxide concentration in the final solutions never exceeded 0.5%, at which concentration the vehicle per se did not affect cytotoxicity and whole-cell Cl^- currents of parietal cells. 2′,7′-Bis(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) and EGTA were obtained from Dojindo Laboratories (Kumamoto, Japan). ATP disodium salt (Oriental Yeast, Tokyo, Japan), N^2,$O^{2'}$-dibutyryl guanosine 3′,5′-cyclic monophosphate (DBcGMP) sodium salt (Yamasa, Choushi, Japan) and sodium nitroprusside (SNP) dihydrate (Wako Pure Chemical Industries) were dissolved in appropriate solutions just before use.

2.2. Isolation of gastric glands

The procedures described below were performed in accordance with the guidelines presented by the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University. Gastric glands were prepared from male Japanese white rabbits (weighing 1–3 kg) as described previously (Berglindh et al., 1980). Rabbits were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and had free access to water and food until the day of the experiment. Animals were killed by the intraperitoneal administration of an overdose of urethane (> 2 g/kg). The stomach was removed and the gastric mucosa was digested with 170 U/ml of collagenase (Wako Pure Chemical Industries) at 35–37°C for 40 min. Isolated glands were suspended in a respiratory medium. This respiratory medium contained (in mM): 132.4 NaCl, 5.4 KCl, 5 Na_2HPO_4, 1 NaH_2PO_4, 1.2 $MgSO_4$, 1 $CaCl_2$, 10 HEPES (pH 7.35), 11 glucose, and 0.029 bovine serum albumin (BSA).

2.3. Preparation of the parietal cell-rich suspension

Isolated gastric glands prepared as described above were treated with 4000 tyrosine U/ml Actinase E (Kaken Pharmaceutical, Tokyo, Japan) at 35–37°C for 50 min. Then, parietal cells in the suspension were separated by use of a continuous Percoll gradient as described elsewhere (Kashiwagura et al., 1990). The gradient was formed by centrifuging a mixture of Percoll medium (Pharmacia, Uppsala, Sweden), the respiratory medium (pH 7.0) and 1.5 M NaCl solution (the mixing ratio, 45:50:5 in v/v/v) at 23,000 × *g* for 50 min. The fraction of parietal cells was further purified with a Beckmann J2-21M elutriator centrifuge (Soll, 1978). The cell preparation consisted of 86 ± 1% parietal cells (mean ± S.E.M., 30 animals). Parietal cells had at least twice the diameter of other kinds of cells in the purified cell suspension. Therefore, the volume ratio of parietal cells (percent of total cells) was calculated to be about 99%.

2.4. Cytotoxicity assay

EtOH-induced cytotoxicity of isolated parietal cells was assessed by monitoring the release of a fluorescent dye,

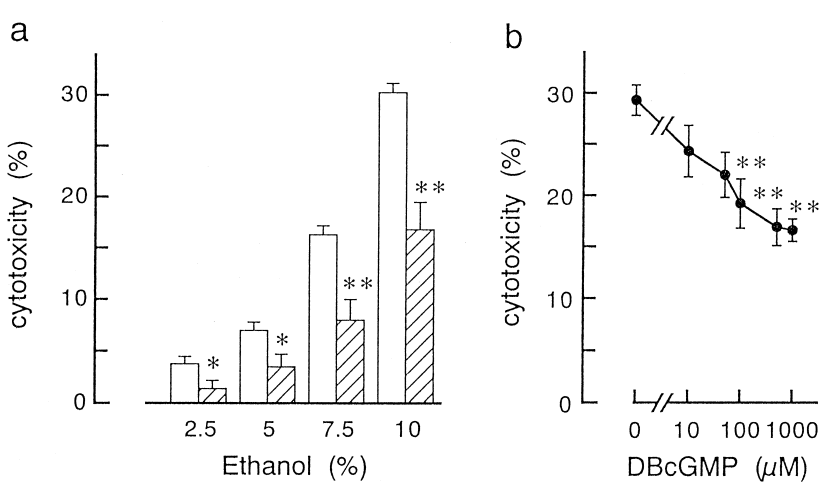

Fig. 1. (a) Protective effect of DBcGMP against EtOH-induced cytotoxicity in rabbit isolated gastric parietal cells. Parietal cell-rich suspensions were incubated in the presence (*hatched bins*) or absence (*open bins*) of 500 μM DBcGMP for 15 min at 35°C, followed by incubation with EtOH (2.5–10%, v/v) for 10 min ($n = 4$). * and ** Significantly different from the value in the absence of DBcGMP ($P < 0.05$ and 0.01, respectively). (b) Concentration-dependent protective effect of DBcGMP against EtOH (10%)-induced cytotoxicity. The experimental protocol was the same as that described for (a) ($n = 5–9$). **Significantly different from the value at 0 μM DBcGMP ($P < 0.01$).

