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CO_2 chemosensing in rat oesophagus

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Abstract

Background—Acid in the oesophageal lumen is often sensed as heartburn. It was hypothesised that luminal CO_2, a permeant gas, rather than H^+, permeates through the epithelium, and is converted to H^+, producing an afferent neural signal by activating chemosensors.

Methods—The rat lower oesophageal mucosa was superfused with pH 7.0 buffer, and pH 1.0 or pH 6.4 high CO_2 (P_{CO2} = 260 Torr) solutions with or without the cell-permeant carbonic anhydrase (CA) inhibitor methazolamide (MTZ, 1 mM), the cell-impermeant CA inhibitor benzolamide (BNZ, 0.1 mM), the transient receptor potential vanilloid 1 (TRPV1) antagonist capsazepine (CPZ, 0.5 mM) or the acid-sensing ion channel (ASIC) inhibitor amiloride (0.1 mM). Interstitial pH (pH_{int}) was measured with 5′,6′-carboxyfluorescein (5 mg/kg intravenously) loaded into the interstitial space, and blood flow was measured with laser-Doppler.

Results—Perfusion of a high CO_2 solution induced hyperaemia without changing pH_{int}, mimicking the effect of pH 1.0 perfusion. Perfused MTZ, BNZ, CPZ and amiloride all inhibited CO_2-induced hyperaemia. CA XIV was expressed in the prickle cells, with CA XII in the basal cells. TRPV1 was expressed in the stratum granulosum and in the muscularis mucosa, whereas all ASICs were expressed in the prickle cells, with ASIC3 additionally in the muscularis mucosa.

Conclusions—The response to CO_2 perfusion suggests that CO_2 diffuses through the stratum epithelium, interacting with TRPV1 and ASICs in the epithelium or in the submucosa. Inhibition of the hyperaemic response to luminal CO_2 by CA, TRPV1 and ASIC inhibitors implicates CA and these chemosensors in transduction of the luminal acid signal. Transepithelial CO_2 permeation may explain how luminal H^+ equivalents can rapidly be transduced into hyperaemia, and the sensation of heartburn.

Symptomatic gastro-oesophageal reflux disease (GORD) encompasses a spectrum of diseases, ranging from severe complicated erosive oesophagitis to a syndrome of reflux

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symptoms experienced without demonstrable mucosal disease. The latter syndrome, termed non-erosive reflux disease (NERD), is an important source of morbidity in that its sufferers experience decrements in their quality of life, use excessive healthcare resources and often require chronic treatment with medications.[12] One factor inhibiting its ready diagnosis is that, in the absence of any disease visible with endoscopy or biopsy, subjective symptom scales are used to quantify the efficacy of interventions.[34]

The oesophagus responds consistently to acid perfusion by augmenting its intrinsic defence mechanisms, such as by increasing the thickness of the pre-epithelial gel layer, increasing bicarbonate secretion (in humans, pigs and frog, but not in rodents or rabbits) and increasing mucosal blood flow. Of these, the augmentation of mucosal blood flow is the most consistent, and can be measured relatively easily in experimental animals and humans alike.[56] Acid-related hyperaemia is probably related to activation of submucosal “acid sensors” on afferent nerves, such as the capsaicin receptor, transient receptor potential vanilloid-1 (TRPV1). These sensors can activate reflex efferent responses, such as the release of the vasodilatory mediators calcitonin gene-related peptide and nitric oxide.[6–8]

Although GORD symptoms are related to the presence of oesophageal acid reflux, the amount of acid permeation, as measured by acidification of the epithelial interstitium, across the tight and thick normal squamous oesophageal mucosa in vivo is negligible.[5] This observation is supported by the high electrical resistance in oesophageal mucosa, which at 1000–2500 Ω cm^2 is similar to that of the stomach, but much higher than in the duodenum,[9] suggesting that H^+ permeation through the oesophageal mucosa is quite low. Nevertheless, the oesophagus responds promptly and robustly to luminal acid by increasing blood flow and pre-epithelial gel thickness, consistent with rapid transduction of the luminal acid signal to submucosal efferent responses.[5] To explain this apparent inconsistency, we propose that CO_2, the permeant gas generated from gastric HCl combined with bicarbonate in the stomach or lower oesophagus and secreted by the pancreas, duodenum, stomach and oesophageal glands, may penetrate the oesophageal epithelium. Notably, the bicarbonate- and mucus-secreting oesophageal glands are anatomically present in humans.[1011] Submucosal CO_2 would then stimulate submucosal afferent nerves, enabling the subject to sense luminal acid. Sensitisation or potentiation of the acid/CO_2-sensing afferents may be related to the abnormal sensitivity of NERD patients to luminal acid, as measured with standard acid perfusion tests, such as the Bernstein test.[12] Since TRPV1 activation characteristically produces burning-like pain,[13] we hypothesise that GORD-related symptoms are also transduced by TRPV1 or another acid sensor, and that the acid-related hyperaemic response is a surrogate for this sensation. Furthermore, since CO_2 diffusion into the duodenal mucosa and CO_2-induced hyperaemia in the duodenum are dependent on epithelial cytosolic and ecto-carbonic anhydrase (CA) activity,[1415] we hypothesise that CAs are involved in CO_2-induced chemosensing in the oesophageal mucosa.

We therefore investigated the effect of the perfusion of a high CO_2 solution on interstitial pH (pH_{int}) and blood flow in rat oesophagus using an established ratio fluorometric method.[5] Furthermore, we examined the effect of inhibition of epithelial CAs and acid sensors on CO_2-induced mucosal responses, in order to test the hypothesis that luminal acid is

propagated through the epithelium by successive H^+–CO_2 interconversion facilitated by CA, and is sensed by epithelial and submucosal acid sensors.

METHODS

Chemicals and animals

5′,6′-Carboxyfluorescein (CF) was obtained from Molecular Probes (Eugene, Oregon, USA). Benzolamide (BNZ) was kindly provided by Dr Erik Swenson, VA Puget Sound Medical Center, Seattle, Washington, USA. A selective Na^+/H^+ exchanger 1 (NHE1) inhibitor, [1-(quinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl] guanidine (CP-597396; zoniporide, ZP)[16] was kindly provided by Pfizer Inc. (Groton, Connecticut, USA). S3226, a selective Na^+/H^+ exchanger 3 (NHE3) inhibitor,[17] was a kind gift of Aventis Pharma Deutschland (Frankfurt am Main, Germany). Methazolamide (MTZ), capsazepine (CPZ), capsaicin, amiloride (AML), HEPES and other chemicals were obtained from Sigma Chemical (St Louis, Missouri, USA). Krebs solution contained (in mM) 136 NaCl, 2.6 KCl, 1.8 $CaCl_2$ and 10 HEPES at pH 7.0. For pH 6.4 high CO_2 perfusion, 50 mM $NaHCO_3$/105 mM NaCl solution and 20 mM HCl/135 mM NaCl solution, prewarmed at 37°C, were equally vigorously mixed 1 min before perfusion, those generating isotonic (310 mOsm) pH 6.4 saline solution having P_{CO2} = 260 Torr at 37°C as previously described.[14,15,18] The pH 1.0 saline solution (P_{CO2} ~0 Torr) was made from 1 N HCl by adding NaCl to adjust isotonicity. Each solution was prewarmed to 37°C using a water bath, and temperature was maintained with a heating pad during the experiment. Capsaicin and CPZ solutions were prepared as previously described.[19] For vehicle perfusion, Krebs solution with 0.1% solvents or dimethylsulfoxide (DMSO) was used. BNZ, MTZ, AML, ZP or S3226 were dissolved in DMSO and stored at −20°C until use.

All studies were performed with approval of the Veterans Affairs Institutional Animal Care and Use Committee (VA IACUC). Male Sprague–Dawley rats weighing 200–250 g (Harlan, San Diego, California, USA) were fasted overnight, but had free access to water.

Measurement of blood flow

Under isoflurane anaesthesia (1.5–2.0%) using a rodent anaesthesia inhalation system (Summit Medical Systems, Bend, Oregon, USA), rats were placed supine on a heating block system warmed with recirculating water (Summit Medical) to maintain body temperature at 36–37°C, as monitored by a rectal thermistor. Prewarmed saline was infused via the right femoral vein at 1.08 ml/h using a Harvard infusion pump (Harvard Apparatus, Holliston, Massachusetts, USA); blood pressure was monitored via a catheter placed in the left femoral artery using a pressure transducer (Kent Scientific, Torrington, Connecticut, USA). The lower oesophageal mucosa was exposed as previously described,[5] and the lower oesophageal area within 1 cm above the oesophageal–cardiac junction was observed (Supplementary fig 1A). A concave stainless steel disk (16 mm diameter and 1–2 mm deep) with a 3 mm central aperture was fixed watertight on the mucosal surface with a silicone plastic adherent (Silly Putty, Binney & Smith, Easton, Pennsylvania, USA). The serosal surface of the oesophagus was supported with the right-angle laser-Doppler flow probe (R-type, Transonic, Ithaca, New York, USA) just below the chambered mucosa. A thin plastic coverslip was fixed to the

disk with the silicone adherent to permit closed superfusion with solutions at a rate of 0.25 ml/min by means of a Harvard infusion pump. Two PE-50 polyethylene perfusion lines were inserted into the chamber to enable rapid changes of the perfusate.

Oesophageal blood flow was measured as the voltage output of the laser-Doppler instrument (model BLF21, Transonic) and expressed relative to the stable level (the basal level) ~30 min after the perfusion was started. Blood flow was continuously recorded with a strip chart recorder and read every 5 min.

