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Gap Junctional Channels Regulate Acid Secretion in the Mammalian Gastric Gland

K. Radebold[1], E. Horakova[1], J. Gloeckner[1], G. Ortega[1], D.C. Spray[2], H. Vieweger[1], K. Siebert[1], L. Manuelidis[3], J.P. Geibel[1,4]

[1]Department of Surgery, Yale University School of Medicine, BML 263, 310 Cedar St., New Haven, CT 06510, USA
[2]Department of Neuroscience and Medicine, Albert Einstein College of Medicine, Bronx, NY, USA
[3]Section of Neuropathology, Yale University School of Medicine, New Haven, CT, USA
[4]Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT, USA

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Abstract. Gap junction channels are regarded as a primary pathway for intercellular message transfer, including calcium wave propagation. Our study identified two gap junctional proteins, connexin26 and connexin32, in rat gastric glands by RT-PCR, Western blot analysis, and immunofluorescence. We demonstrated a potential physiological role of the gap junctional channels in the acid secretory process using the calcium indicator fluo-3, and microinjection of Lucifer Yellow. Application of gastrin (10^{-7} M) to the basolateral membrane resulted in the induction of uniphasic calcium signals in adjacent parietal cells. In addition, single parietal cell microinjections in intact glands with the cell-impermeant dye Lucifer Yellow resulted in a transfer of dye from the injected cell to the adjacent parietal cell following gastrin stimulation, demonstrating gastrin-induced cell-to-cell communication. Both calcium wave propagation and Lucifer Yellow transfer were blocked by the gap junction inhibitor 18α-glycyrrhetinic acid. Our studies demonstrate that functional gap junction channels in gastric glands provide an effective means for rapid cell-to-cell communication and allow for the rapid onset of acid secretion.

Key words: Stomach — Intracellular calcium — Calcium signaling — Parietal cells — Cell-to-cell communication — Lucifer Yellow

Introduction

During evolution, two forms of intercellular communication developed in metazoan animals. The first, which is present even in unicellular organisms, involved humoral communication as mediated by neurotransmitters, growth factors, and hormones. This resulted in the implementation of receptors responding appropriately to humoral messages. A second form of communication operates via gap junctions, which are involved in diverse cellular processes such as homeostasis, morphogenesis, cell differentiation, and growth regulation (Spray, 1998).

The connexin family of gap junction proteins forms transmembrane channels that directly link neighboring cells. These connections allow molecules up to 1000 Daltons in size to readily pass through the 10–15 Å pore of gap junction channels (Hertzberg, Lawrence & Gilula 1981), permitting the exchange of low-molecular weight metabolites, ions, and signaling molecules such as cAMP, Ca^{2+}, and inositol 1,4,5-triphosphate (IP_3) (Saez et al., 1993; Giaume & McCarthy, 1996).

Although many cell types possess both extracellular and intercellular routes for Ca^{2+} wave propagation, direct gap junction-mediated Ca^{2+} waves occur as a result of IP_3 and/or Ca^{2+} diffusion (Scemes, Dermietzel & Spray, 1998).

Gap junction channels are abundant in heart, vessels, kidneys, and brain, and are also found in both endocrine and exocrine glands (Rozental, Carvalho & Spray, 2000). In exocrine organs such as the exocrine pancreas and salivary glands, gap junctions primarily formed of Cx32 and Cx26 appear to play major roles in coordinated secretion. In the gastrointestinal tract, gap junctions were first identified in the liver where they are a major component of appositional membranes between hepatocytes (Hertzberg, Spray & Bennett, 1985). In addition, connexins are expressed in the gastric mucosa where alterations in expression have been associated with several pathological states (Ohkusa et al., 1993; Ueda et al.,

Correspondence to: J.Geibel; email: John.Geibel@yale.edu

1994; Uchida et al., 1995; Iwata et al., 1998). In recurrent gastric ulcer disease there were significantly fewer gap junctions as compared to healthy patients (Ohkusa et al., 1993). In gastric cancer, connexin32 was not observed at all (Ohkusa et al., 1995). Despite these observations, the physiological role of gap junctions in the stomach remains unclear. One potential function of these proteins is in providing a solution to the problem facing the gastric gland in transferring messages along the gland axis to cause synchronized cellular activation and acid secretion. It is well known that the gastric gland responds to the release of gastrin by promoting the release of histamine from the ECL cells, which activates parietal cells and promotes acid secretion (Sachs, 1997). However, the number of endocrine cells is small when compared to parietal cells, which leaves the gland with having to design a method to rapidly produce synchronized activation of the acid secretory machinery.

