Inhibition of acid secretion by the nonsteroidal anti-inflammatory drugs diclofenac and piroxicam in isolated gastric glands: analysis of a multifocal mechanism

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Salvatella, María, Irma Rossi, Juan C. Del Valle, Yolanda Gutiérrez, Carmen Pereda, Begoña Samper, and Juan E. Feliu.
Inhibition of acid secretion by the nonsteroidal anti-inflammatory drugs diclofenac and piroxicam in isolated gastric glands: analysis of a multifocal mechanism. Am J Physiol Gastrointest Liver Physiol 286: G711–G721, 2004. First published December 23, 2003; 10.1152/ajpgi.00305.2003.—In nonsimulated rabbit gastric glands, acetylsalicylic acid (10–500 μM) and indomethacin (3–300 μM) did not significantly modify the basal rate of acid secretion, whereas diclofenac and piroxicam (10–1,000 μM each) caused a marked and dose-dependent inhibitory effect (EC50 = 138 and 280 μM, respectively). In gastric glands stimulated by histamine (100 μM), diclofenac also reduced the rate of acid formation in a dose-dependent manner. In contrast, acetylsalicylic acid, indomethacin, and piroxicam exerted a biphasic effect; thus low concentrations (3–100 μM) of these three agents significantly increased the rate of histamine-stimulated acid secretion (10–20% over the corresponding control value) by a cAMP-independent mechanism, whereas higher concentrations reduced the rate of acid formation. With respect to underlying biochemical mechanisms that could mediate inhibitory effects of NSAIDs on gastric acid formation, it was observed that both diclofenac and piroxicam, but not acetylsalicylic acid or indomethacin, decreased the glandular content of ATP, inhibited hydrolytic activity of gastric gland microsomal H+–K+–ATPase, and reduced the rate of H+–K+–ATPase-dependent proton transport across microsomal membranes in a dose-dependent manner. Furthermore, diclofenac and piroxicam also significantly increased passive permeability of microsomal membranes to protons. In conclusion, our work shows that diclofenac and piroxicam cause a significant reduction in the rate of basal and histamine-stimulated acid formation in isolated rabbit gastric glands and that these mechanisms involve in these inhibitory effects appear to be multifocal and include different states of stimulus-secretion coupling.

rabbit gastric glands; H+–K+–ATPase; proton transport; proton permeability

THERAPEUTIC USE OF NONSTERoidal anti-inflammatory drugs (NSAIDs) is frequently associated with gastrointestinal alterations, which include gastric erosion, peptic ulcer formation and perforation, major upper gastrointestinal hemorrhage and inflammation, and change in the permeability of the intestine and lower bowel (5, 55). All of these effects have long been associated with the inhibition of cyclooxygenase activity with the subsequent blockade of prostaglandin biosynthesis in the gastrointestinal mucosa (39, 49, 53, 55). However, the molecular basis of NSAID toxicity appears to be more complex, and the exact pathogenic mechanism remains to be elucidated. Moreover, the mechanisms responsible for the gastrointestinal damage may differ from one region of the digestive system to another (16, 49, 55).

Gastric damage provoked by NSAIDs seems to be due not only to their ability to suppress prostaglandin biosynthesis but also to topical irritant effects of NSAIDs on the gastric epithelium. These topical irritant effects are predominantly observed with acidic NSAIDs, which can be accumulated inside gastric cells because of the phenomenon of ion trapping (5, 47, 55). However, other side effects of NSAIDs, like disruption of the mucosal barrier (16, 47), uncoupling of oxidative phosphorylation (32, 49), neutrophil-dependent microvascular injuries (55, 56), and oxygen radical-mediated lipid peroxidation (38, 55), may also be important primary events that lead to gastric mucosal injury.

In connection with the phenomenon of ion trapping (5, 47, 55), gastric acid has been shown to play an important permissive role in NSAID-associated mucosal injury (46). Most NSAIDs are weak acids that selectively concentrate inside gastric cells at low intragastric pH (5, 47). The intracellular accumulation of NSAIDs alters ionic fluxes across the gastric mucosa, with sodium and potassium ions being released into the luminal fluid, whereas hydrogen ions diffuse back from the lumen to the mucosa, potentiating the gastric damage (49, 55).

Because of these effects, the influence of NSAIDs on the rate of gastric acid secretion has been a relevant matter of interest for many authors. Different in vivo studies carried out in humans have demonstrated that both acetylsalicylic acid and indomethacin are able to increase the basal rate of acid formation (8, 12, 21, 29) and to potentiate acid secretion stimulated by histamine (21, 29). Furthermore, in vitro studies carried out in isolated rabbit fundic glands (48), in preparations of rat gastric mucosa (41), or in isolated rabbit parietal cells (27, 28, 37) have demonstrated that micromolar concentrations of aspirin and other NSAIDs, like indomethacin, piroxicam, and naproxen, may potentiate gastric acid secretion stimulated by histamine, dibutyryl cAMP, or forskolin without affecting the basal rate of acid formation. The biochemical mechanism of this potentiation is not well established. However, it appears to

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be dependent on the presence of Ca\(^{2+}\) in the incubation medium and occurs without significant changes in either cAMP or inositol 1,4,5-trisphosphate levels or in H\(^{+}\)-K\(^{-}\)-ATPase activity (27, 48).