Measurement of pH_{int}

To measure pH_{int}, the pH-sensitive, fluorescent indicator CF (5 mg/kg) in saline was injected intravenously 5 min before the start of the experiment as previously described.[5] CF injected intravenously quickly distributed into the interstitial space of the oesophagus, which enabled us to measure pH_{int} (Supplementary fig 1B). Fluorescence of the microscopically observed chambered area of the oesophageal mucosa at 515 nm emission was recorded as previously described.[515] Capillary loops above the network of submucosal microvessels, which are assumed to be located in the subepithelial or basal epithelial layer of the rat oesophagus, were used to identify the focal plane (Supplementary fig 1B). Readings were taken ~10 s before and after each measured time point. The paired readings at 495 and 450 nm excitation needed to calculate a fluorescence ratio were thus taken at a maximum of 20 s apart. The paired images were captured every 5 min and analysed by selecting three areas of oesophageal submucosa between microvessels, which were followed throughout the experiment. In vitro calibration using an aqueous solution containing 0.2 μM CF was made. We have confirmed that the fluorescence ratio is stable over 60 min despite the gradual decrease of amplitude over time, consistent with previous measurements.[5]

To correlate pH_{int} and arterial blood pH in vivo, we produced acute respiratory acidosis.[20] After a 10 min basal period as described below, the inhalation gas was switched from 100% O_2 to 5% CO_2/95% O_2 or to 10% CO_2/90% O_2 for 10 min (challenge period), followed by 100% O_2 inhalation for 15 min (recovery period). For constant anaesthesia, isoflurane (2%) was continuously inhaled throughout the experiments. pH_{int} was measured every 5 min; arterial blood (0.2 ml) was collected in a heparinised syringe followed by a 0.2 ml flush of 1% heparin/saline via a catheter inserted in the left femoral artery for blood pressure measurement, at t = 10 min (at the end of the basal period), t = 20 min (at the end of CO_2 inhalation) and t = 35 min (at the end of the recovery period). We confirmed that blood withdrawal and the subsequent flush had no effect on blood pressure. Arterial blood pH and P_{CO2} were measured with a blood gas analyser (ABL5, Radiometer, Copenhagen, Denmark). We found that pH_{int} was stable during the basal period, whereas CO_2 inhalation dose-dependently acidified pH_{int}, accompanied by acute systemic acidosis (Supplementary fig 1C,D). Linear regression analysis revealed a slope ≈1 between arterial pH (assumed to correspond to pH_{int}), and pH_{int} calculated from the in vitro calibration curve (Supplementary fig 1E). These results strongly suggest that pH_{int} measured in vivo by ratiometry corresponds closely to the actual pH_{int} in the oesophagus.

Experimental protocol

The exposed oesophageal mucosa was superfused with solutions via a mucosally placed perfusion chamber. Blood flow was stabilised during continuous perfusion of pH 7.0 Krebs buffer, after which the time was set as t = 0 min. At t = −5 min, CF was injected intravenously. The oesophageal mucosa was then superfused with pH 7.0 Krebs buffer from t = 0 until t = 10 min (basal period); the perfusate was then changed to pH 6.4 saline (P_{CO2} ~0 Torr), the high CO_2 solution (P_{CO2} = 260 Torr; CO_2 challenge) or a pH 1.0 acid solution (acid challenge) from t = 10 until t = 20 min (challenge period), with or without the antagonists or inhibitors described below. The perfusate was changed to the pH 7.0 solution from t = 20 until t = 35 min (recovery period).

All of the luminally applied inhibitors, except for BNZ, were administered for 10 min during the basal period prior to the perfusion of the high CO_2 solution, to enable adequate time for the compounds to permeate through the epithelial layers. To examine the effect of the inhibition of cytosolic CA on oesophageal blood flow, the oesophageal mucosa was preperfused with MTZ (1 mM), dissolved in pH 7.0 Krebs buffer for 10 min, during the basal period. We have previously reported that preperfusion with 1 mM MTZ prior to perfusion of a high CO_2 solution inhibits CO_2-induced duodenal bicarbonate secretion, epithelial acidification, the hyperaemic response and transmucosal CO_2 movement, consistent with inhibition of cytosolic and membrane-bound CA activity.[14,15,18] We perfused the relatively cell-impermeant CA inhibitor BNZ (0.1 mM) in a high CO_2 perfusate to inhibit extracellular CA activity selectively, with conditions in which its cellular permeation is low.[21] BNZ in the concentrations used did not affect the pH of the high CO_2 solution.

The TRPV1 antagonist CPZ (0.5 mM)[15,19] was perfused from t = 0 to t = 10 min (pretreatment). Ablation of capsaicin-sensitive afferent nerves was accomplished with high-dose capsaicin pretreatment (125 mg/kg subcutaneously), as previously described.[19] The oesophageal mucosa of capsaicin-treated or vehicle-treated rat was superfused with a high CO_2 solution as described above. To determine the involvement of the acid-sensing ion channel (ASIC) in CO_2-induced hyperaemia, AML (0.1 mM) was perfused from t = 0 to t = 10 min (pretreatment), followed by the perfusion of high CO_2 solution for 10 min. AML was used to inhibit ASICs non-specifically, since no specific small molecule inhibitor for ASIC is available, although AML inhibits Na^+ channels such as the epithelial Na^+ channel (ENaC) in the micromolar range and NHE in the millimolar range.[22]

Since AML may inhibit NHE1 activity expressed in oesophageal mucosa,[23] we examined the effect of the selective NHE1 inhibitor zoniporide (ZP, 0.1 mM) or the selective NHE3 inhibitor S3226 (10 μM) on pH_{int} and blood flow. ZP or S3226 was perfused from t = 0 to t = 10 min (pretreatment), followed by the perfusion of high CO_2 solution for 10 min.

Expression of CA isoforms and acid sensors in rat oesophagus Reverse transcription-PCR (RT-PCR)

To investigate the expression of membrane-bound CAs and acid sensors in the rat oesophagus, real-time RT-PCR analysis was performed for CA isoforms, TRPV-1 and ASIC isoforms. Three rats were euthanised by terminal exsanguination under sodium pentobarbital

anaesthesia (50 mg/kg, intraperitoneally). The lower oesophagus, fundus and antrum of the stomach, proximal duodenum, Th1–Th8 dorsal root ganglia (DRGs) and nodose ganglia (NGs) were removed and used for RT-PCR as previously described.[1524] Furthermore, to sublocalise the presence of the target mRNA in the oesophagus, stomach and duodenum, these tissues were separated from the muscle layers and the mucosa by sharp dissection under a Zeiss stereomicroscope in ice-cold RNA stabilising solution (RNA-Later, Qiagen, Valencia, California, USA) followed by RNA extraction. The PCR primers used in the present study are listed in Supplementary table 1. The PCR primers for rat β-actin were used as an internal control. An aliquot of the reverse transcription reaction product served as a template in 40 cycles with 30 s at 95°C, 30 s at 58–64°C and 30 s at 72°C.

Immunofluorescence

Cryostat sections of the lower oesophagus and DRGs fixed with 4% paraformaldehyde were cut at 8 μm thickness, then immunofluorescent staining was performed as previously described.[1524] Primary antibodies (Abs) were used as follows; anti-TRPV1 C-terminus Ab (rabbit polyclonal, 1:100, Alomone Labs, Jerusalem, Israel), anti-ASIC1, 2 and 3 Abs (rabbit polyclonal, 1: 100, Alomone Labs) or anti-CA IV, IX, XII and XIV Abs (goat polyclonal, 1:100, R&D Systems, Minneapolis, Minnesota, USA). The sections were rinsed, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit or goat immunoglobulin G Ab (1:1000, Chemicon International, Temecula, California, USA). Negative controls were examined by omitting the primary antibody or by preabsorption with the immunising peptide antigen. The sections were observed using a confocal laser microscope (Leica TCS-SP Inverted, Leica Microsystems, Germany) or a fluorescence microscope (Zeiss, Jena, Germany).

Western blotting

The lower oesophagus, gastric fundus and DRGs were removed and immediately processed for western blotting. Some samples were also removed from vehicle- or capsaicin-treated rats. The tissues were homogenised in ice-cold 0.1 M Tris/5 mM EDTA buffer pH 7.5 containing a protease inhibitor cocktail (Sigma) and 1% Triton X-100. After centrifugation at 100 000 *g* for 30 min, the supernatant was collected and its protein concentration was measured using a Qubitprotein assay kit (Invitrogen, Carlsbad, California, USA). The samples were diluted with Laemmli sample buffer containing 5% β-mercaptoethanol, electrophoresed in 4–20% gradient ready gels, and blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary Abs as described above at 1:200–1:1000 dilution, followed by incubation with alkaline phosphatase-conjugated secondary Abs (Chemicon) at 1:1000–1:3000. The bound antibody was colourised using an alkaline phosphatase detection kit (Sigma). Primary Ab for β-actin (monoclonal, Sigma) was used as a loading control.

Transmucosal CO_2 movement

To elucidate whether luminal CO_2 diffuses into the interstitium and affects portal venous (PV) blood gas and to examine the role of epithelial CA in CO_2 diffusion, we perfused a high CO_2 solution through the oesophageal loop with or without a CA inhibitor. After the abdomen was opened as described above, the pyloric ring was ligated with a nylon ligature

to prevent the gastric content, including the secreted acid and the perfusate (see below), from entering into the duodenum, since acid or CO_2 exposure in the duodenum acidifies PV blood.[14] The lower oesophagus was cannulated with a 23-gauge metal cannula connected to a PE-50 tube, where it was secured with a nylon ligature under the serosal sheath in order to avoid compromising the vessels and vagal nerves. The forestomach was incised and a polyethylene tube was inserted, where it was secured, to drain the perfusate. The strip of gauze was also inserted to drain gastric juice. The tube tip was placed near the junction of the oesophagus and stomach, but not secured, since the large vessels and nerves in this area cannot be eliminated. The resultant 1 cm long oesophageal loop was perfused with prewarmed pH 7.0 saline at 1 ml/min using a peristaltic pump. The perfusate was collected without gastric fluid accumulation during ~1 h experiments. Since the gastric mucosa does not absorb H^+ and CO_2,[25] we predict little effect of gastric residual high CO_2 solution on PV blood gas measurements. Furthermore, before preparing the oesophageal loop, the gastroduodenal branch of the PV, which drains the lower oesophagus as well, was cannulated with a 23-gauge metal cannula connected to a PE-50 tube as previously described.[14] The catheter was fixed with cyanoacrylate glue and the tube was filled with heparinised saline enabling repeated blood sampling (each 0.1 ml). Portal blood samples were collected as described below, and pH and P_{CO2} were measured with a blood gas analyser (ABL5).

After ~30 min stabilisation with saline perfusion, the first sample of PV blood was taken and the time was set as t = 0. The second PV sample was taken at t = 30 min followed by the perfusion of a high CO_2 solution for 10 min, then the third PV sample was taken at t = 40 min at the end of a 10 min CO_2 exposure. To examine the effect of CA inhibition, MTZ (1 mM) in pH 7.0 Krebs solution was pretreated for 10 min from t = 20 to 30 min. The lumen was gently flushed with the perfusate at t = 20 and 30 min for rapid change of the perfusate.

Statistics

All data from six rats in each group were expressed as means (SEM). Comparisons between groups were made by one-way analysis of variance (ANOVA) followed by Fischer least significant difference test. p Values of 0.05 were taken as significant.

RESULTS

Effect of the luminal high CO_2 on oesophageal pH_{int} and blood flow

Oesophageal pH_{int} (fig 1A) and blood flow (fig 1B) were stable during perfusion with pH 7.0 Krebs (basal period), pH 6.4 saline ([CO_2] ~0) during the challenge period, used as control for the high CO_2 solution, and pH 7.0 during the recovery period. Perfusion of the acid solution (pH 1.0, P_{CO2} ~0) had no significant effect on pH_{int}, whereas blood flow was increased during acid perfusion and sustained during the recovery period, as previously described.[5] Similarly, the high CO_2 solution (pH 6.4, P_{CO2} = 260 Torr) increased oesophageal blood flow during the challenge period and sustained it during the recovery period without pH_{int} change (fig 1A,B), showing that luminal CO_2 mimics luminal acid-induced response in the oesophagus.

