Am J Physiol Gastrointest Liver Physiol
280: G1331–G1340, 2001.

Impairment of H^+-K^+-ATPase-dependent proton transport and inhibition of gastric acid secretion by ethanol

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Received 18 May 2000; accepted in final form 11 January 2001

Del Valle, Juan C., María Salvatella, Irma Rossi, Ramón Andrade, Yolanda Gutiérrez, Carmen Pereda, Begoña Samper, and Juan E. Felíu. Impairment of H^+-K^+-ATPase-dependent proton transport and inhibition of gastric acid secretion by ethanol. *Am J Physiol Gastrointest Liver Physiol* 280: G1331–G1340, 2001.—Ethanol (1–20% vol/vol) caused a dose-dependent reduction in the basal rate of acid formation in isolated rabbit gastric glands with a calculated EC_{50} value of 4.5 ± 0.2%. Ethanol also reduced ATP levels in isolated gastric glands and in cultured parietal cells (EC_{50}: 8.8 ± 0.4% and 8.5 ± 0.2%, respectively) and decreased both basal and forskolin-stimulated cAMP levels. In studies carried out in gastric gland microsomes, ethanol inhibited the hydrolytic activity of H^+-K^+-ATPase (EC_{50}: 8.5 ± 0.6%), increased passive proton permeability (EC_{50}: 7.9%), and reduced H^+-K^+-ATPase-dependent proton transport (EC_{50}: 3%). Our results show that the inhibition of gastric acid secretion observed at low concentrations of ethanol (≤5%) is mainly caused by the specific impairment of H^+-K^+-ATPase-dependent proton transport across cell membranes rather than inhibition of the hydrolytic activity of H^+-K^+-ATPase, reduction in the cellular content of ATP, or increase in the passive permeability of membranes to protons, although these changes, in combination, must be relevant at concentrations of ethanol ≥7%.

gastric glands; proton permeability; parietal cells

IN VIVO AND IN VITRO EXPERIMENTS have shown that ethanol exerts a dose-related biphasic effect on gastric acid secretion in humans and in different animal models (11, 30, 33). Thus, in humans, low topical concentrations of ethanol (1.4–4% vol/vol) have been shown to moderately stimulate acid production, whereas concentrations of 5–40% have no effect or, rather, an inhibitory effect (33). Similarly, in experiments carried out in isolated toad gastric mucosa, ethanol applied at low concentrations (2%-10% vol/vol) to either the luminal or the submucosal side was a potent stimulator of HCl secretion, whereas high concentrations (≥20%) were inhibitory (11). Furthermore, in isolated rabbit gastric glands stimulated by histamine, low concentrations of ethanol (0.2%-5% vol/vol) significantly potentiated the rate of acid formation as well as the glandular concentration of cAMP, whereas the presence of 10% ethanol markedly decreased both HCl secretion and cAMP levels (30).

Different experimental evidence suggests that the rise in gastric acid secretion in vivo induced by low concentrations of ethanol may be caused by its stimulation of the release of humoral agents such as histamine (2, 16, 28) or gastrin (6, 17). Moreover, because ethanol has a fluidizing effect on membrane phospholipids in intact cell systems (20), a specific potentiation of the interaction between histamine receptor, activated by endogenous histamine, and parietal cell adenylate cyclase has also been implicated in the stimulatory effects of low ethanol concentrations on gastric acid secretion in vivo (30).

On the other hand, the mechanisms by which ethanol impairs gastric acid secretion have been related to a variety of cellular processes involved in stimulus-secretion coupling. Possible targets of ethanol toxicity that may impair the secretory function of the parietal cell are, among others, the membrane-bound histamine receptor, the adenylate cyclase complex, polymerization of actin and fusion of cytosolic microsomes with the apical membrane, H^+-K^+-ATPase activity, membrane permeability to certain ions, and cell energy charge (26, 30).

In this work, we have carried out a systematic study on the influence of different concentrations of ethanol (1–20% vol/vol) on the rate of acid formation in isolated rabbit gastric glands. This in vitro model allows us to investigate direct effects of ethanol on gastric acid formation in the absence of neural or endogenous hormonal influences. The accompanying changes in gastric gland cell viability, ATP and cAMP levels, as well as the effect of ethanol on H^+-K^+-ATPase activity and on both passive and H^+-K^+-ATPase-dependent transport of protons across microsomal membranes, have also been investigated. Our results show that 1% (vol/vol) ethanol did not significantly affect the rates of basal and forskolin-stimulated acid formation in iso-

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lated rabbit gastric glands. In contrast, concentrations of ethanol ≥5% caused a marked reduction in the rate of acid production in both nonstimulated and forskolin-treated gastric glands, inhibition being almost complete at 10% ethanol. Although ethanol caused a dose-dependent reduction in both ATP and cAMP levels in gastric glands and parietal cells, inhibited the hydrolytic activity of H^+-K^+-ATPase, and increased the passive proton permeability of microsomal membranes, the inhibition of the rate of acid formation elicited by low concentrations of ethanol (<5%) seemed to be mainly related to an impairment of the H^+-K^+-ATPase-dependent transport of protons across the cell membranes.

MATERIALS AND METHODS

Animals. Male rabbits (New Zealand White, 1.5–2.5 kg body wt) from our inbred colony were used. The animals were fed on a standard chow (N-25; Moragón, Toledo, Spain) and water ad libitum and were housed in animal quarters at constant temperature (23°C) with a fixed (12 h) light cycle. The animal experimentation described was conducted in accordance with the highest standards of humane animal care. All animals were anesthetized with pentobarbital sodium (30 mg/kg body wt iv) immediately before the experiments.

Reagents. Collagenase A from *Clostridium histolyticum* (type I), forskolin, IBMX, *N*,*N*′-dicyclohexylcarbodiimide (DCCD), ouabain, oligomycin, nigericin, valinomycin, and acridine orange were purchased from Sigma (St. Louis, MO). [Dimethyl-amine-^{14}C]aminopyrine (100–120 mCi/mmol) and the cAMP ^{125}I-radioimmunoassay kit were obtained from Amersham International (Little Chalfont, UK). Matrigel was purchased from Becton Dickinson (Bedford, MA). Percoll was obtained from Pharmacia Biotech (Uppsala, Sweden). Ethanol was from Merck (Darmstadt, Germany). The remaining reagents, all of analytical grade, were from Boehringer Mannheim (Mannheim, Germany), Sigma, or Merck.