The present study was undertaken to investigate the influence of different NSAIDs, like acetylsalicylic acid, indomethacin, diclofenac, and piroxicam, on the rate of acid formation, in isolated rabbit gastric glands incubated either under basal conditions or in the presence of histamine (100 \(\mu\)M). The assayed NSAIDs were used in a wide range of concentrations, including those calculated to be present in the gastric lumen of patients treated with these drugs. We also tried to correlate the resulting changes in gastric acid secretion elicited by the NSAIDs with modifications in the glandular content of ATP and cAMP, as well as with the effect of these agents on the H\(^{+}\)-K\(^{-}\)-ATPase activity and on the rate of passive permeability of microsomal membranes to protons.

Our findings show that at the concentrations used, both piroxicam and diclofenac, but not acetylsalicylic acid or indomethacin, exert a marked and dose-dependent inhibition of the basal and histamine-stimulated rates of acid formation in isolated rabbit gastric glands. The inhibitory effects elicited by both piroxicam and diclofenac occur in parallel with a diminution in the glandular content of ATP. Furthermore, concentrations of these two agents able to block acid formation in isolated gastric glands cause a significant inhibition of the hydrolytic activity of H\(^{+}\)-K\(^{-}\)-ATPase, a reduction in the H\(^{+}\)-K\(^{-}\)-ATPase-dependent proton transport across microsomal membranes, and an increase in the passive permeability of these membranes to protons. On the other hand, low concentrations (3–100 \(\mu\)M) of acetylsalicylic acid, indomethacin, and piroxicam cause a small increase in the rate of histamine-stimulated acid formation, apparently through a cAMP-independent mechanism.

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; Morago, Toledo, Spain), were given water ad libitum, and were housed in animal quarters at a constant temperature of 23°C with a fixed 12:12-h light-dark cycle. The animal experimentation described was approved by the Ethics in Human and Animal Experimentation Committee of the Universidad Autónoma de Madrid. All animals were anesthetized with pentobarbital sodium (30 mg/kg body wt iv) immediately before the experiment.

Reagents. Collagenase A from Clostridium histolyticum (type 1), histamine dihydrochloride, acetylsalicylic acid, piroxicam, diclofenac, indomethacin, nigericin, IBMX, N,N’-dicyclohexylcarbodiimide (DCCD), valinomycin, and acridine orange were obtained from Sigma (St. Louis, MO). [Dimethylamine-\(^{14}\)C]aminopyrine (100–120 mCi/mmol) and the cAMP \(^{125}\)I-labeled radioimmunossay kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The remaining reagents, all of analytical grade, were from Roche Molecular Biochemicals (Mannheim, Germany), Sigma, or Merck (Darmstadt, Germany).

Gastric gland isolation. Gastric glands were prepared basically according to the method reported by Berglindh and Ohrberg (2), as described by Rossi et al. (44). Briefly, the stomach of an anesthetized rabbit was perfused in situ with phosphate-buffered saline (in mM: 149.6 NaCl, 3 K\(_2\)HPO\(_4\), and 0.64 NaH\(_2\)PO\(_4\), 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\(_2\)HPO\(_4\), 1 NaH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 1 CaCl\(_2\), 0.5 dithiothreitol, 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. NSAIDs were dissolved in 5 mM NaOH. Control incubations of gastric glands were carried out in the presence of NaOH at the same final concentrations. No significant differences were observed between control and saline (0.9% NaCl) incubations with respect to any assayed metabolic parameter.

Isolated rabbit gastric glands had the typical rodlike appearance (0.3–0.7 mm in length). More than 95% of the different cells present in the glands excluded the 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.

Measurement of acid production. Acid secretion by isolated gastric glands was determined by accumulation of [dimethylamine-\(^{14}\)C]aminopyrine in the canalicular compartment, following the method described by Berglindh et al. (1), with the modifications made by Del Valle et al. (6).

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 containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 15,000 g for 15 min. The resulting supernatant was then collected and centrifuged again at 105,000 g for 60 min. 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.

\(\text{ATP and cAMP assays.}\) The glandular content of ATP was determined fluorometrically according to the method described by Lowry and Passonneau (31). Briefly, after appropriate incubations in the presence of different concentrations of NSAIDs for 30 min at 37°C, aliquots of the gland suspension were treated with 5% HClO\(_4\) and centrifuged. The supernatants were neutralized with neutralize/K\(_2\)CO\(_3\) (0.3 M/3 M) and centrifuged again. ATP was measured in these neutralized extracts.

Glandular content of cAMP was determined by using a radioimmunologic method as previously described (7). Gastric glands were incubated at 37°C for 15 min with different concentrations of NSAIDs in the absence or presence of histamine (100 \(\mu\)M). After deproteinization with 5% trichloroacetic acid and subsequent centrifugation (3,000 g for 15 min), cAMP was determined in the resulting supernatants.