Effect of the inhibition of acid sensors on pH_{int} and CO_2-induced hyperaemia

The mucosa was preperfused with CPZ (0.5 mM), at a dose inhibiting acid- and CO_2-induced hyperaemia in rat duodenum,[15,19] and AML (0.1 mM), in order to inhibit the acid sensors TRPV1 and ASICs, respectively. CPZ or AML had no effect on basal pH_{int} and blood flow, whereas their pretreatment abolished CO_2-induced hyperaemia and further decreased blood flow during the recovery period (fig 2B) while irreversibly acidifying the interstitium (fig 2A). These studies suggest that the response to luminal CO_2 perfusion is TRPV1 dependent and ASIC dependent, and that pH_{int} is maintained by mucosal blood flow.

Since the IC_{50} (concentration that reduces the effect by 50%) for ENaC is ~0.1 μM, whereas the IC_{50} for ASICs is 10–100 μM,[26] and since ENaC is expressed in the oesophageal epithelium,[27] we examined the effect of low dose AML on oesophageal parameters in order to evaluate the involvement of ENaC in CO_2-sensing mechanisms. Luminal AML (1 μM) had no effect on pH_{int} (Supplementary fig 3A) and blood flow (Supplementary fig 3B) during CO_2 exposure, distinct from the effect of a high dose of AML (0.1 mM) (fig 2A,B), suggesting that ENaC may not be involved in the CO_2-induced oesophageal response in our experimental system.

We further examined the effect of the selective NHE1 inhibitor ZP, due to the known expression of NHE1 in the oesophagus. ZP had no effect on baseline pH_{int} and blood flow, whereas ZP inhibited and delayed the CO_2-induced hyperaemic response with no change in pH_{int} (Supplementary fig 3C,D). In contrast, S3226, a selective NHE3 inhibitor, used as a negative control, since the oesophagus does not express NHE3,[23] had no effect on CO_2-induced hyperaemia and pH_{int}. Thus, the effect of selective NHE1 inhibition was distinct from that of AML, suggesting that AML at a concentration that confers selectivity for ion channels was inhibiting ASIC rather than NHE1.

To examine the involvement of capsaicin-sensitive afferent nerves in CO_2-induced hyperaemia, we perfused the oesophagus of capsaicin-treated rats with the high CO_2 solution. Capsaicin deafferentation abolished the CO_2-induced hyperaemia observed in vehicle-pretreated rats (fig 3B), while reversibly acidifying the interstitium (fig 3A). This result strongly implicates capsaicin-sensitive afferent nerves in the hyperaemic response to luminal CO_2.

Effect of the CA inhibition on pH_{int} and CO_2-induced hyperaemia

We used MTZ (1 mM) and BNZ (0.1 mM) to inhibit cytosolic and extracellular CA activity, respectively. MTZ perfused with pH 7.0 Krebs during the basal period had no effect on basal pH_{int} and blood flow, whereas its pretreatment abolished CO_2-induced hyperaemia (fig 4B), and progressively decreased pH_{int} (fig 4A). BNZ co-perfusion with the high CO_2 solution abolished the hyperaemic response (fig 4B), accompanied by partial, reversible interstitial acidification (fig 4A). Our data suggest that epithelial CA activity is related to the regulation of blood flow and pH_{int} and that extracellular CA in the oesophageal epithelium is partially involved in the maintenance of pH_{int} and CO_2-induced hyperaemia.

Expression of membrane-bound CAs and acid sensors in the oesophagus

RT-PCR analysis revealed that cytosolic CA II and four membrane-bound CAs (IV, IX, XII and XIV), TRPV1 and three ASIC isoforms were expressed in oesophageal mucosa as well as in the muscle layer (Supplementary fig 3). This is the first description of oesophageal mucosal expression of CA XIV, TRPV1 and ASICs, with the expression of the other CA paralogues generally consistent with prior studies.[28–30] The stomach and proximal duodenum expressed these CAs and acid sensors, consistent with our results.[15] DRGs and NGs, used as positive controls for TRPV1 and ASICs, also expressed all of the above proteins, except for CA IX as previously reported.[15]

Immunofluorescence demonstrated differential expression of membrane-bound CA isoforms and acid sensors in the oesophagus. CA IV was not detected in the oesophageal mucosa (fig 5A), inconsistent with a prior study[29] and the PCR result as described above. CA IX expression was not observed (fig 5B), consistent with prior studies of normal human oesophagus[3132] despite the detection of mRNA expression.[30] In contrast, CA XII expression was clearly observed on the plasma membrane of the basal cells in oesophageal mucosa (fig 5C), consistent with the focal expression in the basal cells of the epidermis,[30] whereas CA XIV was recognised in the plasma membrane of the prickle cells of the stratum spinosum (fig 5D).

Unexpectedly, the acid sensors were detected in the oesophageal epithelium as well as in the afferent nerves (fig 6). All ASIC paralogues were observed in the prickle cell layer in oesophageal mucosa (fig 6A,D,G). Furthermore, ASIC3 was expressed in nerve fibres in the muscularis mucosa (fig 6G), and TRPV1 was localised in the muscularis mucosa as previously reported,[33] consistent with its functional involvement in oesophageal chemosensitivity and mechanosensitivity.[3435] Interestingly, TRPV1 was clearly recognised in the granular cells in the stratum granulosum (fig 6J), between the prickle cell layer and stratum corneum, consistent with the expression of TRPV1 in the human epidermis, where TRPV1 is localised in granular cells (most intense), prickle cells and basal cells.[3637] Immunoreactivity for ASIC1–ASIC3 or TRPV1 in the oesophageal mucosa was completely blocked by preabsorption with the corresponding immunising peptide antigen, as a negative control (fig 6B,E,H,K). Furthermore, immunoreactivity for ASIC1–ASIC3 and TRPV1 was recognised in DRGs, as a positive control (fig 6C,F,I,L). These findings suggest that not only neuronal acid sensors, but also epithelial acid sensors and epithelial CAs contribute to luminal H^+/CO_2 sensing in the oesophagus.

Western blot analysis confirmed, at least, the detection of CA XII at 55 kDa, ASIC2 at 85 kDa and ASIC3 at 85 kDa, as the expected molecular size, and TRPV1 at ~60 kDa in the oesophageal mucosa (fig 7A–D). Nevertheless, we failed to detect CA IV, IX, XIV and ASIC1 (data not shown). The molecular size of TRPV1 was ~60 kDa in the oesophageal mucosa, whereas it was an ~95 kDa protein in DRGs (fig 7E), as expected.[13] Detection was blocked by preabsorption with the immunising peptide antigen, suggesting the presence of a possible epithelial splice variant[38] or post-transcriptional modification. Direct DNA sequencing of the PCR product for TRPV1 revealed that the sequence of TRPV1 in DRGs was identical to the published cDNA of TRPV1. The C-terminus of oesophageal TRPV1 cDNA was identical to that of DRGs, whereas the N-terminus of oesophageal TRPV1 was

not detected, supporting our hypothesis regarding truncation of the gene product in rat oesophagus (data not shown).

Furthermore, the expression of ~95 kDa TRPV1 was decreased in DRGs of capsaicin-treated rats compared with those of vehicle-treated rats, whereas the expression of TRPV1 at ~60 kDa was unchanged in the oesophageal mucosa of both groups (fig 7F).

CO_2 diffusion through oesophageal epithelium

We next examined whether luminal CO_2 diffuses into the epithelium, acidifying the PV. We perfused an oesophageal loop while measuring PV blood gases. PV pH and P_{CO2} were stable during the basal period (t = 0–30 min); luminal CO_2 challenge did not alter PV pH and P_{CO2} (fig 8A,B). In contrast, MTZ (1 mM) pretreatment, which inhibited epithelial CA, acidified PV blood and increased P_{CO2} after CO_2 exposure (t = 30 min), although MTZ itself had no significant effect on basal PV pH and P_{CO2} (t = 20 min). MTZ inhibited CO_2-induced hyperaemia, lowered pH_{int} and then increased PV acidification, strongly implicating epithelial CA activity in the generation of the hyperaemic response, which in turn helps maintain pH_{int}. Epithelial CA activity is thus necessary for mucosal chemosensing and pH homeostasis.

DISCUSSION

We demonstrated that luminal CO_2 challenge, similarly to luminal acid challenge, induced hyperaemia without pH_{int} change in rat oesophagus. The CO_2 response was inhibited by the TRPV1 antagonist CPZ, the ASIC inhibitor AML, ablation of capsaicin-sensitive afferent nerves, the cell-permeant CA inhibitor MTZ and the relatively cell-impermeant CA inhibitor BNZ. Inhibition of CO_2-induced hyperaemia by any means was associated with interstitial acidification with progressive decrease of oesophageal blood flow. CAs and acid sensors were localised in the oesophageal epithelium as well as in the submucosa. Furthermore, CA inhibition during luminal CO_2 exposure induced PV acidification. These results suggest that luminal CO_2 rather than H^+ diffuses into the stratum epithelium, interacts with epithelial membrane-bound and cytosolic CAs, TRPV1 and ASICs, and conducts the signals to capsaicin-sensitive afferent nerves via activation of acid sensors, producing hyperaemia. Epithelial CA activity impeded transmucosal movement of acid or acid equivalents. This is the first study to demonstrate the presence and role of CAs and acid sensors in the oesophageal epithelium in the regulation of mucosal blood flow and pH_{int} during luminal CO_2 challenge.

We propose that CO_2, not H^+, is the permeant gas generated from acid mixed in the duodenum, stomach or lower oesophagus, with bicarbonate secreted by the pancreas, duodenum, stomach or oesophageal glands (when present). Transmucosal CO_2 transport increases submucosal P_{CO2}, stimulating submucosal afferent nerves, producing the sensation of luminal acidity.[5] We also proposed that CO_2/H^+ sensing in the oesophagus might be mediated by epithelial CA activity and acid sensors, as observed in the duodenum.[1415] Nevertheless, the duodenum actively absorbs CO_2, whereas CO_2 absorption is not a known oesophageal function. Luminal CO_2 challenge and acid challenge increased oesophageal blood flow without changing pH_{int}, suggesting that either acid- or CO_2-induced hyperaemia

and pH_{int} are independently regulated, or that pH_{int} changes during acid or CO_2 challenge are undetectable. Inhibition of CO_2-induced hyperaemia by MTZ, BNZ, CPZ and AML supports our hypothesis that sensing of luminal CO_2 is mediated by cytosolic and extracellular CAs and the acid sensors TRPV1 and ASICs. Furthermore, capsaicin pretreatment abolished CO_2-induced hyperaemia, suggesting that capsaicin-sensitive sensory afferent nerves play a role in oesophageal CO_2 sensing in the oesophagus. Since inhibition of the hyperaemic response to luminal CO_2 challenge lowered pH_{int}, mucosal blood flow probably strongly influences pH_{int}. In the duodenum, epithelial cellular acidification predicts subsequent hyperaemia,[1519] consistent with the present findings. We propose that acid sensors in the oesophagus and duodenum sense the pH of the extracellular interstitium, which is dependent on blood flow, which, as has long been hypothesised, delivers HCO_3^- to and removes H^+ from the epithelium.[3940] Thus, disruption of the hyperaemic response to CO_2 challenge decreases pH_{int}, presumably due to CO_2/H^+ accumulation and reduced HCO_3^- delivery.