Gastric gland isolation. Gastric glands were prepared according to the method reported by Berglindh and Öbrink (8) as previously described by Rossi et al. (31). Briefly, the stomach of an anesthetized rabbit was perfused in situ with phosphate-buffered saline (in mM: 149.6 NaCl, 3 K_2HPO_4 and 0.64 NaH_2PO_4, pH 7.4) at 37°C. The mucosa from the gastric corpus was freed from the muscle layers, minced, and incubated with collagenase (0.5 mg/ml) at 37°C for 30–45 min. The resulting digest was filtered through a nylon mesh, rinsed three times, and resuspended at a final concentration of 2–5 mg dry weight/ml in *medium A* (in mM: 132.4 NaCl, 5.4 KCl, 5 Na_2HPO_4, 1 NaH_2PO_4, 1.2 $MgSO_4$, 1 $CaCl_2$, 15 HEPES, and 10 glucose with 10 mg/l phenol red and 1 mg/ml bovine serum albumin, at pH 7.3). The incubations were performed with agitation (100 strokes/min) at 37°C in an 95% O_2-5% CO_2 atmosphere. Isolated rabbit gastric glands had the typical rodlike appearance (0.3–0.7 nm in length). More than 98% of the different cells present in the glands excluded trypan blue dye (0.1% wt/vol). The dry weight of the gastric gland suspensions was calculated in 1-ml aliquots; after centrifugation (10,000 *g* for 3 min), the pellets were dried overnight at 60°C and then weighed.

Gastric parietal cell isolation. Rabbit gastric parietal cells were isolated according to the method reported by Beales and Calam (5). Perfusion of the stomach and isolation of the gastric mucosa was carried out as described in *Gastric gland isolation*. After the mucosa was minced into small fragments, it was subjected to sequential digestion by collagenase (0.35 mg/ml) and EDTA as previously reported (5) to obtain a crude suspension of isolated gastric cells. Parietal cells were enriched from the crude cell suspension by centrifugal elutriation in a Beckman J6-MC elutriator using a Beckman JE 5.0 rotor (5). Further purification of the parietal cells was performed by density gradient centrifugation in 30% Percoll (20 min at 2,000 *g*). The resulting top cell layer, which contained >90% purified parietal cells (by Giemsa staining), was recovered, washed, and resuspended in culture medium (1:1 mixture of Ham's F12-DMEM supplemented with 15 mM HEPES pH 7.4, 7% fetal bovine serum, 100 μg/ml gentamicin sulfate, and 8 μg/ml insulin). Parietal cells were plated (0.4 million cells/well) on 12-well cell culture clusters (Corning Costar, Cambridge, MA) coated with Matrigel (1:9 in sterile water). The cultured cells were maintained in a 95% O_2-5% CO_2 atmosphere at 37°C for 24 h.

Measurement of acid production. Acid secretion by isolated gastric glands was determined by accumulation of the weak base [^{14}C]aminopyrine in the canalicular compartment, following the method described by Berglindh et al. (7) with minor modifications. Thus 1.5-ml samples of the gland suspensions were incubated in *medium A*, as indicated in *Gastric gland isolation*, in the presence of 0.88 μM [^{14}C]aminopyrine (0.1 μCi/ml) and different concentrations of ethanol with or without 1 μM forskolin. After 30-min incubation, 1-ml aliquots of the gland suspensions were taken and immediately centrifuged (10,000 *g* for 20 s). The supernatants were quickly removed, and the pellets were rinsed twice with *medium A*, dried, and dissolved in 250 μl of 60% HNO_3 at 50°C for 15 min. The radioactivity of the supernatants and those of acid extracts were counted in a liquid scintillation spectrometer. Results are expressed as the ratio of intraglandular (acid space) to extraglandular [^{14}C]aminopyrine concentration, which was calculated as described elsewhere (7). Correction for the [^{14}C]aminopyrine trapped in the pellet was performed by incubating glands in the presence of 10 mM thiocyanate (12).

Preparation of gastric gland microsomes. Nonstimulated rabbit gastric glands were homogenized with 20 strokes of a motor-driven (2,500 rpm) Teflon pestle in a Potter homogenizer in a medium consisting of 0.25 M sucrose and 10 mM Tris at pH 7.4. The homogenate was centrifuged at 15,000 *g* for 15 min, and the supernatant was then collected and centrifuged again at 105,000 *g* for 1 h. The microsomal pellet obtained was resuspended in the homogenization medium and kept in liquid nitrogen until use. All of these procedures were carried out at 0–4°C.

Enzymatic assays. Glandular lactate dehydrogenase (EC 1.1.1.27) and phosphoglucose isomerase (EC 5.3.1.9) activities were measured by a standard spectrophotometric method (9). H^+-K^+-ATPase (EC 3.6.1.3) activity was assayed in gastric gland microsomes, basically according to the method reported by Hersey et al. (21). Thus gastric gland microsomes (0.1 mg of protein), previously incubated with or without different concentrations of ethanol (0.5–20% vol/vol) for 10 min at 37°C, were added to an assay mixture containing 150 mM KCl, 10 mM PIPES, 1 mM $MgSO_4$, 5 mM MgATP, 10 μg/ml valinomycin, 10 μM DCCD, 2.5 μg/ml oligomycin, 1 mM EGTA, and 0.1 mM ouabain, pH 7.2, and the indicated ethanol concentrations. The reaction was carried out at 37°C for 20 min, and was stopped by adding one volume of 10% ice-cold trichloroacetic acid. After centrifugation (10,000 *g* for 2 min), inorganic phosphate was measured in the resulting supernatant (18). Nonspecific ATPase activity (measured under similar conditions but in the absence of valinomycin and substituting 250 mM sucrose for the 150

mM KCl in the reaction mixture) was subtracted from the estimated H^+-K^+-ATPase activity.

H^+ transport assays. H^+-K^+-ATPase-dependent transport of protons across microsomal membranes was assayed using the fluorescent amine acridine orange as described elsewhere (23, 24). In this fluorometric assay, H^+-K^+-ATPase activity was coupled to H^+ accumulation in microsomal vesicles via a K^+/H^+ exchange process. In response to the pH gradient generated by the H^+-K^+-ATPase activity, binding of acridine orange to intravesicular membrane sites increased, quenching the fluorescence of the probe. The assay was performed at room temperature (21–23°C) in a Perkin-Elmer LS-5B spectrofluorometer. Microsomes (40 μg of protein/ml) were suspended in a medium containing (in mM) 10 PIPES, 150 KCl, 1 $MgCl_2$, 0.1 EDTA , and 1 ATP with 10 μM valinomycin, pH 7.2, in the absence or presence of different concentrations of ethanol (1–10% vol/vol). The excitation and emission wavelengths used were 493 and 530 nm, respectively. When the influence of ethanol on passive H^+ permeability of microsomal membranes was studied, an ATP-dependent proton gradient across the microsomal membranes was generated first by the addition of 10 μM valinomycin. H^+-K^+-ATPase activity was then blocked by the rapid hydrolysis of ATP. This was achieved by the incorporation of glucose (5 mM) and hexokinase (18 U) into the reaction mixture immediately before the addition of either saline or ethanol (30).