2′,7′-bis(carboxyethyl)-4 or 5-carboxylfluorescein (BCECF), which is retained by viable cells only (Kolber et al., 1988; Suzuki et al., 1992; Tabuchi et al., 1995). This method was previously found to give cytotoxicity values comparable to those obtained by measuring ^{51}Cr release (Kolber et al., 1988). In the present study, the purified isolated cells in the BSA-free respiratory medium (2×10^6 cells/ml) were loaded with 5 μM BCECF-AM for 40 min at 22°C and washed. Then, the BCECF-loaded cells in the medium (0.5–1 ml) were treated with various reagents or combinations of them. For example, they were incubated in the presence or absence of DBcGMP or SNP for indicated periods at 35°C, followed by incubation with EtOH for 10 min. When indicated, high K^+ (124 mM K^+) and/or low Cl^- (2, 25 or 60 mM Cl^-) respiratory medium was used. In these solutions, Na^+ was replaced by K^+, and Cl^- by aspartate. For determination of BCECF release after various treatments, the cell suspension was centrifuged at $120 \times g$ for 2 min. Then, the supernatant was collected and the cells were washed and lysed with 0.5% polyoxyethylene octylphenyl ether (1 ml). When NPPB was used, the samples were treated with 10 mM $Na_2S_2O_4$ for 15 min to clarify the yellowish color of NPPB because NPPB without this treatment interfered with the fluorescence of BCECF. Necessary corrections for the decrease in BCECF fluorescence induced by this clarification (for example, 7% decrease at 500 μM NPPB) were made. The fluorescence intensities of BCECF in the supernatant (F_{super}) and in the lysed solution of cells (F_{cell}) were measured at an excitation wavelength of 440 nm with an emission wavelength of 535 nm, using a spectrofluorophotometer (Shimadzu RF-5000, Kyoto, Japan). Under these conditions, the fluorescence was insensitive to H^+ concentration (Takeguchi et al., 1993). The spontaneous release of the dye (F_{spont}) from cells was determined by incubating cells with medium alone. Cytotoxicity (%) was calculated from the following equation (Kolber et al., 1988; Suzuki et al., 1992; Tabuchi et al., 1995).

$$\text{Cytotoxicity (\%)} = \frac{F_{super} - F_{spont}}{F_{super} + F_{cell} - F_{spont}} \times 100$$

2.5. Patch-clamp experiments

In whole-cell patch-clamp experiments, the isolated gastric glands were treated with 500 tyrosine U/ml of Actinase E at 25–27°C for 5 min. As a result of this digestion, parietal cells protruded from the base of glands and had no leaky connection between the intracellular canaliculi and the lumen (Sakai et al., 1992). The patch-clamp technique was applied to the basolateral membrane of parietal cells