Since AML is a non-selective Na^+ channel inhibitor, the inhibitory effect of AML can be explained either by the inhibition of NHE1, which helps regulate epithelial pH_i,[4142] or by inhibition of non-selective cation channels and ENaC, regulating Na^+ absorption at a rate dependent on luminal pH.[43] Our study suggests that the inhibitory effect of AML is due to the inhibition of ASIC rather than inhibition of NHE1 or ENaC. NHE1 may also be involved in hyperaemic signalling, possibly by extruding H^+ into the interstitium. Nevertheless, the lack of an available ASIC-selective small molecule inhibitor prevents our directly testing this possibility.

Multiple CAs, ASIC isoforms and TRPV1 were detected in the oesophageal mucosa and the muscle layer by RT-PCR, immunohistochemistry and western blot. Differences in the expression of ASICs and TRPV1 in DRGs and the oesophagus in our study and those reported in the literature may be due to species, organ and antibody specificity differences. Since we predicted acid sensors to be expressed only on afferent nerves, the epithelial expression, combined with our functional data, supports the hypothesis that epithelial CAs and acid sensors regulate the propagation of the CO_2/H^+ signal from lumen to the submucosal afferents. Increasing H^+ diffusion from the lumen to the mucosa by barrier disruption, using bile salts or pepsin during acid perfusion, failed to induce the hyperaemic response to luminal acid,[44] further supporting our hypothesis. Nevertheless, decreased expression of TRPV1 in DRGs, but not in the oesophageal mucosa in capsaicin-treated rats, accompanied by impairment of CO_2-induced hyperaemia, suggests that afferent acid sensors more profoundly regulate pH_{int} and blood flow than do epithelial acid sensors.

The contribution of vagal and spinal afferents to CO_2/H^+ sensation is still unknown. Vagal and spinal afferent innervation is present in rat oesophagus,[45] with vagal afferents contributing to acid chemosensation and to distention-induced mechanosensation.[3435] The role of spinal afferents in chemosensitivity to acid in the oesophagus has not been studied. Since spinal afferents are believed to mediate nocioception, including heat and pain sensation, oesophageal CO_2/H^+ sensing might be due to the spinal afferent activation, producing the sensation of heartburn.

Serosal acidification of oesophageal epithelium is associated with the irreversible injury.[46] Although this serosal acidification has been thought to occur as a result of enhanced transmucosal H^+ diffusion, our data support more the hypothesis that interstitial acidification occurs as a result of disruption of afferent chemosensors and the resultant hyperaemic response. Since interstitial acidification may cause oedema, injury and noxious sensation, disruption of CO_2/H^+ chemosensing via CAs and acid sensors, irreversibly reducing pH_{int} may augment injury and inflammation, producing the sensation of pain during luminal challenge.

The mechanism by which luminal CO_2/H^+ diffuses through the oesophageal epithelium is unknown. The most accepted concept is dilation of intercellular spaces concomitant with the increase of paracellular permeability and decrease of shunt resistance induced by strong acid (pH ~1.0) in subjects with GORD.[4748] Our finding that the response to luminal CO_2 (pH 6.4) challenge closely resembles the response to luminal acid challenge suggests alternatively that CO_2 rather than H^+ actually traverses the electrically high resistance epithelium, propagated by sequential, CA-facilitated CO_2–H^+ interconversion. In humans, HCO^{3-} secreted by the oesophageal glands and refluxed duodenal HCO_3^- contribute to the luminal high P_{CO2} environment. Since CO_2 is far more permeant than H^+ in biological systems,[1449] the proposed CO_2–H^+ conversion combined with control of pH_{int} by blood flow explains how acid signals traverse the epithelium without acidifying the interstitium.

The source of HCO_3^- in the oesophagus perfused with the low CO_2–acid solution is not obvious, since rodent oesophagus has no HCO_3^--secreting oesophageal glands. The expression of HCO_3^- transporters, such as Cl^-/HCO_3^- exchangers and the Na^+: HCO_3^- co-transporters in oesophageal epithelial cells,[5051] suggests that HCO_3^- is transported from the interstitium to the lumen. In the stratified layers of gland-free oesophageal mucosa, H^+ may traverse a few layers from the lumen before being neutralised by extracellular HCO_3^- secreted from underlying epithelial cells, producing CO_2. We propose that the oesophageal stratified epithelium acts as a buffering zone to prevent direct diffusion of luminal H^+ into the interstitium, since, unlike gastric or duodenal mucosa, the oesophageal mucosa has no known pre-epithelial buffering microclimate.[52] Another question is how epithelial acid sensors play a role in conductance of luminal CO_2/H^+ concentration to epithelial or submucosal afferent neurons. Since TRPV1 and ASIC are non-selective cation channels for mostly Ca^{2+} and Na^+, respectively, activation of acid sensors in the epithelium may release mediators to modulate the blood flow response.

The role of CAs in the oesophagus is different from that in the duodenum. CAs in the duodenal epithelium facilitate transmucosal CO_2 diffusion,[1415] which induces hyperaemia, whereas oesophageal CAs help maintain mucosal pH by regulating mucosal blood flow in response to luminal CO_2. These dissimilar acid responses reflect the physiological functions of each organ in that the duodenum absorbs gastric H^+ as CO_2,[14] whereas in the oesophagus, H^+ sensing can increase lower sphincter pressure, impairing further reflux episodes.[53]

By studying the mechanism by which the luminal acid signal is transduced into an efferent neural reflex response, we hope to understand further the signalling pathways mediating the response to luminal challenge, in order to identify novel molecular targets. Furthermore,

efferent responses such as blood flow increase, easily measured endoscopically, could also be used as a surrogate for dyspepsia and other sensations, providing an additional and potentially objective and reproducible dimension to clinical studies of oesophageal acid perfusion and sensitivity.

In conclusion, luminal CO_2 challenge increased blood flow, mediated by epithelial CA activity, acid sensors and NHE1, thereby maintaining pH_{int}. Alteration of one or more of these molecular targets may help explain why NERD subjects, without evidence of gross or microscopic injury, nevertheless experience heartburn and dyspepsia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Quigley EM. The spectrum of GERD: a new perspective. Drugs Today (Barc). 2005; 41(Suppl B):3–6. [PubMed: 16200225]
2. Dickman R, Fass R. Functional heartburn. Curr Treat Options Gastroenterol. 2005; 8:285–291. [PubMed: 16009029]
3. Lee YC, Wang HP, Chiu HM, et al. Patients with functional heartburn are more likely to report retrosternal discomfort during wireless pH monitoring. Gastrointest Endosc. 2005; 62:834–841. [PubMed: 16301022]
4. Nakamura T, Shirakawa K, Masuyama H, et al. Minimal change oesophagitis: a disease with characteristic differences to erosive oesophagitis. Aliment Pharmacol Ther. 2005; 21(Suppl 2):19–26. [PubMed: 15943842]
5. Tanaka S, Chu S, Hirokawa M, et al. Direct measurement of acid permeation into rat oesophagus. Gut. 2003; 52:775–783. [PubMed: 12740330]
6. Bass BL, Trad KS, Harmon JW, et al. Capsaicin-sensitive nerves mediate esophageal mucosal protection. Surgery. 1991; 110:419–425. [PubMed: 1858050]
7. McKie LD, Dunkin BJ, Pennanen MF, et al. Esophageal mucosal blood flow: a central role for calcitonin gene-related peptide. Surgery. 1994; 116:409–417. [PubMed: 7519367]
8. Sandler AD, Schmidt C, Richardson K, et al. Regulation of distal esophageal mucosal blood flow: the roles of nitric oxide and substance P. Surgery. 1993; 114:285–293. [PubMed: 7688153]
9. Orlando RC, Lacy ER, Tobey NA, et al. Barriers to paracellular permeability in rabbit esophageal epithelium. Gastroenterology. 1992; 102:910–923. [PubMed: 1537527]
10. Abdulnour-Nakhoul S, Nakhoul NL, Wheeler SA, et al. HCO_3^- secretion in the esophageal submucosal glands. Am J Physiol Gastrointest Liver Physiol. 2005; 288:G736–G744. [PubMed: 15576627]
11. Sarosiek J, McCallum RW. Mechanisms of oesophageal mucosal defence. Baillieres Best Pract Res Clin Gastroenterol. 2000; 14:701–717. [PubMed: 11003804]
12. Bernstein L, Baker L. A clinical test for esophagitis. Gastroenterology. 1958; 34:760–781. [PubMed: 13538145]
13. Tominaga M, Caterina MJ, Malmberg AB, et al. The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron. 1998; 21:531–543. [PubMed: 9768840]