ATP and cAMP assays. The ATP levels of gastric gland and parietal cells were determined fluorometrically according to the method reported by Lowry and Passonneau (25). Gastric gland suspensions or cultured parietal cells were incubated in the absence or presence of ethanol (1–20% vol/vol) at 37°C for 30 or 60 min; after addition of $HClO_4$ (5%), the acid extracts were centrifuged and the supernatants were neutralized with imidazol-K_2CO_3 (0.3 M-3 M) and centrifuged again. ATP was measured in these neutralized extracts.

cAMP was determined in trichloroacetic acid extracts of cultured parietal cells using a radioimmunological method as previously described (15). Gastric parietal cells cultured for 24 h were incubated at 37°C for 15 min with different concentrations of ethanol in the absence or presence of either IBMX (0.5 mM) or forskolin (1 μM) or both. After treatment with 5% trichloroacetic acid and subsequent centrifugation (3.000 *g* for 15 min), cAMP was determined in the resulting supernatants.

Statistical analysis. Statistical significance of differences was calculated by the paired Student's *t*-test. The differences were considered statistically significant when the *P* value was <0.05. The calculated concentrations of ethanol corresponding to the half-maximal effects (EC_{50}) were calculated by the computer program Fig.P (Fig.P Software, Durham, NC).

RESULTS

Effect of ethanol on trypan blue exclusion in gastric gland cells. In the first part of the experiments, we studied the influence of ethanol on the integrity of the cells present in the isolated rabbit gastric glands. For this purpose, the percentage of cells stained with trypan blue (0.1%) as well as the amount of both lactate dehydrogenase and phosphoglucose isomerase released into the incubation medium were estimated in gastric gland suspensions treated with different concentrations of ethanol. As shown in Fig. 1, >98% of the cells excluded the trypan blue stain in gastric glands incubated in the absence of ethanol. The presence of

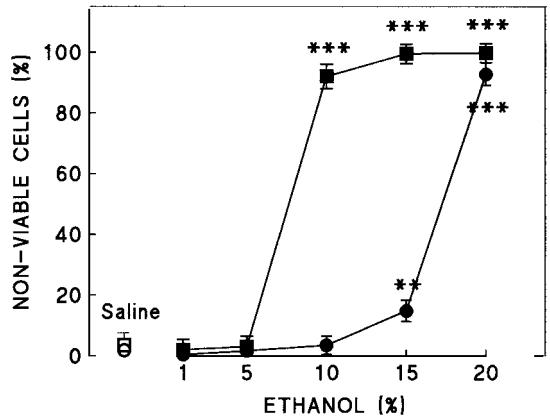

Fig. 1. Influence of ethanol on trypan blue exclusion in gastric gland cells. Isolated rabbit gastric glands were incubated as indicated in MATERIALS AND METHODS in the absence (open symbols) or presence (filled symbols) of different concentrations of ethanol (1–20% vol/vol) for 30 (circles) or 60 (squares) min. The percentage of nonviable cells (stained by 0.1% trypan blue) was estimated in at least 20 gastric glands per experiment. Results are expressed as means ± SE of 12 separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the corresponding saline value.

ethanol (1–20% vol/vol) in the incubation medium caused a time- and dose-dependent increase in the percentage of stained cells. It should be pointed out that treatment of gastric glands with 10% ethanol for 30 min was not associated with a significant increase in the number of stained cells (1.0 ± 0.2% and 3.5 ± 2.0% of stained cells, respectively, for gastric glands incubated without and with 10% ethanol; $P > 0.05$; n = 12 experiments). However, higher concentrations of ethanol (15 and 20%) significantly raised the amount of stained cells, 19.3 ± 0.3% (n = 12 experiments) being the calculated EC_{50} value. Likewise, after a longer period (60 min) of incubation, concentrations of ethanol of ≥10% caused a marked increment in the percentage of stained cells (92.1 ± 0.2% of stained cells for 10% ethanol); under these conditions, the calculated EC_{50} value for ethanol was 8.2 ± 0.2%.

The influence of ethanol on the release of cytosolic enzymes from the glandular cells was also studied (Fig. 2). Ethanol accelerated the release of lactate dehydrogenase and phosphoglucose isomerase into the incubation medium in a time- and dose-dependent manner. Thus incubation of gastric gland suspensions in the presence of concentrations of ethanol of up to 10% (vol/vol) for 30 min did not significantly increase the release of the above-mentioned cytosolic enzymes into the incubation medium compared with the spontaneous leakage estimated in gastric glands incubated without ethanol. However, higher ethanol concentrations (15 and 20%) or longer periods of incubation (60 min) of the gastric glands in the presence of ethanol significantly stimulated enzyme leakage into the medium; the maximal effect was observed when the glands were treated with 20% ethanol for 30 or 60 min. In gastric gland suspensions incubated for 30 min, the calculated EC_{50} values for ethanol eliciting lactate dehydrogenase and phosphoglucose isomerase release were 13.9 ± 0.4% and 13.5 ± 0.2%, respectively.

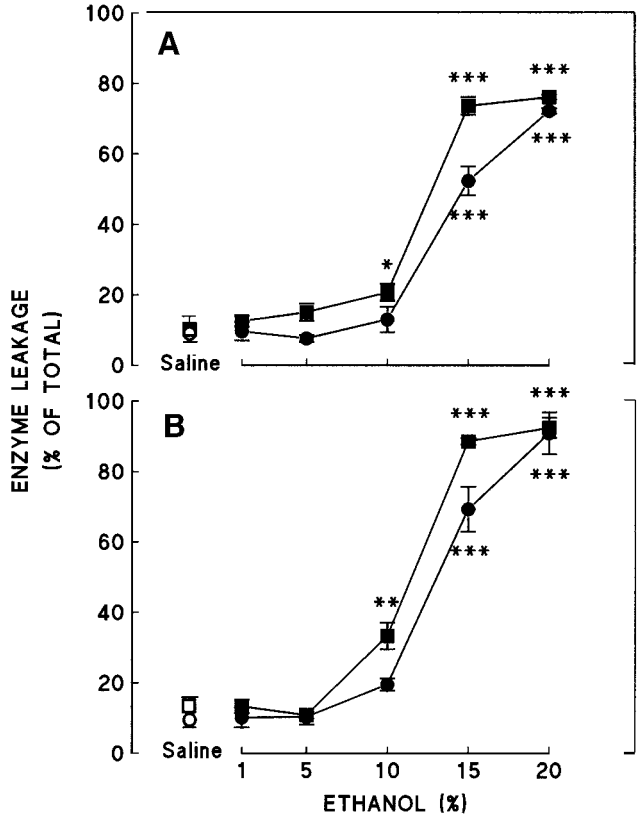

Fig. 2. Effect of ethanol on lactate dehydrogenase (*A*) and phosphoglucose isomerase (*B*) release from isolated rabbit gastric glands into the incubation medium. Isolated rabbit gastric glands were incubated in the absence (open symbols) or presence (filled symbols) of different concentrations of ethanol (1–20% vol/vol) for 30 (circles) or 60 (squares) min. Samples of the gastric gland suspensions were then processed as indicated in MATERIALS AND METHODS. Values represent means ± SE of 3 separate experiments carried out in duplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. the corresponding saline value.