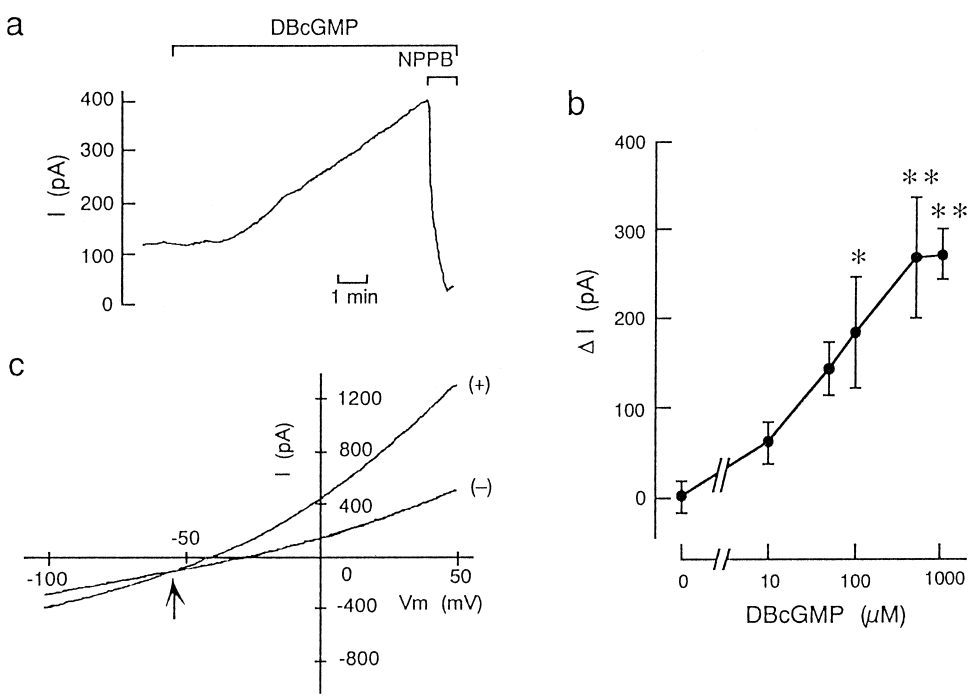

Fig. 2. (a) DBcGMP-induced increase in the whole-cell Cl^- current recorded from a single gastric parietal cell in isolated gastric glands. A representative trace of the Cl^- current recorded at 0 mV is shown. The cells were perfused with bathing solution containing 1 mM DBcGMP and subsequently with bathing solution containing 1 mM DBcGMP plus 500 μM NPPB as indicated. (b) Concentration–response curve for activation of the whole-cell Cl^- current by DBcGMP. The increased current (ΔI) was assessed 8 min after the addition of various concentrations of DBcGMP or vehicle ($n = 4$–6). * and ** Significantly different from the value at 0 μM ($P < 0.05$ and 0.01, respectively). (c) Reversal potential of the DBcGMP-elicited current. A typical relation between whole-cell currents (I) and membrane potential (V_m) from three similar experiments is shown. The currents in response to voltage ramps were recorded before (−) and 6 min (+) after the addition of 500 μM DBcGMP. The reversal potential at which two ramps cross is indicated by the arrow.

in rabbit isolated gastric glands. Whole-cell currents recorded from the parietal cells were previously established to be due to the current through the basolateral membrane (Sakai et al., 1992; Sakai and Takeguchi, 1993). An EPC-7 patch-clamp system (List Electronic, Darmstadt, Germany) was used for the recording. The resistance of patch electrodes filled with the pipette solution was about 5 MΩ. The cell resistance was 0.2–1 GΩ in the whole-cell configuration. Series resistance and capacitance were compensated electronically as best as possible. The liquid junction potential between pipette and bathing solutions was experimentally determined and corrected (Hamill et al., 1981; Sakai et al., 1992). The following extracellular bathing and intracellular pipette solutions were used (Sakai et al., 1989; Sakai et al., 1996). The bathing solution contained (in mM): 133 KCl, 7 NaCl, 1 sodium aspartate, 1 $MgCl_2$, 1 $CaSO_4$, 0.1 ouabain and 10 HEPES (pH 7.3). The pipette solution contained (in mM): 133 potassium aspartate, 7 NaCl, 3 $MgCl_2$, 0.062 $CaSO_4$, 0.1 EGTA, 2 ATP and 10 HEPES (*p*Ca 7, pH 7.3). After whole-cell configuration was achieved, the seal between the cell and the patch electrode and the cell's activity were checked by measuring the cell resistance and the membrane potential, respectively. Whole-cell Cl^- currents were recorded continuously at a holding potential of 0 mV, the zero-current potential for K^+ and non-selective cation channels under the present ionic conditions of the bathing and pipette solutions; that is, both solutions contained 133 mM K^+ and 7 mM Na^+ (Sakai and Takeguchi, 1994; Sakai et al., 1995, 1996). To determine the reversal potential for the current opened by DBcGMP, we recorded whole-cell currents in response to voltage ramps from −100 to +50 mV for 1 s in both the absence and the presence of DBcGMP, and obtained the reversal potential at which two ramps crossed. The ramp pulses were generated by using the pCLAMP 6 program (Axon Instruments, Foster City, CA, USA) installed on an IBM AT-compatible computer. The computer was equipped with Digidata 1200A (Axon Instruments), which has D/A output and A/D input channels. Experiments were performed at 35–37°C.

2.6. Statistics

Results are presented as the means ± S.E.M. Differences between groups were analyzed by one-way analysis of variance (ANOVA), and correction for multiple compar-