14. Mizumori M, Meyerowitz J, Takeuchi T, et al. Epithelial carbonic anhydrases facilitate PCO_2 and pH regulation in rat duodenal mucosa. J Physiol. 2006; 573:827–842. [PubMed: 16556652]
15. Akiba Y, Ghayouri S, Takeuchi T, et al. Carbonic anhydrases and mucosal vanilloid receptors help mediate the hyperemic response to luminal CO_2 in rat duodenum. Gastroenterology. 2006; 131:142–152. [PubMed: 16831598]
16. Knight DR, Smith AH, Flynn DM, et al. A novel sodium–hydrogen exchanger isoform-1 inhibitor, zoniporide, reduces ischemic myocardial injury in vitro and in vivo. J Pharmacol Exp Ther. 2001; 297:254–259. [PubMed: 11259552]
17. Furukawa O, Bi LC, Guth PH, et al. NHE3 inhibition activates duodenal bicarbonate secretion in the rat. Am J Physiol Gastrointest Liver Physiol. 2004; 286:G102–G109. [PubMed: 12881227]
18. Furukawa O, Hirokawa M, Zhang L, et al. Mechanism of augmented duodenal HCO_3^- secretion after elevation of luminal CO_2. Am J Physiol Gastrointest Liver Physiol. 2005; 288:G557–G563. [PubMed: 15499081]
19. Akiba Y, Guth PH, Engel E, et al. Acid-sensing pathways of rat duodenum. Am J Physiol Gastrointest Liver Physiol. 1999; 277:G268–G274.
20. Unwin R, Stidwell R, Taylor S, et al. The effects of respiratory alkalosis and acidosis on net bicarbonate flux along the rat loop of Henle in vivo. Am J Physiol. 1997; 273:F698–F705. [PubMed: 9374832]
21. Wetzel P, Hasse A, Papadopoulos S, et al. Extracellular carbonic anhydrase activity facilitates lactic acid transport in rat skeletal muscle fibres. J Physiol. 2001; 531:743–756. [PubMed: 11251055]
22. Kleyman TR, Cragoe EJ Jr. Amiloride and its analogs as tools in the study of ion transport. J Membr Biol. 1988; 105:1–21. [PubMed: 2852254]
23. Shallat S, Schmidt L, Reaka A, et al. NHE-1 isoform of the Na^+/H^+ antiport is expressed in the rat and rabbit esophagus. Gastroenterology. 1995; 109:1421–1428. [PubMed: 7557121]
24. Akiba Y, Kato S, Katsube K, et al. Transient receptor potential vanilloid subfamily 1 expressed in pancreatic islet beta cells modulates insulin secretion in rats. Biochem Biophys Res Commun. 2004; 321:219–225. [PubMed: 15358238]
25. Gamble J, Ross S. The factors in the dehydration following pyloric obstruction. J Clin Invest. 1925; 1:403–423. [PubMed: 16693658]
26. Kellenberger S, Schild L. Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. Physiol Rev. 2002; 82:735–767. [PubMed: 12087134]
27. Awayda MS, Bengrine A, Tobey NA, et al. Nonselective cation transport in native esophageal epithelia. Am J Physiol Cell Physiol. 2004; 287:C395–C402. [PubMed: 15197006]
28. Christie KN, Thomson C. The distribution of carbonic anhydrase II in human, pig and rat oesophageal epithelium. Histochem J. 2000; 32:753–757. [PubMed: 11254091]
29. Christie KN, Thomson C, Xue L, et al. Carbonic anhydrase isoenzymes I, II, III, and IV are present in human esophageal epithelium. J Histochem Cytochem. 1997; 45:35–40. [PubMed: 9010466]
30. Ivanov S, Liao SY, Ivanova A, et al. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol. 2001; 158:905–919. [PubMed: 11238039]
31. Pastorekova S, Parkkila S, Parkkila AK, et al. Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementary DNA sequence and expression in human and rat alimentary tracts. Gastroenterology. 1997; 112:398–408. [PubMed: 9024293]
32. Turner JR, Odze RD, Crum CP, et al. MN antigen expression in normal, preneoplastic, and neoplastic esophagus: a clinicopathological study of a new cancer-associated biomarker. Hum Pathol. 1997; 28:740–744. [PubMed: 9191010]
33. Boudaka A, Worl J, Shiina T, et al. Key role of mucosal primary afferents in mediating the inhibitory influence of capsaicin on vagally mediated contractions in the mouse esophagus. J Vet Med Sci. 2007; 69:365–372. [PubMed: 17485923]
34. Page AJ, Brierley SM, Martin CM, et al. Different contributions of ASIC channels 1a, 2, and 3 in gastrointestinal mechanosensory function. Gut. 2005; 54:1408–1415. [PubMed: 15987792]
35. Bielefeldt K, Davis BM. Differential effects of ASIC3 and TRPV1 deletion on gastroesophageal sensation in mice. Am J Physiol Gastrointest Liver Physiol. 2008; 294:G130–G138. [PubMed: 17975130]

36. Stander S, Moormann C, Schumacher M, et al. Expression of vanilloid receptor subtype 1 in cutaneous sensory nerve fibers, mast cells, and epithelial cells of appendage structures. Exp Dermatol. 2004; 13:129–139. [PubMed: 14987252]
37. Bodo E, Kovacs I, Telek A, et al. Vanilloid receptor-1 (VR1) is widely expressed on various epithelial and mesenchymal cell types of human skin. J Invest Dermatol. 2004; 123:410–413. [PubMed: 15245445]
38. Schumacher MA, Moff I, Sudanagunta SP, et al. Molecular cloning of an N-terminal splice variant of the capsaicin receptor. Loss of N-terminal domain suggests functional divergence among capsaicin receptor subtypes. J Biol Chem. 2000; 275:2756–2762. [PubMed: 10644739]
39. Fiddian-Green RG, Silen W. Mechanisms of disposal of acid and alkali in rabbit duodenum. Am J Physiol. 1975; 229:1641–1648. [PubMed: 2019]
40. Silen W, Machen TE, Forte JG. Acid–base balance in amphibian gastric mucosa. Am J Physiol. 1975; 229:721–730. [PubMed: 2015]
41. Tobey NA, Reddy SP, Keku TO, et al. Mechanisms of HCl-induced lowering of intracellular pH in rabbit esophageal epithelial cells. Gastroenterology. 1993; 105:1035–1044. [PubMed: 8405846]
42. Layden TJ, Schmidt L, Agnone L, et al. Rabbit esophageal cell cytoplasmic pH regulation: role of Na^+–H^+ antiport and Na^+-dependent HCO_3^- transport systems. Am J Physiol. 1992; 263:G407–G413. [PubMed: 1329529]
43. Tobey NA, Argote CM, Vanegas XC, et al. Electrical parameters and ion species for active transport in human esophageal stratified squamous epithelium and Barrett's specialized columnar epithelium. Am J Physiol Gastrointest Liver Physiol. 2007; 293:G264–G270. [PubMed: 17431220]
44. Bass BL, Schweitzer EJ, Harmon JW, et al. H^+ back diffusion interferes with intrinsic reactive regulation of esophageal mucosal blood flow. Surgery. 1984; 96:404–413. [PubMed: 6463868]
45. Dutsch M, Eichhorn U, Worl J, et al. Vagal and spinal afferent innervation of the rat esophagus: a combined retrograde tracing and immunocytochemical study with special emphasis on calcium-binding proteins. J Comp Neurol. 1998; 398:289–307. [PubMed: 9700572]
46. Tobey NA, Orlando RC. Mechanisms of acid injury to rabbit esophageal epithelium. Role of basolateral cell membrane acidification. Gastroenterology. 1991; 101:1220–1228. [PubMed: 1936791]
47. Carney CN, Orlando RC, Powell DW, et al. Morphologic alterations in early acid-induced epithelial injury of the rabbit esophagus. Lab Invest. 1981; 45:198–208. [PubMed: 7265916]
48. Tobey NA, Hosseini SS, Argote CM, et al. Dilated intercellular spaces and shunt permeability in nonerosive acid-damaged esophageal epithelium. Am J Gastroenterol. 2004; 99:13–22. [PubMed: 14687135]
49. Stewart AK, Boyd CA, Vaughan-Jones RD. A novel role for carbonic anhydrase: cytoplasmic pH gradient dissipation in mouse small intestinal enterocytes. J Physiol. 1999; 516:209–217. [PubMed: 10066935]
50. Tobey NA, Reddy SP, Khalbuss WE, et al. Na^+-dependent and -independent Cl^-/HCO_3^- exchangers in cultured rabbit esophageal epithelial cells. Gastroenterology. 1993; 104:185–195. [PubMed: 8419242]
51. Bernardo AA, Kear FT, Stim JA, et al. Renal cortical basolateral Na^+/HCO_3^- cotransporter: IV. Characterization and localization with polyclonal antibodies. J Membr Biol. 1996; 154:155–162. [PubMed: 8929289]
52. Quigley EM, Turnberg LA. pH of the microclimate lining human gastric and duodenal mucosa in vivo. Studies in control subjects and in duodenal ulcer patients. Gastroenterology. 1987; 92:1876–1884. [PubMed: 3569763]
53. Giles GR, Humphries C, Mason MC, et al. Effect of pH changes on the cardiac sphincter. Gut. 1969; 10:852–856. [PubMed: 5350111]

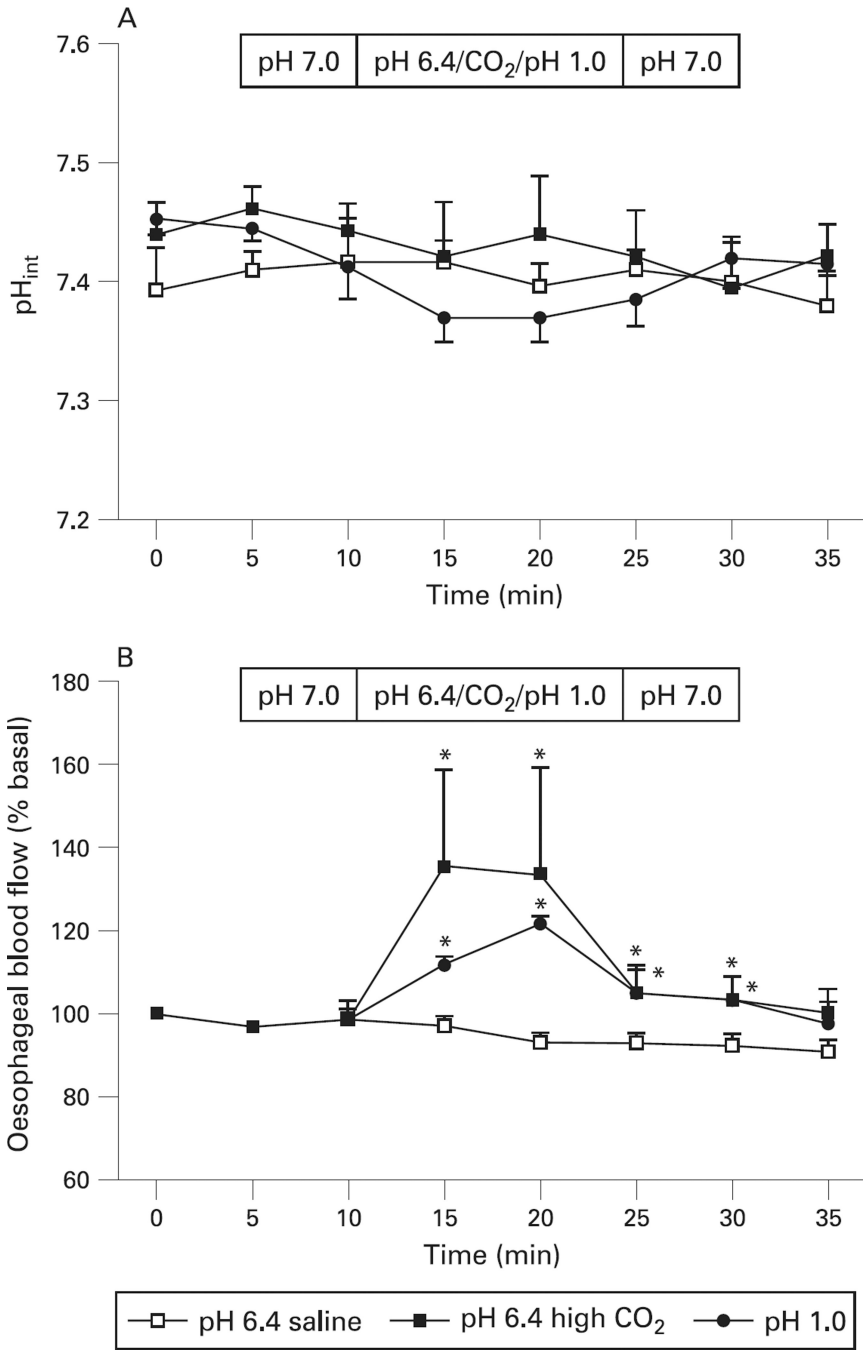

Figure 1.