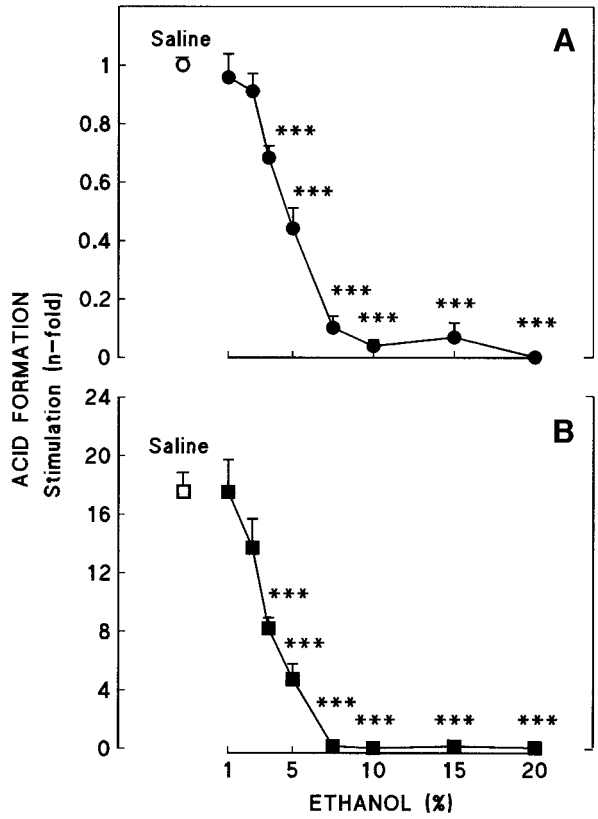

Fig. 3. Influence of ethanol on the rate of acid secretion in isolated rabbit gastric glands. Isolated rabbit gastric glands were incubated for 30 min at 37°C either under basal conditions (*A*) or with 1 μM forskolin (*B*) in the absence (open symbols) or presence (filled symbols) of different concentrations of ethanol as indicated in MATERIALS AND METHODS. The [^{14}C]aminopyrine concentration ratio under basal conditions (1.94 ± 0.23) was considered as 1-fold stimulation. Values represent means ± SE of 3 separate experiments carried out in duplicate. $***P < 0.001$ vs. the corresponding saline value.

Influence of ethanol on gastric acid secretion. When the influence of different concentrations (1–20%) of ethanol on the rate of acid formation was examined (Fig. 3), it was observed that 1% ethanol did not significantly affect the rate of acid secretion in gastric glands incubated either in the absence of secretagogues or in the presence of forskolin (1 μM). However, 5% ethanol caused a significant reduction in the rate of acid formation under both conditions (58% and 63%, respectively). Maximal inhibition was already observed at 10% ethanol, and the calculated EC_{50} values for this alcohol were 4.5 ± 0.2% and 3.5 ± 0.2%, respectively, in nonstimulated and forskolin-stimulated gastric glands.

Effect of ethanol on ATP and cAMP levels in gastric glands and in cultured parietal cells. As shown in Fig. 4, incubation of gastric glands with 1% ethanol for either 30 or 60 min did not significantly affect the concentration of ATP in isolated gastric glands. However, concentrations of ethanol ≥5% caused a dose-dependent and statistically significant decrease in ATP levels. It is of note that treatment of gastric glands with 10% ethanol for 30 min reduced the glandular

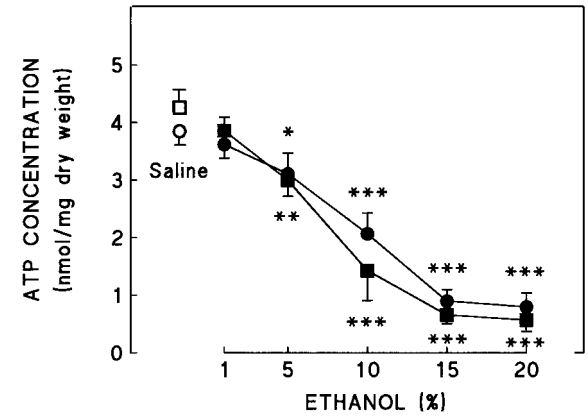

Fig. 4. Effect of ethanol on the ATP content of isolated rabbit gastric glands. Isolated rabbit gastric glands were incubated in the absence (open symbols) or presence (filled symbols) of different concentrations of ethanol (1–20% vol/vol) for 30 (circles) or 60 (squares) min. Samples of the gastric gland suspensions were then processed as indicated in MATERIALS AND METHODS. Values represent means ± SE of 3 separate experiments carried out in duplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. the corresponding saline value.

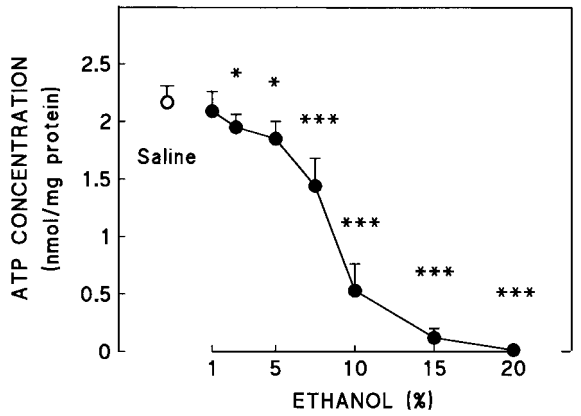

Fig. 5. Effect of ethanol on the ATP content of cultured gastric parietal cells. Gastric parietal cells cultured for 24 h were incubated in the absence (○) or presence (●) of different concentrations of ethanol (1–20% vol/vol) for 30 min. Values represent means ± SE of 3 separate experiments carried out in duplicate. $*P < 0.05$, $***P < 0.001$ vs. the corresponding saline value.

concentration of ATP by 47% compared with that measured in control gastric glands (2.0 ± 0.3 and 3.8 ± 0.2 nmol/mg dry wt, respectively; $P < 0.001$, $n = 3$ experiments). The maximal effect (a reduction of ~80% in glandular ATP content) was observed in the presence of 20% ethanol, with 8.8 ± 0.4% and 6.7 ± 0.1% being the corresponding EC_{50} values estimated in gastric gland suspensions treated for 30 and 60 min, respectively, with this alcohol.