Enzyme assays. Glandular lactate dehydrogenase (EC 1.1.1.27) and phosphoglucose isomerase (EC 5.3.1.9) activities were measured by standard spectrophotometric methods (51, 54). 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. (20). For the enzyme assay, gastric gland microsomes (0.1 mg of protein), previously incubated with or without different concentrations of NSAIDs for 10 min (vol/vol) at 37°C, were added to an assay mixture containing (in mM) 150 KCl, 10 PIPES, 1 MgSO\(_4\), 5 Mg ATP, 1 EGTA, and 0.1 ouabain, at pH 7.2, and 10 \(\mu\)g/ml valinomycin, 10 \(\mu\)M DCCD, 2.5 \(\mu\)g/ml oligomycin, and the indicated NSAID 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 (14). Nonspecific ATPase activity (measured under similar conditions but in the absence of valinomycin and substituting 150 mM KC\(_2\) with 250 mM sucrose in the external mixture) was subtracted from the estimated H\(^{+}\)-K\(^{-}\)-ATPase activity.

Proton transport assays. H\(^{+}\)-K\(^{-}\)-ATPase-dependent transport of protons across microsomal membranes was assayed by using the
fluorescent amine acridine orange method (24, 25) as described by Del Valle et al. (7). The assay was performed at room temperature (21–23 °C) in a Perkin-Elmer LS-5B spectrophotometer. Microsomes (80 μg of protein/ml) were suspended in a medium containing (in mM) 10 PIPES, 150 KCl, 1 MgCl₂, 0.1 EDTA, 1 ATP, and 10 μM valinomycin, at pH 7.2, in the absence or in the presence of different concentrations of NSAIDs. Excitation and emission wavelengths used were 493 and 530 nm, respectively.

The influence of diclofenac and piroxicam on passive H⁺ permeability of microsomal membranes was studied, as previously described (7). For this purpose, an ATP-dependent proton gradient across the microsomal membranes was generated first by the addition of 10 μM valinomycin. Then, the H⁺-K⁺-ATPase activity was blocked by the rapid hydrolysis of ATP. This was achieved by the incorporation of glucose (5 mM) and hexokinase (18 U) to the reaction mixture immediately before the addition of either saline or NSAIDs (42).

Statistical analysis. Statistical significance of differences was calculated by the paired Student’s t-test. Differences were considered statistically significant when the P value was <0.05. The calculated concentrations of NSAIDs corresponding to the half-maximal effects (EC₅₀) were calculated by the computer program Fig.P (Fig.P Software, Durham, NC).

RESULTS

Effects of NSAIDs on the cellular integrity of the isolated gastric glands. In the first part of the study, we examined the influence of different concentrations of NSAIDs 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 amounts of both lactate dehydrogenase and phosphoglucone isomerase released into the incubation medium were estimated in gastric gland suspensions treated for 30 min with different concentrations of NSAIDs. The amount of cells excluding the trypan blue stain in gastric glands incubated in the absence of NSAIDs was 98 ± 0.5%, and this value was not significantly modified by the presence of either acetylsalicylic acid, diclofenac, piroxicam (10–1,000 μM each), or indomethacin (3–300 μM) in the incubation medium (data not shown). With respect to the influence of NSAIDs on the release of cytosolic enzymes from the glandular cells, it was observed that, among the assayed NSAIDs, only diclofenac at a concentration of 1 mM significantly accelerated the release of both lactate dehydrogenase and phosphoglucone isomerase into the incubation medium (lactate dehydrogenase release: 5.69 ± 0.4 and 9.72 ± 0.8 for saline and 1 mM diclofenac incubations, respectively; phosphoglucone isomerase release: 5.56 ± 0.4 and 11.71 ± 0.4 for saline and 1 mM diclofenac incubations. Values are expressed as percentages of total enzyme activities present in the gastric gland incubations; n = 3 experiments).

Influence of NSAIDs on gastric acid secretion. Addition of acetylsalicylic acid (10–1,000 μM) or indomethacin (3–300 μM) to the incubation medium did not significantly modify the basal rate of acid formation (Fig. 1). In contrast, diclofenac and piroxicam (10–1,000 μM each) significantly reduced the basal rate of acid secretion in a dose-dependent manner; in both cases, the maximal effects (96 and 74% inhibition for diclofenac and piroxicam, respectively) were observed at concentrations of 1 mM, with 138 and 280 μM being the calculated EC₅₀ values, respectively. Treatment of isolated gastric glands with histamine (100 μM) increased the rate of acid formation approximately fourfold (Fig. 1). In the presence of this secretagogue, low concentrations (3–100 μM) of either acetylsalicylic acid or indomethacin enhanced the rate of acid secretion (10–20% over the control value), an effect that disappeared at higher NSAID concentrations. In contrast, diclofenac caused a marked dose-dependent reduction in the rate of acid formation.