Effect of luminal acid or high CO_2 challenge on interstitial pH (pH_{int}) and blood flow in rat oesophagus. (A) pH_{int}. Acid or CO_2 challenge had no effect on pH_{int}. (B) Blood flow. Acid or CO_2 challenge reversibly increased blood flow during the challenge period. Data are expressed as mean (SEM) (n = 6). *$p < 0.05$ vs pH 6.4 saline group.

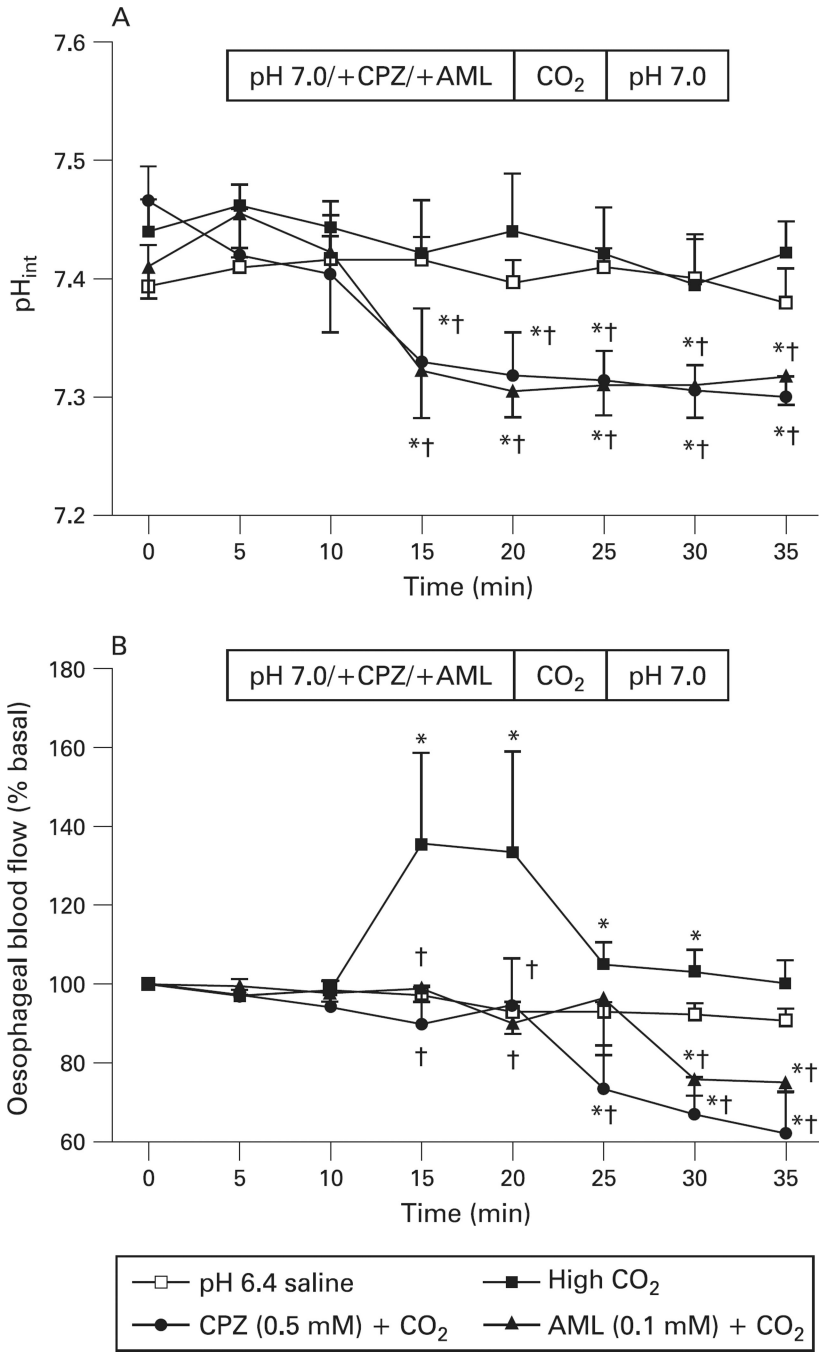

Figure 2.

Effect of capsazepine (CPZ) or amiloride (AML) on interstitial pH (pH_{int}) and blood flow during CO_2 challenge. (A) pH_{int}. Pretreatment with CPZ or AML followed by CO_2 challenge decreased pH_{int}. (B) Blood flow. CO_2 challenge-induced hyperaemia was inhibited by CPZ or AML. Data are expressed as mean (SEM) (n = 6). *p < 0.05 vs pH 6.4 saline group, †p < 0.05 vs high CO_2 group.

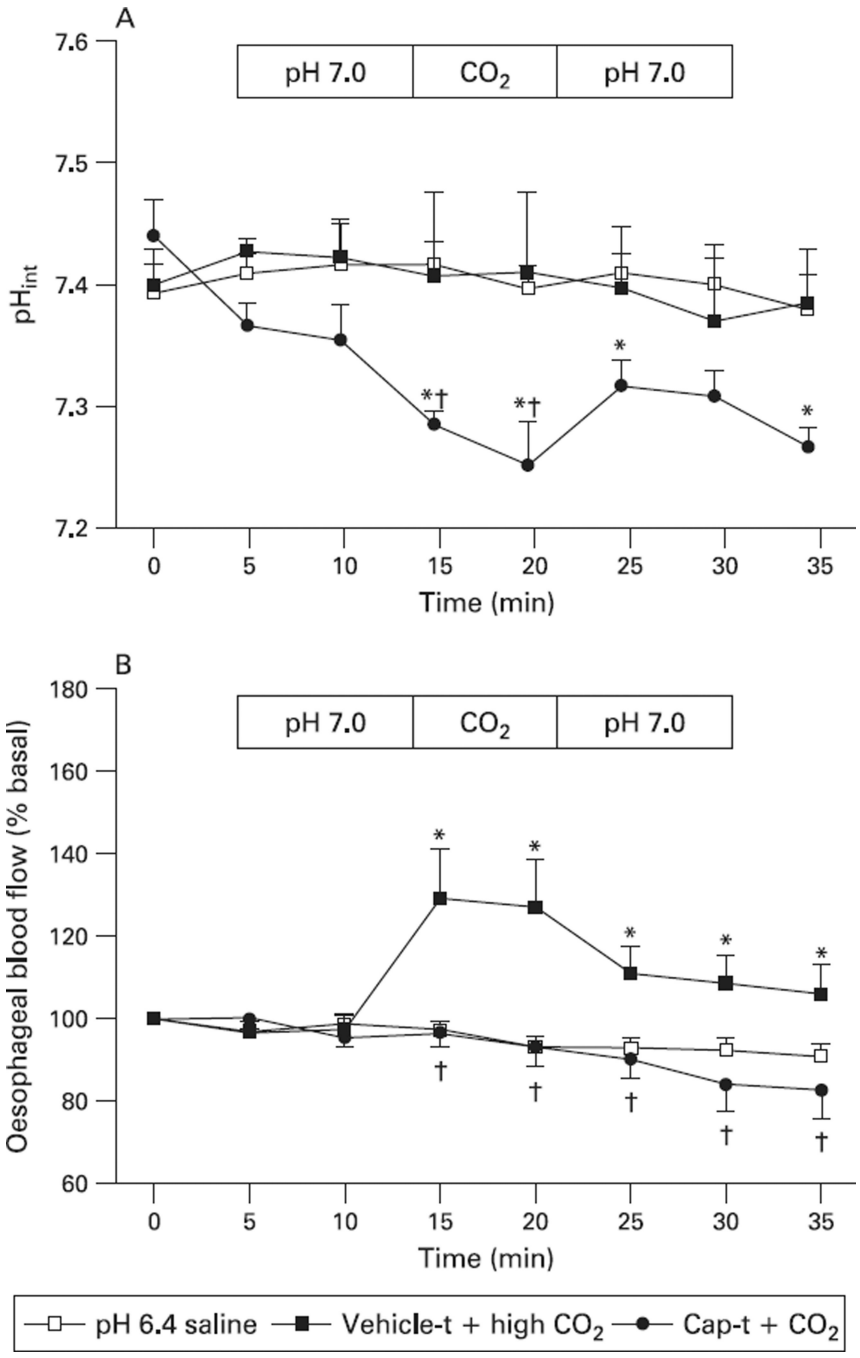

Figure 3.

Effect of afferent denervation on interstitial pH (pH_{int}) and blood flow during CO_2 exposure in rat oesophagus. (A) pH_{int}. In capsaicin-treated rats (Cap-t), perfusion of high CO_2 saline exposure decreased pH_{int}. (B) Blood flow. Oesophageal CO_2 challenge-induced hyperaemia was abolished in Cap-t rats. Data are expressed as mean (SEM) (n = 6). *$p < 0.05$ vs pH 6.4 saline group, †$p < 0.05$ vs vehicle-treated (vehicle-t) + high CO_2 group.