To investigate more thoroughly the influence of ethanol on the energetic metabolism of the gastric acid secretory cell, we have studied the effect of different concentrations of ethanol on the cellular content of ATP in primary cultures of highly purified gastric parietal cells. As shown in Fig. 5, similarly as described for isolated gastric glands, treatment of cultured parietal cells with ethanol for 30 min caused a dose-dependent reduction in the cellular levels of ATP. The maximal effect was observed at 20% ethanol, 8.5 ± 0.2% being the calculated EC_{50} value ($n = 3$ experiments).

We also studied the influence of ethanol on cAMP levels in cultured parietal cells. As shown in Table 1, the cAMP content of parietal cells incubated either under basal conditions or in the presence of IBMX (500 μM) was not significantly modified by the presence of 5% ethanol in the incubation medium. In contrast, this ethanol concentration caused a marked reduction in cAMP levels in parietal cells incubated with forskolin (1 μM) in the absence or presence of IBMX (500 μM). Moreover, 10% ethanol markedly reduced the cellular content of this second messenger in parietal cells incubated under all the assayed conditions (Table 1).

Table 1. *Effect of ethanol on cAMP levels in cultured rabbit parietal cells incubated in absence or presence of secretagogues*

	cAMP, pmol/mg protein		
	Saline	5% Ethanol	10% Ethanol
Saline	1.2 ± 0.1	1.5 ± 0.1	0.8 ± 0.1*
Forskolin (1 μM)	69.2 ± 5.6	31.9 ± 3.5†	4.0 ± 0.6†
IBMX (500 μM)	1.9 ± 0.1	2.4 ± 0.2	1.3 ± 0.1†
Forskolin (1 μM) + IBMX (500 μM)	95.7 ± 13.4	48.1 ± 7.4*	8.2 ± 0.9†

Values are means ± SE of 4 separate experiments. Gastric parietal cells cultured for 24 h were incubated with the indicated agents for 15 min. cAMP was determined in acid extracts of cell monolayers by radioimmunoassay as indicated in MATERIALS AND METHODS. $*P < 0.05$, and $†P < 0.001$ vs. corresponding saline value.

Effect of ethanol on H^+-K^+-ATPase activity. The influence of ethanol on H^+-K^+-ATPase activity was studied in preparations of microsomes derived from nonstimulated gastric glands. As indicated in MATERIALS AND METHODS, microsomal H^+-K^+-ATPase activity was assayed by a colorimetric method that measures the rate of inorganic phosphate release from ATP (21). As shown in Fig. 6, H^+-K^+-ATPase activity was inhibited by the presence of ethanol in the assay mixture in a dose-dependent manner. The maximal inhibitory effect was observed at 20% ethanol, 8.5 ± 0.6% being the calculated EC_{50} value. It is of note that at the lower ethanol concentrations tested (1 and 5%), H^+-K^+-ATPase activity was not significantly affected. This is in contrast with the finding that 5% ethanol caused a marked reduction in the rate of both basal and forskolin-stimulated acid formation (Fig. 3).

Influence of ethanol on H^+-K^+-ATPase-dependent proton transport. The influence of ethanol on the rate of H^+-K^+-ATPase-dependent proton transport into microsomal vesicles is shown in Fig. 7. Addition of ATP to the assay mixture caused a small and restricted reduction of acridine orange fluorescence (Fig. 7, *inset*), possibly due to the presence of limited amounts of K^+ within the microsomal vesicles. The additional presence of the K^+ ionophore valinomycin increased the availability of K^+ to internal sites, maximally stimulating H^+-K^+-ATPase activity. Consequently, the ATP-induced intravesicular H^+ accumulation rose, and the acridine fluorescence decreased to a minimum value

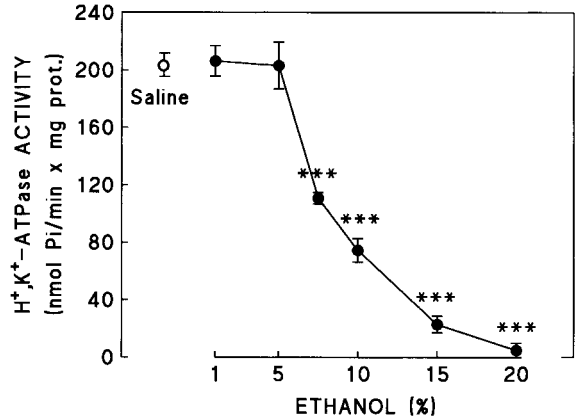

Fig. 6. Effect of ethanol on H^+-K^+-ATPase activity in gastric gland microsomes. H^+-K^+-ATPase activity was assayed by the hydrolysis of ATP in microsomes obtained from nonstimulated rabbit gastric glands as indicated in MATERIALS AND METHODS in the absence (○) or presence (●) of different concentrations of ethanol (1–20% vol/vol). Values represent means ± SE of 6 separate experiments carried out in duplicate. $***P < 0.001$ vs. the saline value.