![Fig. 1. Influence of different NSAIDs on the rate of acid formation. Isolated rabbit gastric glands were incubated at 37°C for 30 min in the absence or presence of different concentrations of NSAIDs under basal conditions (open symbols) or with 100 μM histamine (closed symbols) as indicated in MATERIALS AND METHODS. Values represent means ± SE of 3–4 separate experiments carried out in duplicate. *P < 0.05, **P < 0.01, and ***P < 0.005 vs. the corresponding control histamine value.](http://ajpgi.physiology.org/doi/10.1210/gastro.2004-0839)
in histamine-treated gastric glands; the maximal inhibitory effect (98%) was observed at 1 mM diclofenac, and the calculated EC50 value was 100 μM. However, under these conditions, piroxicam exerted a biphasic effect on the rate of acid formation; thus low concentrations (3–100 μM) of this NSAID caused a small but statistically significant increase (10–20% over the corresponding control value) in the rate of histamine-stimulated acid formation, but higher concentrations showed a clear inhibitory effect. In fact, piroxicam at the highest concentrations tested (1 mM) blocked acid secretion almost completely (that fell below the basal rate); the calculated EC50 value for piroxicam as inhibitor of histamine-stimulated gastric acid secretion was 210 μM.

With respect to the reinforcement of histamine-stimulated acid secretion by low micromolar concentrations of acetylsalicylic acid, indomethacin, and piroxicam, our data show a small but statistically significant effect (10–20% over the corresponding histamine value). These results contrast with those previously reported by Murthy and Levine (37) and Levine et al. (27), showing that acetylsalicylic acid (10 μM) and indomethacin (1–100 μM) reinforced the rate of acid formation in isolated parietal cells treated with 100 μM histamine by ~50–100%. On the other hand, Schwartzel et al. (48) observed that, in isolated rabbit gastric glands, indomethacin (100 μM) increased the rate of aminopyrine uptake stimulated by histamine (1–100 μM) by approximately threefold. We have no clear explanation for the less-marked NSAID reinforcement of histamine-mediated acid secretion observed by us. The possibility that the concentration of histamine used (100 μM) could maximally stimulate the rate of acid formation in our gastric gland preparations, minimizing in this way the potentiation elicited by low concentrations of NSAIDs, should not be overlooked.

Effect of NSAIDs on ATP and cAMP levels in isolated gastric glands. It is well established that gastric acid formation is an ATP-dependent process. To investigate whether or not the inhibition of the rate of acid formation elicited by diclofenac and piroxicam could be due to a reduction in the glandular content of ATP, we measured the levels of this adenine nucleotide in gastric glands incubated for 30 min with different concentrations of the indicated NSAIDs. As shown in Fig. 2, neither acetylsalicylic acid nor indomethacin, which did not inhibit the basal rate of acid formation at the concentrations used, significantly modified the glandular content of ATP. In contrast, diclofenac and piroxicam caused a marked reduction in the ATP levels in a dose-dependent manner. The maximal effect (a reduction of ~85%) was observed in the presence of 1 mM of either diclofenac or piroxicam; the calculated EC50 values were 254 and 361 μM, respectively. As shown in Fig. 3, a statistically significant direct correlation could be established between the basal rate of acid formation and the glandular content of ATP in nonstimulated gastric glands incubated with different concentrations of diclofenac and piroxicam. In the case of diclofenac, this close correlation was most clearly observed at the highest concentrations of NSAID used (0.25–1 mM). In fact, low concentrations of diclofenac (10–100 μM) reduced the basal rate of acid formation without significant changes in the glandular ATP content (Fig. 3A).

When the influence of the different NSAIDs on the glandular content of cAMP was investigated (Table 1), it was observed that none of these agents at the assayed concentrations significantly modified cAMP levels measured in gastric glands incubated either in the absence of secretagogues or in the presence of both histamine (100 μM) and IBMX (0.5 mM). In gastric glands incubated under the latter conditions, only pi-
We studied their effect on the glandular content of cAMP (Table 1).

**Effect of NSAIDs on H\(^+\)-K\(^+\)-ATPase activity.** To gain more insight into the mechanism by which diclofenac and piroxicam inhibit gastric acid formation, we studied their effect on the hydrolytic activity of H\(^+\)-K\(^+\)-ATPase assayed in microsomal preparations obtained from nonstimulated gastric glands. As shown in Table 2, both diclofenac and piroxicam were able to cause a statistically significant inhibition of this enzyme activity in a dose-dependent manner. Maximal inhibitory effects were observed at 1 mM NSAID concentration, diclofenac being more effective than piroxicam (~65 and 38% of inhibition, respectively). In contrast, acetylsalicylic acid and indomethacin, agents that did not show any inhibitory effect on gastric acid secretion at the concentrations used, did not significantly modify the hydrolytic activity of H\(^+\)-K\(^+\)-ATPase.