We have previously found that intracellular calcium levels in isolated gastric glands increased in response to either gastrin or histamine stimulation (Geibel et al., 1995). We have now used this system, as well as microinjections, to examine whether 1) gap junctional proteins are present in gastric glands, 2) whether channels formed by these proteins function as a pathway for the transfer of calcium and other materials between adjacent cells, and 3) how gap junctional proteins are directly involved in gastric acid secretion.

Materials and Methods

Animals

Sprague-Dawley rats weighting 150–225 g (Charles River Laboratories, Wilmington, MA) were housed in climate- and humidity-controlled, light-cycled rooms, fed standard rat chow with free access to water, and handled according to the humane practices of animal care established by the Yale Animal and Use Committee.

Isolated Gastric Glands

The stomach was removed, fundus and antrum isolated, sliced into 0.5-cm square sections and washed several times in a standard 4°C HEPES-buffered Ringer's solution (in mM: 125 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 2 NaH_2PO_4, 2 H_3PO_4, 32.2 Na-HEPES, and 5 glucose) to remove residual food particles. The tissues were then transferred to the stage of a dissecting microscope. Individual glands were isolated using a hand-dissection technique as described previously (Waisbren et al., 1994). After isolation, the glands were allowed to adhere to coverslips precoated with Cell-Tak (Collaborative Research, Bedford, MA).

Immunofluorescence and Laser Scanning Confocal Microscopy

The glands attached to the coverslips were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde solution for 20 minutes. After permeabilization with 0.2% Triton X-100 (20 min), unspecific binding was blocked by incubation with 10% normal goat serum (NGT) in PBS. The glands were then incubated at room temperature for two hours either with 1:100 diluted rabbit polyclonal anti-connexin 26, or 1:100 rabbit polyclonal anti-connexin 32 antibodies (Zymed Laboratories, CA) in PBST and 2% NGT. Thereafter, the slides were incubated with a secondary fluorescent rhodamine (Sigma, St. Louis, MO) 1:1000 diluted anti-rabbit IgG antibody (Calbiochem, La Jolla, CA) for 1 hr at room temperature, and then washed and mounted with 50% glycerol. Controls without the primary antibody accompanied each series of 8 experiments.

Fluorescent distribution of connexins was monitored using a multiline argon laser coupled to a Zeiss LSM 410 microscope (Zeiss, Oberkochen, Germany) with an excitation wavelength of 568 nm and the subsequent fluorescent emission being collected at 575 nm. Parietal cells were positively identified by location, shape, and diameter. The parietal cells are the largest cells in the gastric gland, and have a unique conical shape.

Western Blot Analysis

Glands were harvested from the stomach by rapidly separating the mucosa from the underlying muscularis using a hand dissection technique with ultrasharp forceps dissection. Glands were then transferred into a homogenization buffer with a protease inhibitor (1 mM PMSF), snap-frozen in liquid nitrogen and stored at −70°C until further use. For immunoblotting, the samples were boiled in SDS and resolved on 12% SDS-Page as described (Manuelidis, Valley & Manuelidis, 1985). Protein samples were transferred onto PDVF membranes (Immobilon-P; Millipore, Bedford, MA), blocked with casein, and incubated with the primary antibodies against either Cx26 or Cx32 in a 1:1000 dilution, followed by reaction with peroxidase-conjugated 1:1000 goat-anti-rabbit IgG (Amersham, UK).

Thermo RT-PCR Assays

Total RNA was isolated from stomach tissue and treated with DNase I (Boehringer Mannheim, Indianapolis, IN) to eliminate contamination with residual genomic DNA. One to two μg of total RNA was brought to 10 μl in DEPC-treated water and combined with 1 μl of Oligo (dT) primer (50 μM). The mixture was heated at 65°C for 5 min and then incubated on ice. The remaining ingredients were added to the reaction as follows: 4 μl of 5× RT buffer, 2 μl 10 mM dNTP mix (10 mM each), 1 μl of 0.1 M DTT, 1 μl of RNaseOUT (40 U/μl), and 1 μl Thermoscript RT (15 U/μl) (Life Technologies, Grand Island, NY). The mix was incubated at 50°C for 50 min and then terminated by incubating at 85°C for 5 min. PCR reactions contained 1–2 μg of first strand cDNA, 50 μM of sense and anti-sense primers, 5 μl 10× PCR Buffer, 1 μl of 1.5 mM $MgCl_2$, 1 μl 10 mM dNTP (10 mM each), and 2.5 U Taq Polymerase in a final volume of 50 μl. Thirty cycles were performed on the samples as follows: (1) denaturation at 94°C for 30 sec; (2) annealing at 55°C for 30 sec; (3) extension at 72°C for 30 sec. This was followed by a final extension cycle at 72°C for 8 min and soak cycle at 4°C. Reaction products were analyzed by electrophoresis on 2% agarose gels. The image was acquired using Kodak dS 1D (Kodak, New Haven, CT).