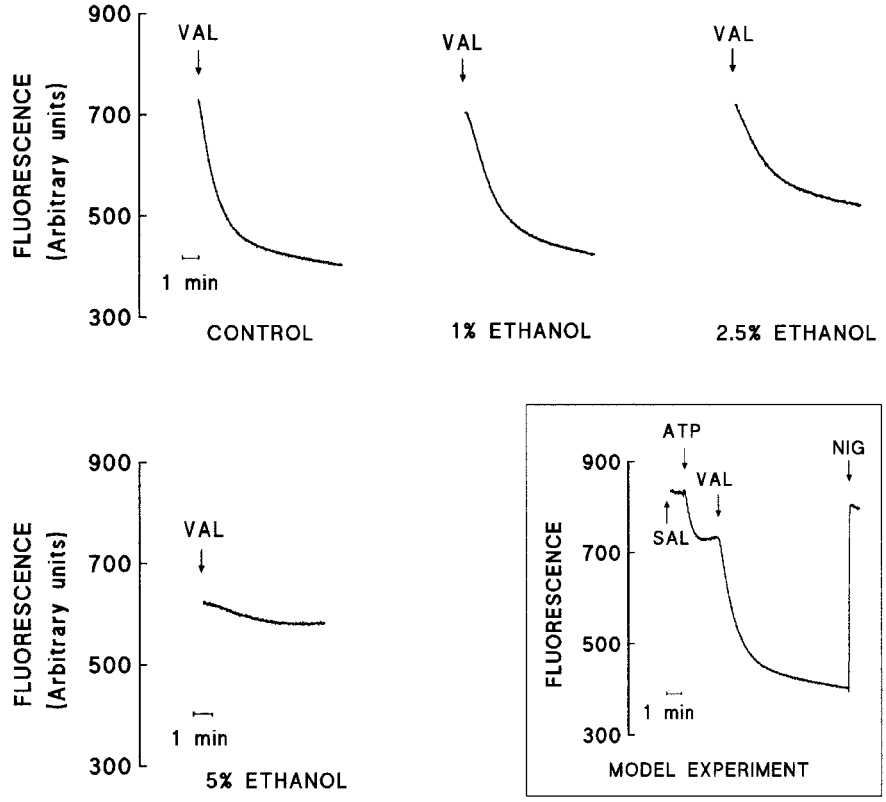

Fig. 7. Effect of ethanol on the rate of H^+-K^+-ATPase-dependent transport of protons in gastric gland microsomes. H^+-K^+-ATPase-dependent transport of protons was assayed in microsomes isolated from nonstimulated rabbit gastric glands by estimation of acridine orange fluorescence as indicated in MATERIALS AND METHODS. The initial rate of valinomycin-induced fluorescence decrease was considered as the rate of H^+-K^+-ATPase-dependent proton transport. Saline (Sal) or ethanol was incorporated into the reaction mixture immediately before the addition of ATP. Data are from a single preparation of gastric glands and are representative of at least 3 separate experiments. Val, 10 μM valinomycin; Nig, 5 μM nigericin.

within a few minutes, remaining at this low level for at least 15 min (data not shown). This was caused by the continuous influx of protons mediated by H^+-K^+-ATPase activity, which was apparently equilibrated with the rate of spontaneous proton leakage from the microsomal vesicles. The initial rate of this valinomycin-induced decrease of fluorescence was considered an index of the rate of H^+-K^+-ATPase-dependent proton transport into microsomal vesicles (23, 24). The subsequent addition of the K^+/H^+ exchange ionophore nigericin to the assay mixture rapidly dissipated the pH gradient generated, and the acridine fluorescence returned to its initial value in a few seconds (Fig. 7, *inset*). As shown in Figs. 7 and 8, the presence of different concentrations of ethanol in the reaction mixture caused a dose-dependent reduction in the rate of H^+-K^+-ATPase-dependent proton transport. The maximal effect was already observed at 7.5% ethanol, 3% being the calculated EC_{50} value.

Effect of ethanol on passive H^+ permeability. To study more specifically the influence of ethanol on passive proton permeability of microsomal membranes, we used the assay described by Reichstein et al. (30). In this approach, an ATP-dependent H^+ gradient across the microsomal membranes was generated first by the addition of valinomycin (Fig. 9, *inset*). Afterwards, H^+-K^+-ATPase activity was blocked by the rapid hydrolysis of ATP through a glucose-hexokinase trap. In a few seconds, spontaneous recovery of acridine orange fluorescence was observed, which corresponded to the rate of passive H^+ leakage from the microsomal intravesicular space (30). In fact, when the maximal fluorescence recovery was attained, nigericin was without further effect (Fig. 9, *inset*). The presence of ethanol increased the rate of passive H^+ leakage in a dose-dependent manner, 7.9% (vol/vol) being the calculated EC_{50} value (Figs. 9 and 10). As expected, nigericin (5 μM) also accelerated the passive leakage of protons from the intravesicular space (Fig. 9).

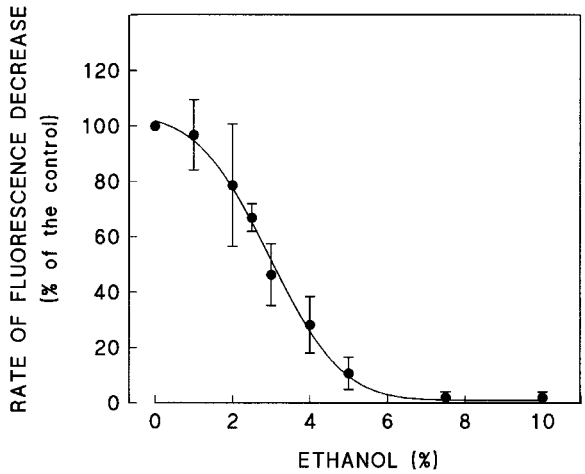

Fig. 8. Dose-response curve of the effect of ethanol on the rate of H^+-K^+-ATPase-dependent transport of protons in gastric gland microsomes. Data are from 3–6 experiments similar to those reported in Fig. 7 and represent the initial rates of valinomycin-induced fluorescence decrease measured in the absence or presence of different concentrations of ethanol. Values are expressed as percentage of the rate of valinomycin-induced fluorescence decrease observed in the absence of ethanol.