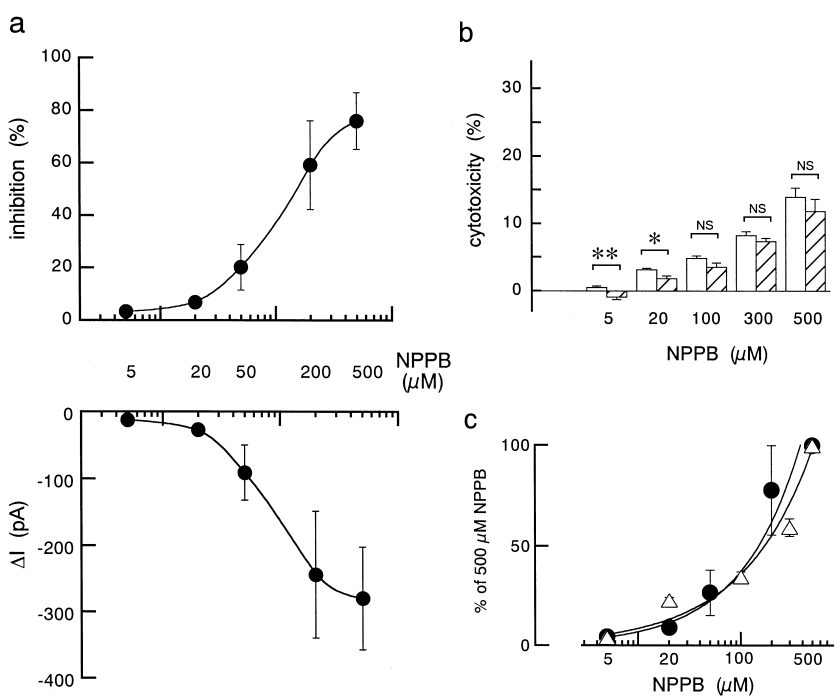

Fig. 3. (a) Concentration–response curve for inhibition of the whole-cell Cl^- current by NPPB. The cells were pre-incubated with 500 μM DBcGMP for 10 min at 32°C. The decreased current (ΔI; *lower*) and inhibition percentage (*upper*) were assessed 30 s after the addition of NPPB ($n = 3$–4). (b) Cytotoxic effect of NPPB for the parietal cells in the absence of EtOH. The cell suspension was incubated in the presence (*hatched bins*) or absence (*open bins*) of 500 μM DBcGMP for 15 min at 35°C, followed by incubation with NPPB (5–500 μM) for only 30 s ($n = 5$). $*P < 0.05$ and $**P < 0.01$; NS: not significantly different ($P > 0.05$). (c) Normalized concentration–response curves for NPPB-induced inhibition of the whole-cell Cl^- current and NPPB-induced cytotoxicity. The data from (a) (●) and (b) (△) are shown as percent (%) of the effect at 500 μM NPPB.

isons was made by using Dunnett's multiple comparison test unless described otherwise. Where indicated, Tukey's multiple comparison test was used. Statistically significant differences were assumed at $P < 0.05$.

3. Results

3.1. Cytoprotective effect of DBcGMP against EtOH-induced damage in rabbit gastric parietal cells

EtOH (2.5–10%, v/v) was cytotoxic to rabbit gastric parietal cells in a concentration-dependent manner (Fig. 1a). The EtOH-induced cytotoxicity was significantly decreased by pre-incubation of the cells with 500 μM DBcGMP (Fig. 1a). The protective effect of DBcGMP (10 μM–1 mM) against EtOH (10%)-induced cytotoxicity was concentration-dependent and showed a saturation at 0.5–1 mM. The half-maximal effect of DBcGMP was observed at 24 μM (Fig. 1b).

3.2. Activation of the basolateral Cl^- channel by DBcGMP

Basolateral Cl^- channel opening and closure were observed as an increase and decrease in the whole-cell Cl^- current, respectively. We previously confirmed that both the basal and cGMP-elicited currents of rabbit gastric parietal cells arose from opening of the same basolateral Cl^- channel (Sakai et al., 1995, 1996). Fig. 2a shows that extracellular addition of 1 mM DBcGMP increased the whole-cell Cl^- current with a slow time course. The effect

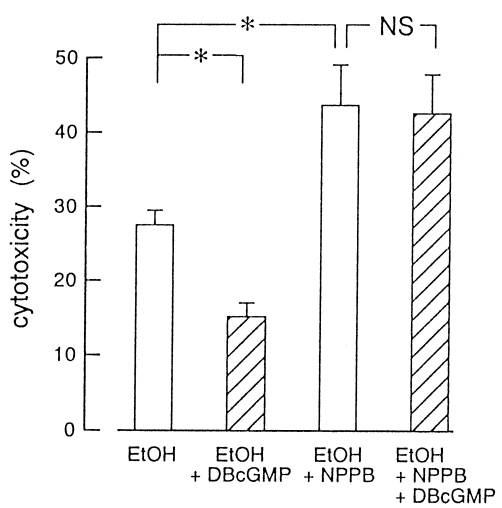

Fig. 4. Effect of NPPB on the DBcGMP-elicited protection against EtOH-induced cytotoxicity. The parietal cell-rich suspension was incubated in the presence (*hatched bins*) or absence (*open bins*) of 500 μM DBcGMP for 15 min at 35°C, followed by a 10-min incubation with 10% EtOH. To see the combined effect of EtOH and NPPB, the cell suspension was incubated with 10% EtOH for 9.5 min, followed by a 0.5-min incubation with 500 μM NPPB and 10% EtOH. The data were obtained from four to six experiments. Tukey's multiple comparison test was used. * $P < 0.05$; NS: not significantly different ($P > 0.05$).