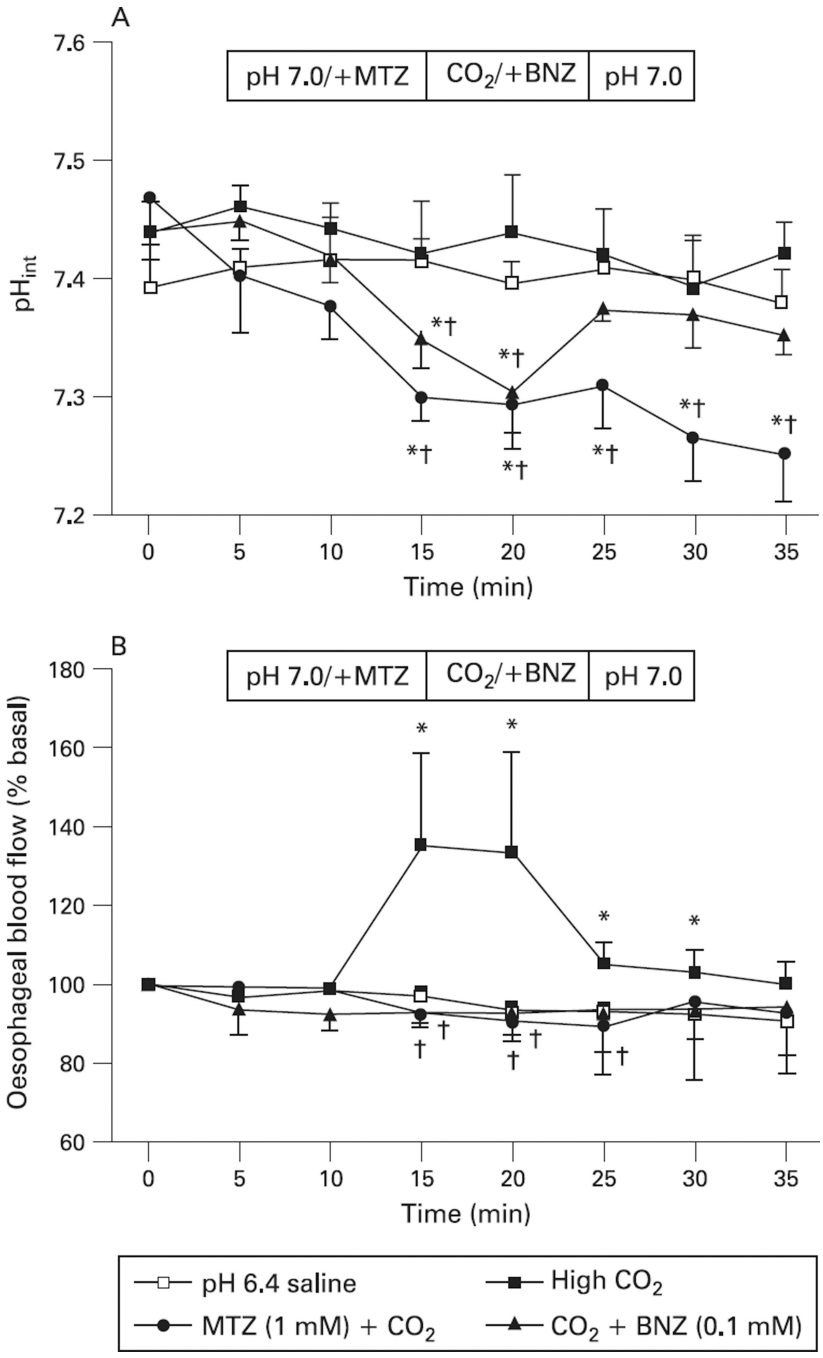

Figure 4.

Effect of carbonic anhydrase inhibitors on interstitial pH (pH_{int}) and blood flow during CO_2 exposure. (A) pH_{int}. Pretreatment with methazolamide (MTZ) followed by CO_2 challenge or luminal benzolamide (BNZ) co-perfused during CO_2 challenge decreased pH_{int}. (B) Blood flow. CO_2-induced hyperaemia was abolished by MTZ or BNZ. Data are expressed as mean (SEM) (n = 6). *$p < 0.05$ vs pH 6.4 saline group, †$p < 0.05$ vs high CO_2 group.

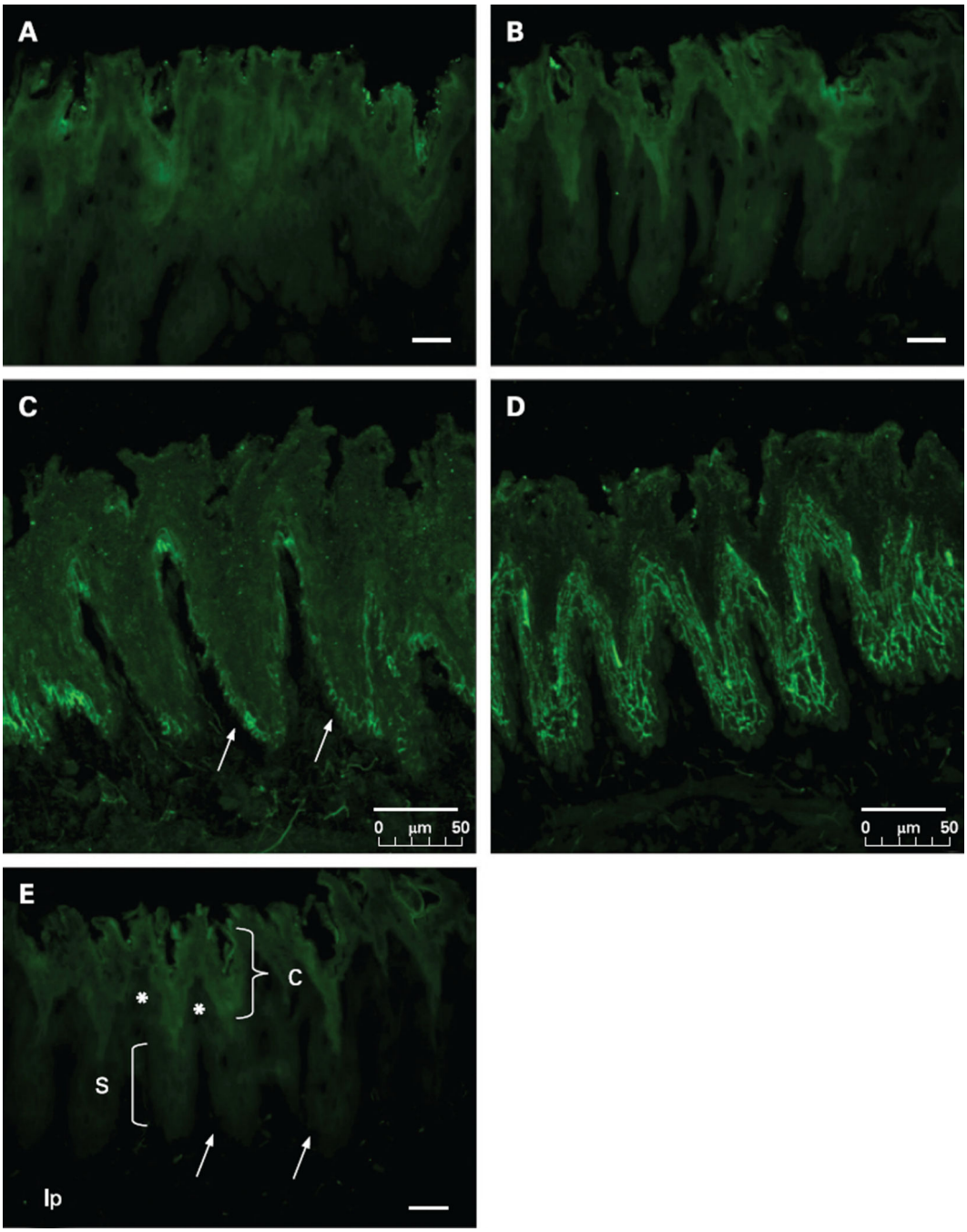

Figure 5.

Expression of membrane-bound carbonic anhydrases (CAs) in rat oesophagus. No specific staining was observed for CA IV (A) and IX (B), compared with the negative control (E). CA XII was clearly localised on the plasma membrane of the basal cells (C, arrows), whereas CA XIV was observed on the membrane of the prickle cells (D). Note that oesophageal epithelium consists of stratum (St) corneum (c), St granulosum (*), St spinosum (prickle cell layer, s), St basale (basal cell layer, arrows) and lamina propria (lp)

(E). Bar = 50 μm. A, B and E: conventional microscopic images; C and D: confocal microscopic images.

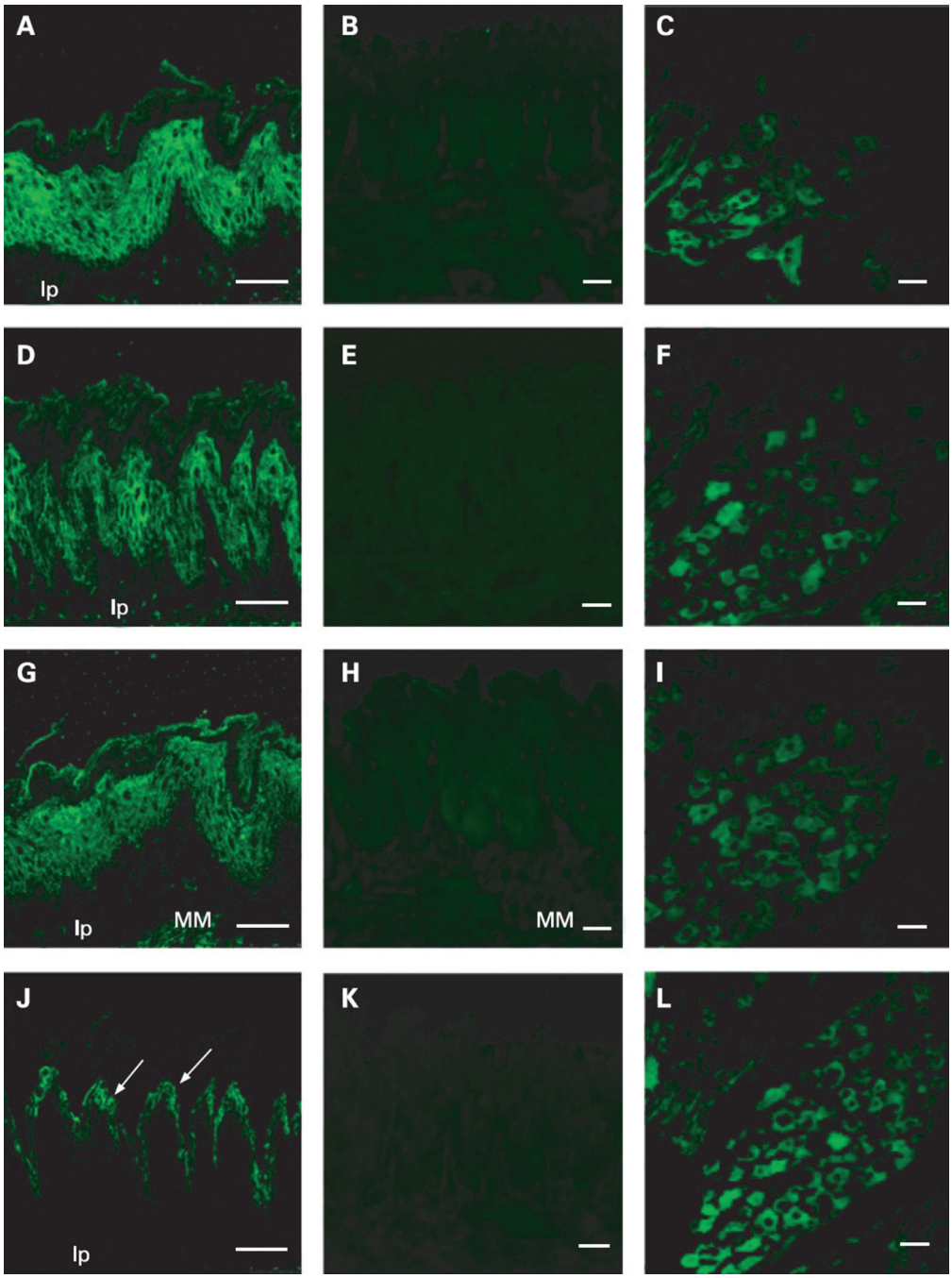

Figure 6.