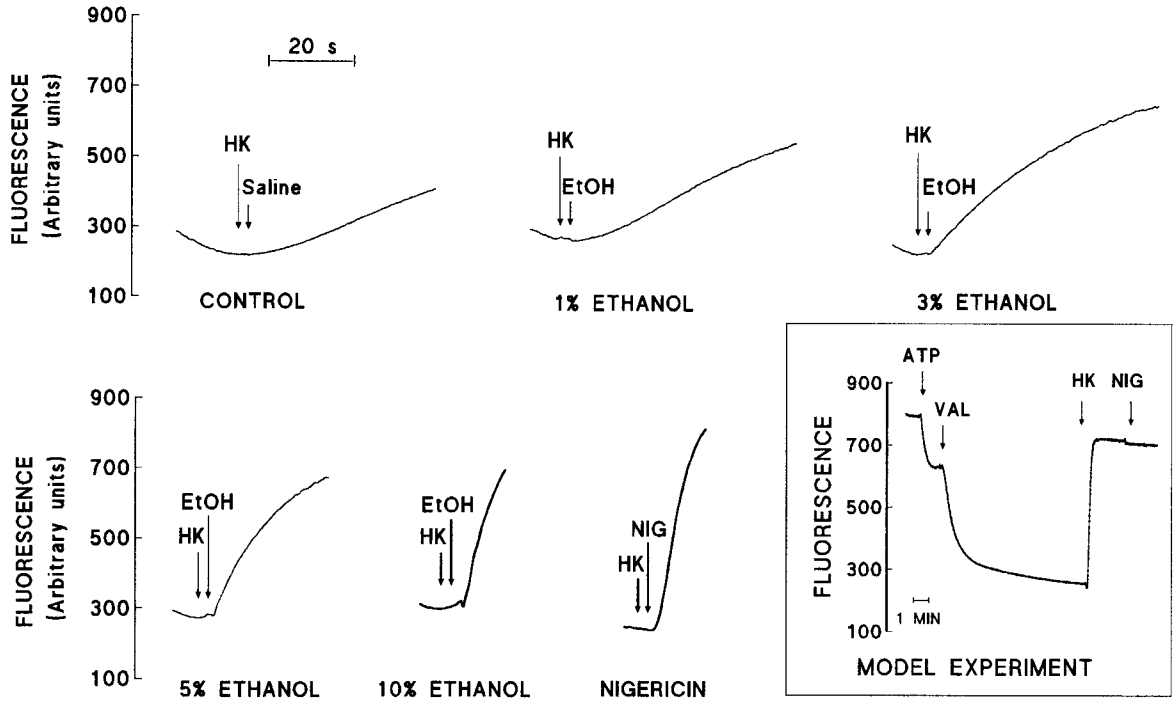

Fig. 9. Influence of ethanol on the rate of passive proton permeability in gastric gland microsomes. As shown in the inset and explained in the text, an ATP-dependent proton gradient was first established in gastric gland microsomes. A glucose/hexokinase trap (HK) was then added to the assay mixture to block H^+-K^+-ATPase activity. Under these conditions, the rate of fluorescence recovery is an index of passive leakage of protons from the gastric gland microsomes. EtOH, ethanol; Val, 10 μM valinomycin; Nig, 5 μM nigericin; ATP concentration was 1 mM. Data are from a single preparation of gastric glands, and are representative of at least 4 separate experiments.

DISCUSSION

It has been estimated that gastric intraluminal concentrations of ethanol may attain values of up to 10% (vol/vol) even in moderate drinkers (4, 25a). As demonstrated by Davenport (14), ethanol concentrations >8% (vol/vol) in the lumen of the stomach may alter the gastric mucosal "barrier" in dogs. Furthermore, studies carried out in animals (16, 22, 32, 34) and in humans (33, 35) demonstrated that exposure of gastric mucosa to concentrations of ethanol ≥10% produces functional and histopathological changes, the extent of the injury being directly related to the concentration of ethanol and to the exposure time. With respect to the toxic effects of ethanol in gastric acid secretion, concentrations of ethanol >10% have been shown to exert a marked inhibitory effect (11, 30, 32, 33, 35) through a multifocal mechanism acting at different points of the stimulus-secretion coupling process (26, 30).

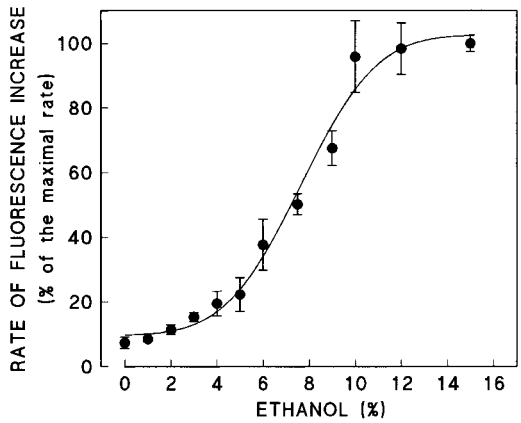

Fig. 10. Dose-response curve of the effect of ethanol on the rate of passive proton permeability in gastric gland microsomes. Data are from 4–6 experiments similar to those reported in Fig. 9. Values are expressed as the percentage of the maximal rate of fluorescence recovery elicited by ethanol.

In this work, we have investigated the influence of different concentrations of ethanol (1–20% vol/vol) on the rate of acid formation in isolated rabbit gastric glands incubated either under basal conditions or in the presence of forskolin (1 μM). Thus we have observed that the presence of ethanol in the incubation medium for 30 min caused a dose-dependent reduction in the rate of acid formation in both nonstimulated and forskolin-stimulated gastric glands; the calculated EC_{50} values for ethanol were, respectively, 4.5 ± 0.2% and 3.5 ± 0.2%, values very similar to those obtained by Mazzeo et al. (26) (4.2% vol/vol) in their study of the inhibition by ethanol of the [^{14}C]aminopyrine uptake ratio in basal parietal cell suspensions. Furthermore, the EC_{50} value for ethanol inhibiting [^{14}C]aminopyrine uptake in forskolin-stimulated gastric glands, calculated from the data reported by Reichstein et al. (30), was also ~4%. In agreement with the latter authors, we have also found that a concentration of 10% ethanol was sufficient to cause maximal inhibition of acid formation in gastric glands incubated either under basal conditions or in the presence of forskolin. The fact that

treatment of gastric glands with 10% ethanol for 30 min did not significantly modify the percentage of cells that excluded the trypan blue stain or the rates of lactate dehydrogenase and phosphoglucose isomerase release into the incubation medium indicates that the blockade in gastric acid formation elicited by this concentration of ethanol was not caused by a reduction in the viability of parietal cells present in the gastric glands.

Because gastric acid secretion is an energy-dependent process, a decrease in the cellular ATP levels caused by ethanol could be implicated in the underlying mechanism by which this alcohol inhibits the rate of acid formation in isolated gastric glands. In fact, ethanol caused a dose-dependent diminution in the ATP content of both gastric glands and parietal cells. However, the reduction in ATP levels did not appear to be the main mechanism involved in the inhibition of the rate of acid formation, at least by low concentrations of ethanol. This conclusion is based on the marked difference observed between the calculated EC_{50} value for ethanol-induced inhibition of acid formation in nonstimulated gastric glands (4.5 ± 0.2%) and those corresponding to ethanol-mediated reduction of ATP levels either in isolated gastric glands or in cultured parietal cells (8.8 ± 0.4% and 8.5 ± 0.2%, respectively).

The generation of cAMP has also been demonstrated to be a key step in the modulation of gastric acid secretion by ethanol. Thus several reports have established a direct relationship between the biphasic effect of ethanol on acid formation—stimulatory at concentrations of ethanol <5% (vol/vol) and inhibitory at higher concentrations—and the ability of this alcohol to elevate or depress the mucosal (29) or glandular (30) content of cAMP. In good agreement with these reports, our results show that ethanol, at concentrations of 5 and 10% (vol/vol), caused a significant reduction of cAMP levels in forskolin-stimulated parietal cells, whereas in cells incubated under basal conditions the decrease in the glandular cAMP content was statistically significant only in the presence of 10% ethanol. However, our findings do not support the concept that reduction in the glandular concentration of cAMP is the main mechanism implicated in the blockade of acid secretion, not only in gastric glands incubated under basal conditions but also in those treated with forskolin. In fact, although 10% ethanol completely blocked acid formation in forskolin-stimulated gastric glands and reduced the content of cAMP in forskolin-treated parietal cells by ~95%, the cellular concentration of this second messenger remained threefold higher than that measured in parietal cells incubated under basal conditions in the absence of ethanol (Table 1).