**Effect of NSAIDs on H\(^+\)-K\(^+\)-ATPase-dependent proton transport.** We also investigated the influence of all the assayed NSAIDs on the rate of H\(^+\)-K\(^+\)-ATPase dependent proton transport into microsomal vesicles, using the fluorometric method previously described (7, 42). In this fluorometric assay (Fig. 4, control incubation), addition of ATP to the reaction mixture caused a small reduction of acridine orange fluorescence, possibly due to the presence of limited amounts of K\(^+\). In contrast, subsequent addition of the K\(^+\) ionophore valinomycin increased the availability of K\(^+\) and significantly increased the acridine orange fluorescence, which returned to its initial value after a few minutes more (data not shown). The subsequent addition of the K\(^+\)-K\(^+\) exchange ionophore nigericin (5 \(\mu\)M) to the assay mixture rapidly dissipated the pH gradient generated, and the acridine fluorescence returned to its initial value in a few seconds. The initial rate of this valinomycin-induced fluorescence decrease was considered an index of the rate of the H\(^+\)-K\(^+\)-ATPase-dependent proton transport into microsomal vesicles (7, 42). In good agreement with the lack of inhibitory effects on gastric acid secretion and on the hydrolytic activity of the H\(^+\)-K\(^+\)-ATPase, neither acetylshalic acid, agents that did not show any inhibitory effect on gastric acid secretion at the concentrations used, did not significantly modify the hydrolytic activity of H\(^+\)-K\(^+\)-ATPase.
tysalicylic acid nor indomethacin modified the rate of H⁺-K⁺-ATPase-dependent proton transport at the assayed concentrations (Fig. 4). In contrast, the presence of different concentrations of either piroxicam or diclofenac in the assay mixture caused a dose-dependent reduction in the rate of proton transport mediated by the H⁺-K⁺-ATPase activity (Figs. 5 and 6). The maximal inhibition elicited by piroxicam was ~54% and was observed at a concentration of 1 mM; the calculated EC₅₀ value was 183 μM (Fig. 5B). In contrast, 1 mM diclofenac completely blocked the H⁺-K⁺-ATPase-dependent proton transport, with 240 μM being the calculated EC₅₀ value (Fig. 6B).

Effect of piroxicam and diclofenac on passive proton permeability. To study more specifically the influence of piroxicam and diclofenac on passive H⁺ permeability of microsomal membranes, we used the assay described by Reichstein et al. (42), with the modifications made by Del Valle et al. (7). As shown in the inset of Fig. 7A, first an ATP-dependent H⁺ gradient across the microsomal membranes was generated by addition of valinomycin. Afterward, H⁺-K⁺-ATPase activity was blocked by the rapid hydrolysis of ATP through a glucose-hexokinase trap. In a few seconds, a spontaneous recovery of acridine orange fluorescence was observed, which corresponded to the basal rate of passive H⁺ leakage from the microsomal intravesicular space. In fact, when the maximal fluorescence recovery was attained, addition of the K⁺/H⁺ exchange ionophore nigericin (5 μM) to the assay mixture was without effect (Fig. 7A, inset).

As shown in Fig. 7, when different concentrations (25–1,000 μM) of piroxicam were incorporated into the assay mixture immediately after the addition of the glucose-hexokinase trap, a significant and dose-dependent increase in the rate of passive H⁺ leakage from the microsomal intravesicular space was observed. Taking the rate of microsomal H⁺ leakage measured in the presence of 5 μM nigericin (Fig. 7A) as 100%, the rate of spontaneous or basal passive H⁺ permeability was 7.6 ± 1.2%. The presence of 1 mM piroxicam in the assay mixture raised the rate of passive H⁺ permeability to 19.4 ± 1.9% (≈2.5-fold increase), 590 μM being the calculated EC₅₀ value. Diclofenac (50–800 μM) also caused a dose-dependent increase in the rate of passive H⁺ leakage from microsomal vesicles (Fig. 8). In this case, and in accord with its more potent inhibitory effect on gastric acid secretion, 0.8 mM diclofenac enhanced the rate of passive H⁺ leakage to 37 ± 2.5% (approximately fivefold increase), 290 μM being the calculated EC₅₀ value for this NSAID. It is interesting to note that in the case of diclofenac the calculated EC₅₀ values for both the inhibition of the H⁺-K⁺-ATPase-dependent proton transport and the stimulation of the passive H⁺ permeability of microsomal membranes were very similar (240 and 290 μM, respectively).