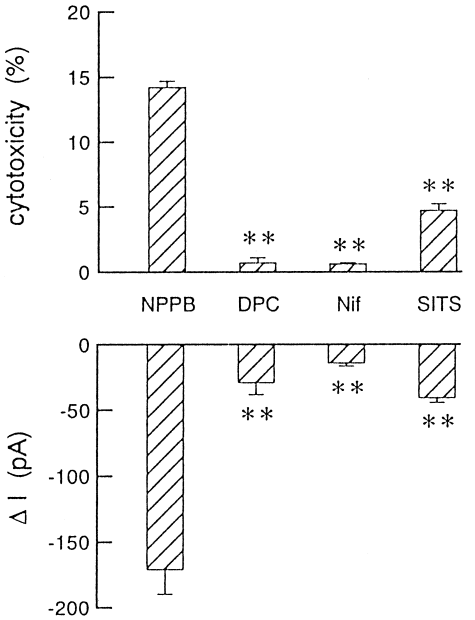

Fig. 5. Effects of other Cl^- channel blockers on cytotoxicity and the whole-cell Cl^- current. (*Upper*) The Cl^- channel blocker-induced cytotoxicity in parietal cells in the absence of EtOH. The cell suspension was pre-incubated for 15 min at 35°C. Then, 500 μM of NPPB, DPC, niflumic acid (Nif) or SITS was added and incubated for only 30 s. The data were obtained from five experiments. (*Lower*) Effects of the blockers on the whole-cell Cl^- current. The decreased current (ΔI) was assessed 30 s after the addition of NPPB, DPC, Nif or SITS at 500 μM ($n = 4$). ** Significantly different from the value of NPPB ($P < 0.01$).

of DBcGMP (10 μM–1 mM) was concentration dependent, and the EC_{50} was 44 μM (Fig. 2b). The reversal potential of the DBcGMP (500 μM)-elicited current was -56.8 ± 3.6 mV ($n = 3$) (Fig. 2c). This value was not significantly different ($P > 0.05$) from the theoretical Cl^- equilibrium potential at 36°C (−64 mV).

3.3. Cl^- channel inhibition and cytotoxic effect induced by NPPB

As shown in Fig. 2a, both the basal and DBcGMP-elicited Cl^- currents were inhibited by 500 μM NPPB, which at high concentrations ($IC_{50} = 300$ μM) inhibited the basolateral Cl^- channel of non-stimulated rabbit parietal cells (Sakai et al., 1992). NPPB even at 500 μM has been shown to be specific for the Cl^- channel of rabbit parietal cells when added for a short period of, for example, less than 1 min (Sakai et al., 1996). In the following experiments, the cells were treated with NPPB for only 30 s to avoid non-specific effects. In Fig. 3a, we tested the inhibitory effect of NPPB on the Cl^- channel of parietal cells which were pre-stimulated by 500 μM DBcGMP. The IC_{50} of NPPB was found to be about 150 μM.

Fig. 3b shows that NPPB had a concentration-dependent cytotoxic effect on parietal cells. In contrast to EtOH-induced cytotoxicity (Fig. 1), pre-incubation with DBcGMP even at 500 μM did not have significant protec-