Propagation of Calcium Signals and Lucifer Yellow Transfer Between Parietal Cells

Isolated gastric glands were superfused with Ringer's solution maintained at 37°C in a thermostatically controlled perfusion chamber.

MusCx26 sense	TTCCCCATCTCTCACATCC
MusCx26 A.sense	GCAGAATGCAAATTCCAGAC
Mus Cx30 sense	CTACAGACATGAAACTGCCC
Mus Cx30 A.sense	CCTCGAAATGAAGCAGTCC
Mus Cx32 sense	GAAGAGGTAAAGAGACACAAGG
Mus Cx32 A.sense	ACGTTGAGGATAATGCAGATG
Mus Cx43 sense	AATCTCCAGGTCATCAGG
Mus Cx43 anti-sense	TACCACGCCACCACTGGC
βActin GH1 sense	ACCGCGAGAAGATGACCCAGATCATGTTT
βActin GH2 A.sense	AGCAGCCGTGGCCATCTCTTGCTCGAAGTC

The glands were exposed to the calcium sensitive fluorescent dye fluo-3-acetomethyl ester (fluo-3-AM; Molecular Probes, Eugene, OR) at 10 μM in Ringer's solution for 10 min at 37°C. Following the loading procedure the glands were washed with Ringer's solution for 5 min to remove any extracellular hydrolyzed dye, as well as to eliminate any residual dye that might be trapped on the cell exterior.

Microinjection Study with Lucifer Yellow

For dye coupling studies, single parietal cells were microinjected with the cell impermeant marker Lucifer Yellow (5×10^{-4} M; Molecular Probes, Eugene, OR) using a hydraulic microinjector with digital control (Model 11C, Narishige). Microinjection was performed through a 0.2–0.5-μm-tip sterile micropipette (WPI, Sarosota, Florida) by a pulse pressure of 400 kPa of 0.1-second duration. The axial and depth limits were set to allow penetration of the micropipette below the cell surface at an angle of 45°. Following microinjection, glands were examined on the stage of the fluorescence microscope to look for successful loading under fluorescent excitation. In addition we could examine for dye leakage at the injection site. Glands that failed to fluoresce or showed leakage were discarded from the study.

Measurements of Intracellular Calcium

Following hand dissection individual glands were loaded with the fluorescent Ca^{2+} indicator fluo-3 AM at a concentration of 10 μM. After incubation the glands were washed in Standard HEPES solution for 5 min at 37°C to remove any residual dye. The glands were then excited at 488 nm with emission measured at 535 nm on a Zeiss LSM410 Confocal Microscope. Fluorescence intensity was determined by measuring pixel values over each area of interest (typically three areas of interest per cell were defined with each having an area of 10 μm^2). Images (512 × 512 × 12 bit deep) were measured over the area before and after superfusion with the solution of interest. Sequential frames were acquired at 2-second intervals, each image comprising an average of 8 frames with 30 seconds between images. Each protocol lasted for 10 minutes, and images were stored on a hard disk. Ten gastric glands were analyzed for calcium wave propagation, and 35 for the fluo-3 fluorescence experiment. Data were expressed in arbitrary fluorescence intensity units (Geibel et al., 1995), summarized as ± SEM, and analyzed by pairing measurements at baseline and during experimental maneuvers, using a paired Student *t*-test with significance taken at $p < 0.05$.

Identification of Hormonally Stimulated Gap Junctional Activation

For all of the aforementioned protocols the glands were treated with either histamine (5×10^{-4} M), or gastrin (5×10^{-7} M). The histamine was applied in the direct vicinity of a parietal cell by means of a perfusion pipette. For studies involving gastrin the hormone was added directly to the perfusion solution. In a separate series, 20 μM of the gap junctional inhibitor (GJIC) 18α-glycyrrhetinic acid (Molecular Probes, Eugene, OR), an uncoupler of gap junctions, was applied. 18α-glycyrretinic acid was chosen as a selective inhibitor of gap junctions proteins. In experiments not shown we also used the nonspecific inhibitor heptanol and recorded similar results.

Results

Immunofluorescent Localization of Cx26 and Cx32

Immunofluorescence experiments indicated that Cx26 and Cx32 were localized to gastric glands (Fig. 1). There was a difference in the expression patterns between Cx32, which was mainly found along the membranes, and Cx26, where staining was predominantly found within the cytosol of parietal cells.

Western Blot Analysis of Connexins

The positive immunofluorescence results for the two connexins were further verified by Western blot analysis (Fig. 2). Connexin 26 (Cx26) was present at 26 kDa, and Connexin 32 (Cx32) at 32kDa, respectively.