DISCUSSION

The therapeutic use of NSAIDs is frequently limited by gastrointestinal side effects (3, 5, 55). These agents are thought to cause injury in gastric mucosa by at least two main mechanisms. Thus by blocking prostaglandin biosynthesis, NSAIDs impair different mucosal protective processes, like mucus and bicarbonate secretion, blood flow, and epithelial cell turnover and repair (53, 55). On the other hand, NSAIDs may also cause
topical irritant effects on gastric mucosa that are exacerbated by acidity, because it promotes the absorption of NSAIDs in their nonionized form (47, 55). Furthermore, when gastric epithelial cells have been injured by either of these two mechanisms, a second phase of damage mediated by luminal gastric acid may aggravate the mucosal lesions (5, 55). In this context, different in vivo animal studies have demonstrated that NSAID-related gastric ulcers are markedly dependent on luminal pH (9, 46, 47). Moreover, gastric acid suppression
Therapies have been shown to prevent gastroduodenal ulcers and erosions in patients taking NSAIDs daily (19, 50, 57). The relevant role that acid secretion seems to play in the NSAID-dependent gastric damage led us to carry out a systematic study on the direct influence of different NSAIDs (acetylsalicylic acid, indomethacin, and diclofenac and piroxicam) on basal and histamine-stimulated acid secretion, in isolated rabbit gastric glands. The assayed NSAID concentrations ranged from those at the low micromolar level ($3 \times 10^{-10} - 3 \times 10^{-8}$ M) that could be attained in plasma of patients treated with these drugs to concentrations that could be reached in the gastric lumen after oral NSAID administration ($10^{-2} - 10^{-1}$ M). It must be said that none of the assayed NSAIDs at the concentrations used significantly modified the percentage of glandular cells that excluded the trypan blue stain or the amount of lactate dehydrogenase and phosphoglucone isomerase released into the incubation medium. Our results confirm previous findings (13, 23, 43) and indicate that the concentrations of NSAIDs used in this study did not affect the cellular integrity of gastric glands. Only diclofenac, at the highest concentration assayed (1 mM), slightly increased the release of glandular lactate dehydrogenase and phosphoglucone isomerase into the medium (~4–6% of the total enzyme activities) compared with the release observed in saline incubations.

When the influence of the selected NSAIDs on the basal rate of acid formation was studied, two different patterns were observed. On the one hand, acetylsalicylic acid ($10^{-1} - 10^{-3}$ M) and indomethacin ($3 - 300$ M) did not significantly modify the rate of $[^{14}C]$aminopyrine uptake in gastric glands incubated under basal conditions. These results confirm previous reports that showed that neither of these two NSAIDs affected the basal rate of acid formation in in vitro studies carried out in isolated rabbit fundic glands (48), in preparations of rat gastric mucosa (41), or in isolated rabbit parietal cells (27, 28, 37). Furthermore, these findings were highly consistent with the

**Fig. 7.** Effect of different concentrations of PIR on the rate of passive proton permeability in gastric gland microsomes. A: as shown in the inset, an ATP-dependent proton gradient was first established in gastric gland microsomes. Then, a glucose/hexokinase trap (HK) was added to the assay mixture to remove ATP and to block H$^+\text{-K}^+$-ATPase activity. Under these conditions, the rate of fluorescence recovery is an index of passive leakage of protons from the gastric gland microsomes. The ATP concentration was 1 mM. VAL, 10 M; NIG, 5 M. Data are from a single preparation of gastric gland microsomes and are representative of at least 4 separate experiments. B: dose-response curve. Data are from 4–6 experiments similar to those reported in A. Values are expressed as the percentage of the maximal rate of fluorescence recovery elicited by NIG (100%).

Therapies have been shown to prevent gastroduodenal ulcers and erosions in patients taking NSAIDs daily (19, 50, 57).

The relevant role that acid secretion seems to play in the NSAID-dependent gastric damage led us to carry out a systematic study on the direct influence of different NSAIDs (acetylsalicylic acid, indomethacin, and diclofenac and piroxicam) on basal and histamine-stimulated acid secretion, in isolated rabbit gastric glands. The assayed NSAID concentrations ranged from those at the low micromolar level ($3 - 100$ M) that could be attained in plasma of patients treated with these drugs to concentrations that could be reached in the gastric lumen after oral NSAID administration ($10 - 1,000$ M). It must be said that none of the assayed NSAIDs at the concentrations used significantly modified the percentage of glandular cells that excluded the trypan blue stain or the amount of lactate dehydrogenase and phosphoglucone isomerase released into the incubation medium. Our results confirm previous findings (13, 23, 43) and indicate that the concentrations of NSAIDs used in this study did not affect the cellular integrity of gastric glands.

**Fig. 8.** Effect different concentrations of DCF on the rate of passive proton permeability in gastric gland microsomes. A: as described for PIR in the legend of Fig. 7A. NIG, 5 M. Data are from a single preparation of gastric gland microsomes and are representative of at least 4 separate experiments. B: dose-response curve. Data are from 4–6 experiments similar to those reported in A. Values are expressed as the percentage of the maximal rate of fluorescence recovery elicited by NIG (100%).

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lack of statistically significant changes in the glandular content of ATP, in the hydrolytic activity of microsomal H^+\text{-}K^+\text{-}ATPase, and in the rate of the H^+\text{-}K^+\text{-}ATPase-dependent proton transport across microsomal membranes observed in response to all the assayed concentrations of either acetylsalicylic acid or indomethacin. Moreover, our data are in good agreement with those previously reported by Levine et al. (27, 28) that showed that neither acetylsalicylic acid nor indomethacin significantly modified the hydrolytic activity of H^+\text{-}K^+\text{-}ATPase assayed in rabbit gastric microsomes.