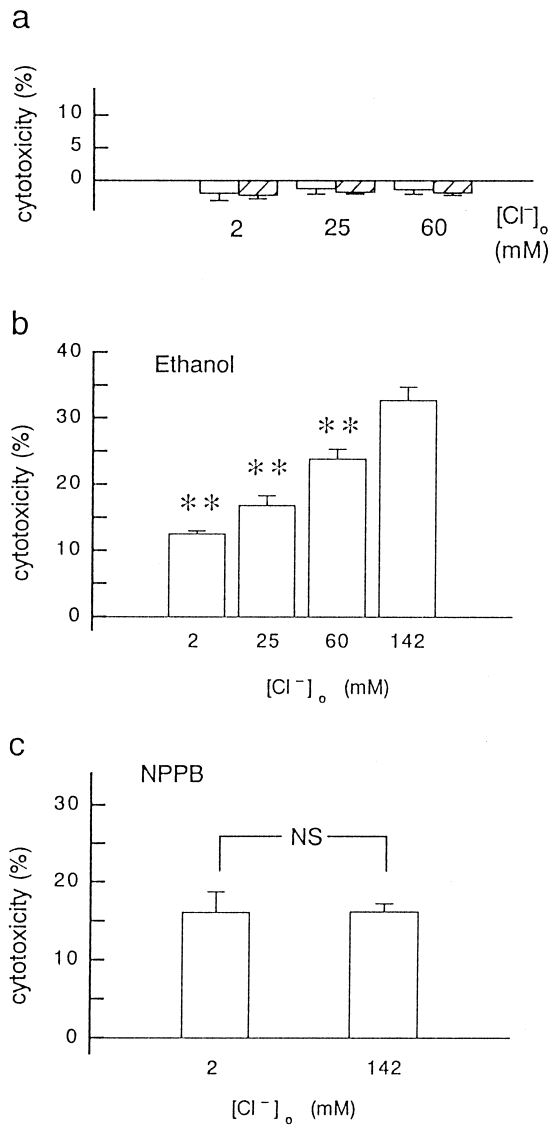

Fig. 6. (a) Effects of extracellular Cl^- concentrations on cytotoxicity. Parietal cell-rich suspensions were incubated with the standard K^+ (5.4 mM)/low Cl^- (2, 25 or 60 mM Cl^-) respiratory medium (*open bins*) or the high K^+ (124 mM K^+)/low Cl^- (2, 5 or 60 mM Cl^-) respiratory medium (*hatched bins*) for 10 min at 35°C, $n = 4$. (b) Effect of extracellular Cl^- concentrations on EtOH-induced cytotoxicity. The cell suspensions in the standard K^+ (5.4 mM)/low Cl^- (2, 25, 60 or 142 mM Cl^-) respiratory medium were incubated in the presence of 10% EtOH for 10 min, $n = 6$–8. ∗∗Significantly different from the value at 142 mM Cl^-. (c) Effects of a 30-s incubation of cells with 500 μM NPPB at two different extracellular Cl^- concentrations (2 and 142 mM), $n = 3$–4. NS: not significantly different ($P > 0.05$).

tive effects against NPPB (100–500 μM)-induced cytotoxicity (Fig. 3b). Since the maximally soluble concentration of NPPB was about 500 μM, we normalized the concentration-dependent effect of NPPB on the whole-cell Cl^- current (Fig. 3a) and cytotoxicity (Fig. 3b) as a function of percentage of the effect at 500 μM NPPB (Fig. 3c). Fig. 3c shows that the two concentration–response curves almost overlapped.

3.4. Combined effects of EtOH and NPPB

When the cells were treated with 10% EtOH, cytotoxicity was about 30%, as described above. Fig. 4 shows that a subsequent incubation with 500 μM NPPB for 30 s significantly increased the cytotoxicity to about 43%, indicating that the effects of EtOH and NPPB were partially additive. Fig. 4 also shows that EtOH-induced cytotoxicity (30%) was significantly prevented by the pre-incubation with 500 μM DBcGMP, and that this protective effect of DBcGMP was completely lost after the subsequent 30-s incubation with 500 μM NPPB.

3.5. Effects of other Cl^- channel blockers on cytotoxicity and the whole-cell Cl^- current

Several other Cl^- channel blockers were tested. At 500 μM, the cytotoxic effects of DPC, niflumic acid and SITS

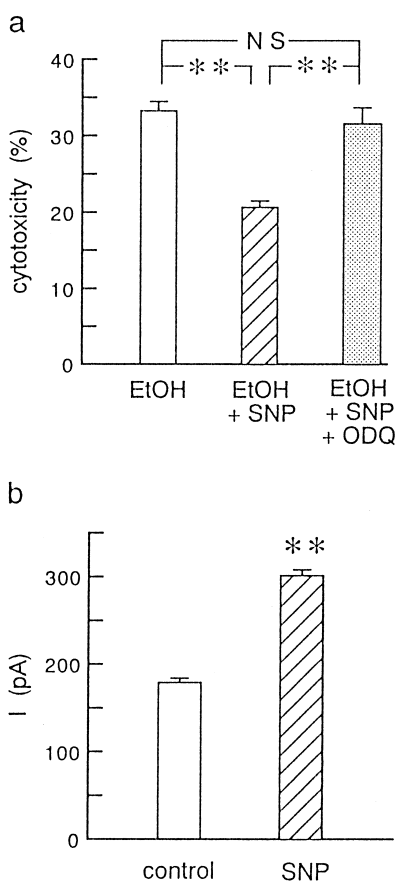

Fig. 7. (a) Protective effect of SNP against EtOH-induced cytotoxicity. The parietal cell-rich suspension was incubated in the presence (*hatched bin*) or absence (*open bin*) of 200 μM SNP or in the presence of 10 μM ODQ plus 200 μM SNP (dotted bin) for 15 min, followed by incubation with 10% EtOH for 10 min ($n = 5$). ∗∗ $P < 0.01$; NS: not significantly different ($P > 0.05$). (b) Increase in the whole-cell Cl^- current by SNP. Values of the current before (*open bin*) and 6 min after the addition of 200 μM SNP. (*hatched bin*) were shown ($n = 3$). ∗∗Significantly different from the value before addition of SNP ($P < 0.01$).

were much less than that of NPPB (Fig. 5, *upper*). In parallel with these results, the inhibitory effects of DPC, niflumic acid and SITS on the whole-cell Cl^- current were much less than that of NPPB (Fig. 5, *lower*).

