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

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; Moragon, 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-14C]aminopyrine (100–120 mCi/mmol) and the cAMP-3H-dicyclohexylcarbodiimide, and was hydrolyzed and were housed in animal quarters at 0–4°C.

Measurement of acid production. Acid secretion by isolated gastric glands was determined by accumulation of the weak base [14C]aminopyrine in the canicular compartment, folowing 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 [14C]aminopyrine (0.1 μCi/ml) and different concentrations of ethanol with or without 1 μM forskolin. After 20 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% HNO3 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 [14C]aminopyrine concentration, which was calculated as described elsewhere (7). Correction for the [14C]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 MgSO4, 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 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 mg/ml) and EDTA as previously reported (5) to obtain a suspension of isolated gastric cells. Parietal cells were enriched from the crude cell suspension by centrifugal elutriation in a Beckman J6-5C 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 g/ml in sterile water). The cultured cells were maintained in a 95% O2-5% CO2 atmosphere at 37°C for 24 h.

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

**H^+ transport assays.** H^+\text{-}K^+\text{-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^+\text{-}K^+\text{-ATPase} activity was coupled to H^+ accumulation in microsomal vesicles via a K^+\text{/H}^+ exchange process. In response to the pH gradient generated by the H^+\text{-}K^+\text{-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 corresponding to the half-maximal effects (EC_{50}) were calculated by the computer program Fig.P (Fig.P Software, Durham, NC).

**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 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.
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 EC50 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. 2. Effect of ethanol on lactate dehydrogenase (A) and phosphoglucone 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.](http://ajpgi.physiology.org/)

![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 14C-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.](http://ajpgi.physiology.org/)

![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.](http://ajpgi.physiology.org/)
3 separate experiments carried out in duplicate. 

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.

Effect of ethanol on cAMP levels in cultured parietal cells. As shown in Table 1, the cAMP content of parietal cells incubated either with saline or ethanol for 30 min caused a dose-dependent reduction in the cellular levels of ATP. The maximal inhibitory effect was observed at 20% ethanol, 8.5 ± 0.6% being the calculated EC50 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).

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

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<tr>
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<th>cAMP, pmol/mg protein</th>
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<tr>
<td></td>
<td>Saline</td>
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<tr>
<td>Saline</td>
<td>1.2 ± 0.1</td>
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<tr>
<td>Forskolin (1 µM)</td>
<td>69.2 ± 5.6</td>
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<tr>
<td>IBMX (500 µM)</td>
<td>1.9 ± 0.1</td>
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<tr>
<td>Forskolin (1 µM) + IBMX (500 µM)</td>
<td>95.7 ± 13.4</td>
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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, †P < 0.001 vs. corresponding saline value.

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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 6 separate experiments carried out in duplicate. *P < 0.05, **P < 0.001 vs. the corresponding saline value.
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 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.

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 EC₅₀ value (Figs. 9 and 10). As expected, nigericin (5 μM) also accelerated the passive leakage of protons from the intravesicular space (Fig. 9).

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

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.
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 $\geq 10\%$ 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).

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 $\mu M$). 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 $\pm$ 0.2% and 3.5 $\pm$ 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 $\sim 4\%$. 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 phosphoglucone 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 EC50 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 EC50 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 (EC50 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 EC50 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 EC₅₀ 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 “dis-ordering” 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 A₂ (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 EC₅₀ 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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