In contrast, both piroxicam and diclofenac (10–1,000 μM each) caused a significant and dose-dependent reduction in the basal rate of acid formation. In both cases, the maximal inhibitory effects (74 and 96% for piroxicam and diclofenac, respectively) were observed at NSAID concentrations of 1 mM. For these two agents, the blockade in the basal rate of gastric acid formation was significantly correlated with a reduction in the glandular content of ATP. Thus a close correlation between these two parameters could be established for piroxicam at all the NSAID concentrations used. However, in the case of diclofenac, the correlation was only clearly observed at the highest assayed concentrations (0.25–1 mM) and not when this NSAID was used at much lower concentrations. In fact, 10–100 μM diclofenac was able to cause a significant reduction in the basal rate of acid secretion without significant changes in the glandular content of ATP. These findings clearly indicate that diclofenac may inhibit gastric acid formation by ATP-dependent and ATP-independent mechanisms. In connection with this, it must be mentioned that gastric acid secretion is an energy-dependent process and a decrease in the ATP content of parietal cells could be implicated in the underlying mechanism by which piroxicam and diclofenac inhibit gastric acid formation, mainly at the higher concentrations of NSAIDs used. Supporting this hypothesis, it should be noted that changes in the content of ATP observed in isolated rabbit gastric glands closely reflect those occurring in cultured gastric parietal cells (7). Furthermore, it has been demonstrated that piroxicam and diclofenac, in the range of the assayed concentrations, exert uncoupling effects on the oxidative phosphorylation in different types of cells, reducing their content of ATP and blocking different cellular ATP-dependent processes (4, 34, 36, 45). However, as shown in Table 3, the calculated EC_{50} values for both piroxicam and diclofenac as inhibitors of gastric acid secretion (280 and 138 μM for basal acid formation, respectively) were clearly lower than those obtained for these two NSAIDs as blockers of ATP formation (361 and 254 μM, respectively). These findings reinforce the concept that ATP-independent mechanisms could also be implicated in the inhibition of gastric acid formation by diclofenac and less evidently by piroxicam.

When the influence of these two NSAIDs on H^+\text{-}K^+\text{-}ATPase activity was assayed in gastric gland microsomes, it was observed that both piroxicam and diclofenac caused a significant and dose-dependent inhibition of the hydrolytic activity of this enzyme, as well as of the rate of H^+\text{-}K^+\text{-}ATPase-mediated proton transport into microsomal vesicles. For these two processes, diclofenac caused a greater inhibitory effect than piroxicam at all the concentrations used. This was in accordance with the higher efficacy showed by diclofenac in reducing gastric acid secretion, compared with that displayed by piroxicam. However, it appears that the inhibition of the H^+\text{-}K^+\text{-}ATPase hydrolytic activity does not completely explain the blockade of gastric acid formation caused by these two NSAIDs. In fact, it must be mentioned that the inhibitory effects elicited by piroxicam and diclofenac at concentrations of 1 mM each on the basal rate of gastric acid formation (74 and 96% inhibition, respectively) were clearly more intense than those caused by identical concentrations of these two NSAIDs on the microsomal H^+\text{-}K^+\text{-}ATPase hydrolytic activity (38 and 65% inhibition, respectively). Moreover, the calculated EC_{50} values for piroxicam and diclofenac as inhibitors of this enzyme activity (333 and >405 μM) were much greater, mainly in the case of diclofenac, than those obtained for these two NSAIDs as blockers of gastric acid formation (Table 3).

In contrast, the inhibitory effect caused by piroxicam on the H^+\text{-}K^+\text{-}ATPase-mediated proton transport across microsomal membranes appeared to be closely related to the reduction in gastric acid formation elicited by this NSAID. Supporting this reasoning, it must be noted that the maximal inhibitory effects elicited by piroxicam (1 mM) on these two processes were quite similar (73 and 60% inhibition, respectively). Moreover, the calculated EC_{50} value for piroxicam as inhibitor of the microsomal H^+\text{-}K^+\text{-}ATPase-mediated proton transport was 183 μM, a value close to, and even lower than, those obtained for this NSAID reducing both basal and histamine-stimulated acid formation in isolated gastric glands (280 and 210 μM, respectively) (Table 3). In the case of diclofenac, although this agent at a concentration of 1 mM was able to completely suppress both gastric acid formation and the H^+\text{-}K^+\text{-}ATPase-mediated proton transport across the microsomal membranes, the accordance between the calculated EC_{50} values for these two processes was not so close. In fact, the corresponding EC_{50} value for the microsomal H^+\text{-}K^+\text{-}ATPase-mediated proton transport (240 μM) was approximately double those obtained for basal and histamine-stimulated gastric acid formation (138 and 100 μM, respectively) (Table 3).