Expression of acid-sensing ion channel (ASIC) isoforms and transient receptor potential vanilloid 1 (TRPV1) in rat oesophageal mucosa. ASIC1 (A), ASIC2 (D) and ASIC3 (G) were expressed in the prickle cells. ASIC3 was also expressed in the muscularis mucosa (MM) (G). TRPV1-like immunoreactivity was observed in the granular cells (arrows) in the stratum granulosum (J). B, E, H, K: preabsorbed antibody with immunising peptide for ASIC1 (B), ASIC2 (E), ASIC3 (H) or TRPV1 (K) was reacted. C, F, I, L: positive control staining in dorsal root ganglia for ASIC1 (C), ASIC2 (F), ASIC3 (I) or TRPV1 (L) was

shown. lp, lamina propria mucosa. Bar = 50 μm. A, D, G and J: confocal microscopic images, B, C, E, F, G and H: conventional microscopic images.

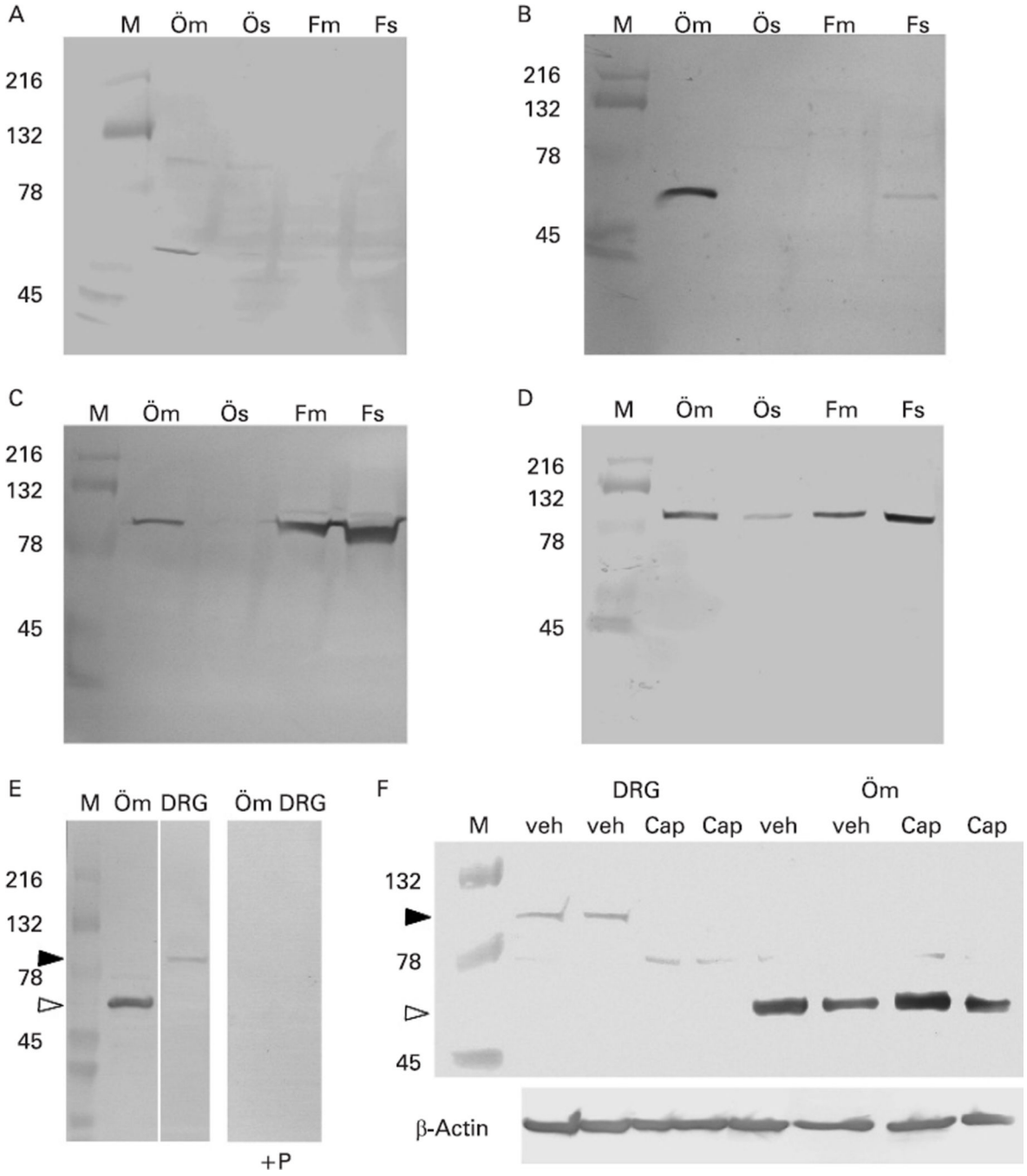

Figure 7.

Western blot analysis for carbonic anhydrase (CA) XII, transient receptor potential vanilloid 1 (TRPV1), acid-sensing ion channel 2 (ASIC2) and ASIC3. Oesophageal mucosa (Öm) expressed CA XII (A, 55 kDa), TRPV1 (B, ~60 kDa), ASIC2 (C, 85 kDa) and ASIC3 (D, 85 kDa), whereas the oesophageal muscle layer (Ös) expressed only ASIC3. Furthermore, the mucosa (Fm) and muscle layer (Fs) of fundic stomach strongly expressed ASIC2 (C) as well as ASIC3 (D). TRPV1 was also recognised in Fs. (E) TRPV1 was recognised at ~60 kDa in Öm (open arrowhead), whereas it was ~95 kDa in dorsal root ganglia (DRGs) (filled

arrowhead). Immunodetection of TRPV1 in Öm and DRGs was blocked by preabsorption with immunised peptide antigen (+P). (F) Effect of vehicle (veh) or capsaicin (Cap) treatment on the expression of TRPV1 in DRGs and Öm. The molecular mass of TRPV1 in DRGs (filled arrowhead) was reduced by Cap treatment, whereas TRPV1 in Öm (open arrowhead) had no change. One of two independent experiments is represented. β-Actin was used as an internal loading control. M, molecular marker.

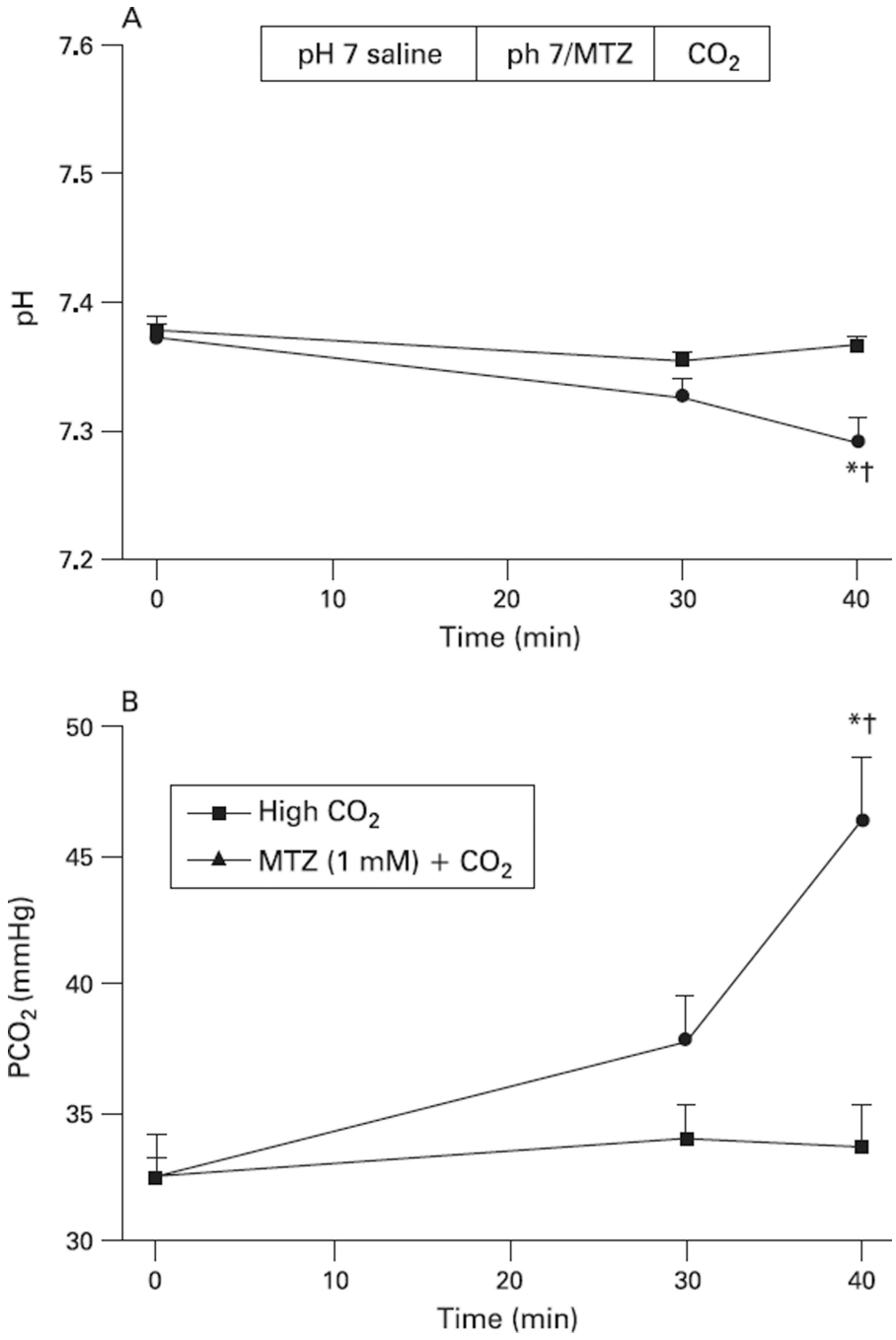

Figure 8.

Effect of high luminal CO_2 solution and carbonic anhydrase inhibitor on portal venous (PV) blood pH and P_{CO2}. PV blood was collected at t = 0, 30 and 40 min to measure PV blood pH and P_{CO2}. During the basal period (t = 0–30 min), PV pH and P_{CO2} were stable, and luminal perfusion with a high CO_2 solution had no effect on PV pH (A) and P_{CO2} (B). Pretreatment with methazolamide (MTZ, 1 mM) followed by CO_2 exposure lowered pH (A) and

increased P_{CO2} (B) in PV blood. Data are expressed as mean (SEM) (n = 6). *p < 0.05 vs the corresponding value at t = 0 min, †p < 0.05 vs the high CO_2 group.