3.6. Effect of extracellular Cl^- concentration on cytotoxicity

The effects of EtOH in the low Cl^- concentration media were studied. When the cells were incubated with the standard K^+ (5.4 mM)/low-Cl^- (2, 25 or 60 mM Cl^-) respiratory media in the absence of EtOH, cytotoxicity was not observed (Fig. 6a, *open bins*). The high K^+ (124 mM K^+)/low Cl^- respiratory medium also did not have a cytotoxic effect (Fig. 6a, *hatched bins*). When the cells were incubated in the presence of 10% EtOH for 10 min, the cytotoxic effect of EtOH decreased as the extracellular Cl^- concentration decreased (Fig. 6b), whereas the cytotoxic effect induced by a 30-s incubation with 500 μM NPPB did not depend on the Cl^- concentration (Fig. 6c).

3.7. Cytoprotection and Cl^- channel activation by SNP

To check whether NO contributes to the cGMP-dependent cytoprotection, SNP, which is a NO donor and a soluble guanylate cyclase activator (Katsuki et al., 1977; Murad, 1986), was used. Fig. 7a shows that pre-incubation of the cells with 200 μM SNP significantly decreased the EtOH-induced cytotoxicity and significantly increased the whole-cell Cl^- current (Fig. 7b). Fig. 7a shows that the nitroprusside-induced cytoprotective effect against EtOH was lost when the cells were pre-incubated for 15 min with 200 μM nitroprusside plus 10 μM ODQ, a NO-sensitive guanylate cyclase inhibitor (Vandecasteele et al., 1998).

4. Discussion

NO has been reported to protect indirectly rat gastric mucosa against EtOH- and HCl-induced tissue damage by increasing blood flow (MacNaughton et al., 1989; Kitagawa et al., 1990; Whittle et al., 1992; Brzozowski et al., 1993; Konturek, 1997). The present study has shown for the first time that NO and cGMP directly protect isolated rabbit parietal cells against EtOH-induced cellular damage. Interestingly, the NO/cGMP pathway is reported to have cytoprotective or cytotoxic effects depending on the cell type studied. For example, in cultured bovine endothelial cells, the NO/cGMP pathway had a cytoprotective effect against cellular injury caused by tumor necrosis factor-α (Polte et al., 1997). In contrast, this pathway-induced cytotoxicity in various types of cells such as mouse cultured cerebral cortical neurons (Frandsen et al., 1992), rat pheochromocytoma PC12 cells (Nakamura et al., 1997), hamster pancreatic B-cell line HIT-T15 (Loweth et al., 1997). It seems that a moderate increase in NO induces cytoprotective effects in some types of cells, whereas a strong increase in NO generally causes cell damage via formation of peroxynitrite.

Prostaglandin E_2 has been reported to partially reduce EtOH-induced cytotoxicity in purified rabbit parietal cell-enriched fractions (Barr et al., 1988) and isolated rabbit gastric glands (Ko and Cho, 1995). Tarnawski et al. (1988) suggested that the cell membrane itself could be a major site of the prostaglandin E_2-induced action. They also showed that there was a threshold concentration of prostaglandin E_2 which provided cell protection and that once the protective threshold concentration was reached, a higher concentration of prostaglandin did not offer additional protection. These authors, however, did not mention the molecular mechanisms of the prostaglandin E_2-induced cytoprotection.

We have recently found that prostaglandin E_2 binds to the EP_3 receptor in the basolateral membrane of the rabbit parietal cell, resulting in an increase in the intracellular free Ca^{2+} concentration, which sequentially activates constitutive NO synthase and guanylate cyclase and increases the intracellular cGMP level. This increased level of cGMP in turn activates the basolateral Cl^- channel of the cells (Sakai et al., 1995, 1996). In the absence of prostaglandin E_2, the basal activity of the Cl^- channel can be observed because the channel is also regulated positively by intracellular NO and cGMP in non-stimulated parietal cells (Sakai et al., 1996). Furthermore, we confirmed that only one kind of open-closed Cl^- channel contributes to both the basal and cGMP-elicited Cl^- currents (Sakai et al., 1995, 1996). In fact, NPPB inhibited both the basal and the DBcGMP-elicited Cl^- currents (Fig. 2a).