In studies carried out in artificial lipid bilayers (15, 18), in cell membranes (18, 30, 35), and in isolated mitochondria (10, 11, 34, 52), it was demonstrated that NSAIDs at concentrations in the high micromolar range may impair membrane permeability for ions and small molecules. In connection with this, we hypothesized that a potential increase in passive H^+ permeability of cell membranes elicited by NSAIDs would dissipate the H^+ gradient generated by the H^+\text{-}K^+\text{-}ATPase activity, resulting in a blockade of gastric acid formation. To learn whether or not changes in membrane permeability to protons

Table 3. Calculated EC_{50} values for piroxicam and diclofenac on different assayed biochemical parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Piroxicam</th>
<th>Diclofenac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal acid secretion</td>
<td>280</td>
<td>138</td>
</tr>
<tr>
<td>Histamine-stimulated acid secretion</td>
<td>210</td>
<td>100</td>
</tr>
<tr>
<td>Glandular ATP levels</td>
<td>361</td>
<td>254</td>
</tr>
<tr>
<td>H^+\text{-}K^+\text{-}ATPase hydrolytic activity</td>
<td>333 &gt;405</td>
<td></td>
</tr>
<tr>
<td>H^+\text{-}K^+\text{-}ATPase-proton transport</td>
<td>183 240</td>
<td></td>
</tr>
<tr>
<td>Passive proton permeability</td>
<td>590</td>
<td>290</td>
</tr>
</tbody>
</table>

Values are given in micromoles. *An “apparent” EC_{50} value because the maximal inhibition was not attained with the highest diclofenac concentration used.

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could be implicated in the inhibitory effects elicited by both piroxicam and diclofenac on gastric acid secretion, we investigated the influence of these two agents on passive permeability to protons in microsomal membranes isolated from non-stimulated gastric glands. Our results clearly show that both piroxicam and diclofenac cause a dose-dependent increase in passive permeability of microsomal membranes to protons. Again, diclofenac was more potent than piroxicam at all the assayed concentrations; this is in good agreement with the higher efficacy shown by diclofenac in reducing the rate of both basal and histamine-stimulated acid formation in isolated gastric glands, as well as in blocking the hydrolytic activity of the H⁺-K⁺-ATPase and the H⁺-K⁺-ATPase-mediated proton transport in gastric gland microsomal membranes. Furthermore, it is interesting to note that in the case of piroxicam, the increase in passive H⁺ permeability is a much less sensitive process (EC₅₀, 590 μM) than the inhibition of acid formation in isolated gastric glands (see the corresponding EC₅₀ values in Table 3). This indicates that the impairment of membrane permeability to protons and small molecules is not the main mechanism by which piroxicam inhibits gastric acid formation. Rather, in view of the EC₅₀ values obtained for the different metabolic parameters modulated by piroxicam (Table 3), it appears that the inhibition of H⁺-K⁺-ATPase-dependent proton transport is the process most closely related to the reduction of gastric acid formation by this NSAID. In contrast, diclofenac shows very similar EC₅₀ values for the increase in passive H⁺ permeability of microsomal membranes (290 μM), for the inhibition of the H⁺-K⁺-ATPase-mediated proton transport into microsomal vesicles (240 μM), and for the reduction of the glandular ATP content (254 μM) (Table 3), suggesting that these three processes are all closely related. In fact, the ability of diclofenac and other NSAIDs to alter the permeability of microsomal and mitochondrial membranes to ions has been demonstrated (10, 11, 18, 34, 52). This impairment of membrane permeability could be the mechanism by which diclofenac dissipates the H⁺ gradient generated by the H⁺-K⁺-ATPase activity as well as the mode by which it uncouples the mitochondrial oxidative phosphorylation. The consequence of these two effects would be a blockade of gastric acid formation. However, as mentioned above, unknown ATP-independent mechanisms could be implicated in the reduction of gastric acid formation by low micromolar concentrations of diclofenac (10–100 μM) and less evidently of piroxicam (10–50 μM). In this respect, the possible synergistic interaction between the effects of these two NSAIDs on H⁺-K⁺-ATPase hydrolytic activity, H⁺-K⁺-ATPase-dependent proton transport, and passive membrane permeability to protons could facilitate the inhibition of gastric acid formation at concentrations of NSAIDs that do not significantly affect the total glandular ATP content.

Finally, we must comment that in recent years diclofenac and other NSAIDs have been demonstrated to cause apoptosis in different types of cells as inducers of mitochondrial membrane permeability transition (MPT) (17, 22, 33, 40). MPT is a nonselective inner mitochondrial membrane permeabilization that may precede necrotic and apoptotic cell death (26), playing an important role in the pathogenesis of NSAID-induced ulcerogenesis (40). With this in mind, the possible induction of the apoptotic process during the 30-min treatment of isolated gastric glands with piroxicam and diclofenac cannot be ruled out. Under these conditions, NSAID inhibition of gastric acid formation could be considered as an early sign of cell death. However, the short-term inhibitory effects of both piroxicam and diclofenac on the glandular content of ATP, the hydrolytic activity of H⁺-K⁺-ATPase, and the H⁺-K⁺-ATPase-dependent proton transport, as well as their effects increasing passive membrane permeability to protons, clearly may explain the short-term blockade of gastric acid formation elicited by piroxicam and diclofenac.

In conclusion, our work supports the concept that both piroxicam and diclofenac inhibit 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 these NSAIDs.

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