We also studied the influence of ethanol on H^+-K^+-ATPase activity in microsomes isolated from nonstimulated rabbit gastric glands. H^+-K^+-ATPase is located at the apical and tubulovesicular membranes of parietal cells and has been identified as the molecular machinery for proton transport. When this enzyme activity was assayed in terms of the hydrolysis of ATP according to the colorimetric method described above, we observed that low concentrations of ethanol (1 and 5%) did not significantly affect this activity. In contrast, higher ethanol concentrations (7.5–20%) caused a significant and dose-dependent inhibition of microsomal H^+-K^+-ATPase activity, 8.5 ± 0.6% being the calculated EC_{50} value for ethanol. Similar findings were reported in studies carried out in rabbit gastric glands (30) and parietal cells (26). In these reports, and in good agreement with our results, the calculated concentrations of ethanol corresponding to the half-maximal inhibitory effects (EC_{50} values) were ~8% and 10.5%, respectively. However, an apparent discordance arose from our data when the effects of ethanol on acid formation and on H^+-K^+-ATPase activity were compared. Thus 5% ethanol was able to reduce acid formation by ~60% in both nonstimulated and forskolin-stimulated gastric glands (Fig. 3), whereas H^+-K^+-ATPase activity was not significantly affected by this ethanol concentration (Fig. 6). This apparent inconsistency was reinforced by the difference found between the calculated EC_{50} values for ethanol as inhibitor of basal acid production and of H^+-K^+-ATPase activity (4.5 ± 0.2% and 8.5 ± 0.6%, respectively). These findings indicate that the inhibition of the hydrolytic activity of microsomal H^+-K^+-ATPase is less sensitive to ethanol than the reduction in acid formation by isolated gastric glands. Consequently, none of these results supports the concept that the inhibition of the hydrolytic activity of H^+-K^+-ATPase is the main mechanism involved in the inhibitory effect of ethanol on gastric acid production. It is of note that a similar discrepancy between the sensitivity to ethanol of aminopyrine uptake ratio and that of K^+-stimulated *para*-nitrophenylphosphatase activity was reported by Mazzeo et al. (26) in isolated rabbit parietal cells (see Fig. 5 of Ref. 26).

When the influence of ethanol on the rate of H^+-K^+-ATPase-dependent proton transport into microsomal vesicles was studied, it was observed that the inhibition of this process caused by ethanol was more effective than that exerted by this alcohol on the hydrolytic activity of H^+-K^+-ATPase itself. In fact, 5% ethanol almost completely reduced (~90%) the estimated rate of H^+-K^+-ATPase-dependent proton transport (Fig. 8) without causing significant changes in the hydrolytic activity of this enzyme (Fig. 6). To explain this apparent discrepancy, we must consider that H^+-K^+-ATPase-dependent proton transport into microsomal membranes was estimated by measuring the initial rate of valinomycin-induced decrease of acridine orange fluorescence (see MATERIALS AND METHODS). With this in mind, the inhibition of this process elicited by low concentrations of ethanol that had no significant effect on the hydrolytic activity of the H^+-K^+-ATPase (e.g., 5% vol/vol) could be caused by *1*) an increase in the passive proton permeability of microsomal membranes, which would dissipate the H^+ gradient generated by the H^+-K^+-ATPase activity, and/or *2*) an alteration of the H^+-K^+-ATPase itself that would specifically impair the ATP-dependent K^+/H^+ translo-

cation process but preserve its hydrolytic activity. With respect to the first hypothesis, we have demonstrated, according to Reichstein et al. (30), that ethanol was able to accelerate the rate of passive H^+ leakage from the microsomal intravesicular space in a dose-dependent manner. However, the difference found between the calculated EC_{50} values for ethanol increasing passive proton leakage (7.9% vol/vol) and inhibiting H^+-K^+-ATPase-dependent proton transport (3% vol/vol) excludes this first hypothesis. In fact, 5% ethanol almost completely blocked the H^+-K^+-ATPase-dependent proton transport (Fig. 8), with only small effects on passive proton leakage (Fig. 10). Concerning the second hypothesis, ethanol has a well-established "disordering" effect in cell membranes (13). Either high or low concentrations of ethanol may selectively disorder certain membrane lipid domains and thereby affect different functional proteins associated with these domains (26). In the case of gastric microsomal H^+-K^+-ATPase, the lipid environment has been shown to play a crucial role in maintaining its activity and stability. Thus changes in membrane-bound phospholipids elicited by lipid-perturbing agents such as ethanol (3, 26) or phospholipase A_2 (27) markedly impair gastric H^+-K^+-ATPase activity. However, the mechanism by which low concentrations of ethanol may inhibit the H^+-K^+-ATPase-dependent transport of protons across microsomal membranes without affecting the hydrolytic activity of H^+-K^+-ATPase is not well understood. Nevertheless, it must be said that a similar phenomenon has been described in studies carried out in isolated gastric vesicles of hog stomachs, in which acetyl phosphate was used as substrate of the H^+-K^+-ATPase (1). These studies showed that acetyl phosphate was able to block the transport of protons in gastric vesicles whereas the hydrolytic activity of the H^+-K^+-ATPase was maintained. Furthermore, it is of note that the corresponding EC_{50} values calculated for ethanol as inhibitor of basal and forskolin-stimulated gastric acid secretion (4.5 ± 0.2% and 3.5 ± 0.2%, respectively) are much closer to those obtained for this alcohol in the assays of H^+-K^+-ATPase-dependent proton transport (3%) than in the passive proton permeability studies (7.9%). This suggests that, at least at low concentrations of ethanol (up to 5% vol/vol), the reduction in gastric acid secretion is mainly due to the inhibition of ATP-dependent K^+/H^+ translocation across the cell membranes and not to an increase of passive proton leakage, which may be relevant at higher concentrations of ethanol.

In conclusion, our results show that ethanol can inhibit gastric acid secretion in isolated rabbit gastric glands by a multifocal mechanism, affecting different cellular processes whose relative relevance in the reduction of acid formation depends on their particular sensitivity to ethanol toxicity. Thus high concentrations of ethanol (≥7% vol/vol) exert a wide variety of toxic effects, including alteration of both passive and H^+-K^+-ATPase-dependent transport of protons across cell membranes, inhibition of the hydrolytic activity of H^+-K^+-ATPase itself, reduction of the cellular contents of both ATP and cAMP, and stimulation of the leakage of cytosolic enzymes, which together may lead to a complete blockade of acid secretion and even to a loss of parietal cell viability. However, at lower concentrations of ethanol (up to 5% vol/vol), the inhibition of H^+-K^+-ATPase-dependent translocation of protons across the cell membranes seems to be the main mechanism involved in decreasing gastric acid formation.

We are grateful to Dr. J. C. Sánchez-Gutiérrez and Dr. J. J. Carrillo for their critical reading of the manuscript.

This work was supported by a grant from Boehringer Ingelheim España S.A. J. C. Del Valle and R. Andrade are fellows of Boehringer Ingelheim España S.A., M. Salvatella is a fellow of Consejo Superior de Investigaciones Científicas, Spain.

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