The NO/cGMP-activated Cl^- channel is present abundantly in the basolateral membrane of rabbit parietal cells (30,000 channels/cell) and dominates the cell membrane potential (Sakai et al., 1989, 1992). Although K^+ and non-selective cation channels are present in the parietal cell, they are not the major determinants of the membrane potential (Sakai et al., 1989).

The present findings suggest that the cGMP-dependent cytoprotection against EtOH-induced cytotoxicity is affected by opening of the basolateral Cl^- channels, because the half-maximal cytoprotective effect of DBcGMP was observed at 24 μM (Fig. 1b), a value close to the ED_{50} for the DBcGMP-induced activation of the Cl^- channel (44 μM, Fig. 2b). One possible working hypothesis for future studies to clarify the role of the basolateral Cl^- channel is that Cl^- channel opening promotes Cl^- efflux from cells, thus reducing the size of the cell from its expanded state caused by EtOH. The present results shown in Fig. 6b may indicate that Cl^- efflux is involved in cytoprotection, although many different types of studies are required to establish this, for example, measurement of the effects of EtOH on cell size, Cl^- efflux, Na^+, K^+-ATPase activity, etc.

The cytotoxic mechanism induced by NPPB (Figs. 3–6c) seems to differ from that induced by EtOH because 1) the two effects were additive (Fig. 4) and 2) the EtOH effect depended on the medium Cl^- concentration whereas the NPPB effect did not (Fig. 6b and c). However, this NPPB-induced effect may be also related to the basolateral Cl^- channel, because (1) the normalized concentration–response curve for NPPB-induced cytotoxicity almost overlapped that for the NPPB-induced inhibition of the Cl^- channel (Fig. 3c) and (2) the cytotoxic effect of NPPB in the parietal cell was much greater than that of other Cl^- channel blockers such as DPC, niflumic acid and SITS, which is in agreement with the inhibitory effect of NPPB on the Cl^- channel being much greater than that of DPC, niflumic acid and SITS (Fig. 5).

At present, we cannot exclude the possibility that NPPB-sensitive targets other than the Cl^- channel are present in gastric parietal cells. But the present very short treatment of the cells with NPPB for only 30 s excludes the possible contribution of other ion channels, as previously reported (Sakai et al., 1996). At present, it is unknown why NPPB binding to this Cl^- channel causes cytotoxicity in such a short time.

Recently, Venkatachalam et al. (1996) reported that glycine and strychnine, which stimulates and inhibits the amino acid-gated Cl^- channel current, respectively, and several Cl^- channel blockers (NPPB, DPC and niflumic acid) had cytoprotective effects against the lethal effects of ATP depletion in Madin–Darby canine kidney epithelial cells. They suggested that a plasma membrane protein related to glycine-gated Cl^- channels plays a significant role in cell injury. So far, such a specific protein related to the basolateral Cl^- channel has not been reported in gastric parietal cells. Further study along this line is necessary.

In the present experiment, DBcGMP and NO significantly reduced EtOH-induced cytotoxicity in parietal cells, but even at their highest concentrations they did not completely prevent it (Fig. 1b). Other cytoprotective pathways may also need to be activated in order to completely overcome EtOH-induced cytotoxicity. For example, EtOH decreases levels of reduced glutathione (GSH) in rat gastric mucosa (Szabo et al., 1981; Takeuchi et al., 1989), and exogenous GSH has a significant cytoprotective effect against EtOH-induced damage in isolated rabbit gastric glands (Ko and Cho, 1995).

A NO donor inhibits histamine-stimulated acid secretion in isolated rat parietal cells (Brown et al., 1993) and rabbit gastric glands (Kim and Kim, 1996). This inhibition is accompanied by an increase in the intracellular cGMP level (Brown et al., 1993; Kim and Kim, 1996) and is prevented by an inhibitor of cGMP-dependent protein kinase (Brown et al., 1993). Thus, the NO/cGMP pathway in parietal cells has two important activities, anti-secretory and cytoprotective. Indeed, the addition of L-arginine induces a concentration-dependent increase in cGMP levels in rabbit isolated glands (Contreras et al., 1997). Interestingly, prostaglandin E_2 is reported to be synthesized mainly in parietal cells rather than in non-parietal cells in rat stomach (Fukuda et al., 1988). Our finding of cGMP-dependent cytoprotection may account, at least in part, for why prostaglandin E_2 shows a protective effect against EtOH-induced cytotoxicity in gastric parietal cells and why a drug such as ecabapide, which increases cGMP concentrations in gastric parietal cells and opens the basolateral Cl^- channel (Sakai et al., 1996), has anti-ulcer activity against EtOH (Asano et al., 1990).

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