RT-PCR

The results show a light positive band for Cx26. Cx32 gave a much stronger signal (Fig. 3). These findings are in accordance with the Western blot results.

Functional Studies in Isolated Gastric Glands

Intracellular Calcium

Parietal cells loaded with the calcium-sensitive dye fluo-3 responded to gastrin and histamine stimulation with increases of intracellular Ca^{2+}. The increase in fluorescence after histamine stimulation was 49% higher than with gastrin [mean arbitrary fluorescent intensity units (AFI) for histamine *vs.* mean AFI for gastrin for all cells examined]. As illustrated in Fig. 4*A*, when gastrin was applied to the gland we were able to observe a phasic activation of the cells in terms of intracellular Ca^{2+} concentration that was not seen with histamine. Pretreatment with the GJIC inhibitor 18α-glycyrrhetinic prevented the gastrin-induced rise of Ca^{2+} in adjacent cells (Fig. 4*B*).

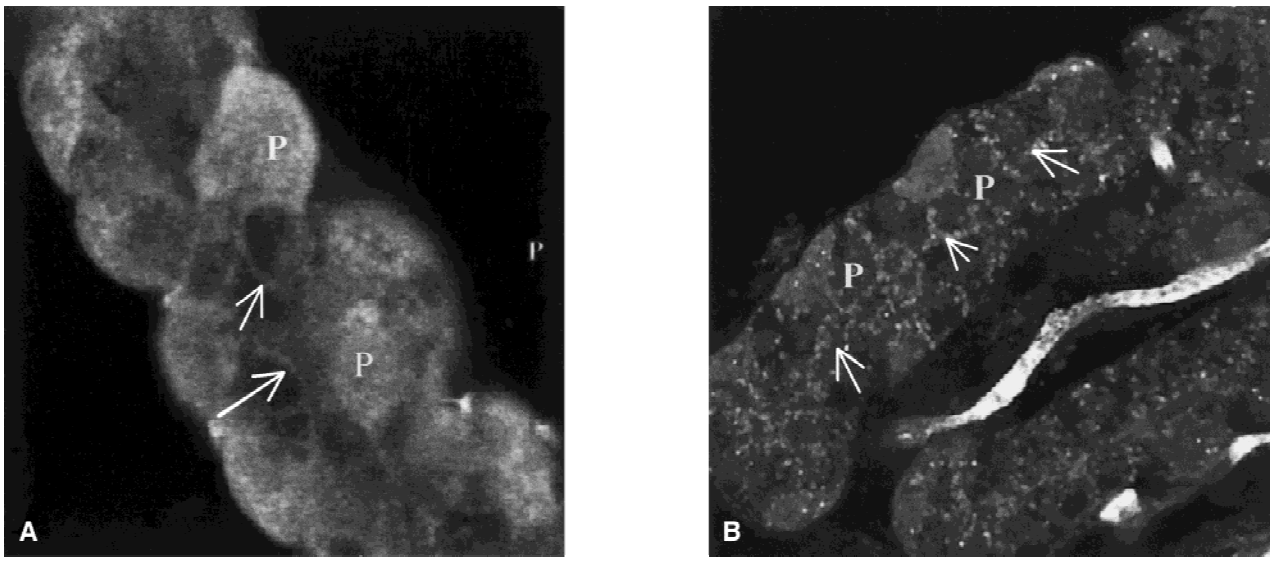

Fig. 1. Isolated gastric glands of the rat under confocal microscopy. Glands stained with anti-connexin Cx26 (*A*) and Cx32 (*B*). Whereas Cx32 is mainly found between parietal cells (*P*) as indicated (arrows), staining for Cx26 was more likely located within cells, preferably parietal cells, which are identified by their unique conical shape. Controls for the two antibodies stained negative.

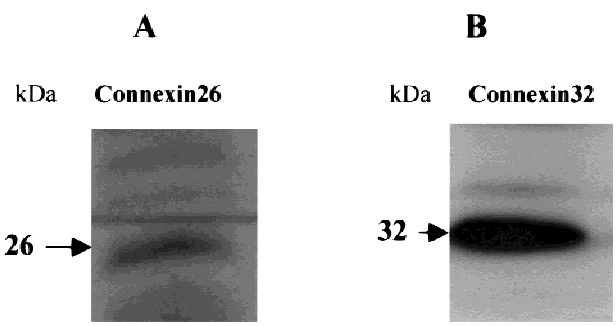

Fig. 2. Western blots of connexin26 (*A*) and connexin32 (*B*) in gastric glands. The nomenclature of each connexin subtype refers to its molecular weight in kDa. The reatively weak band corresponds to the weak expression of connexin26 as detected by RT-PCR.

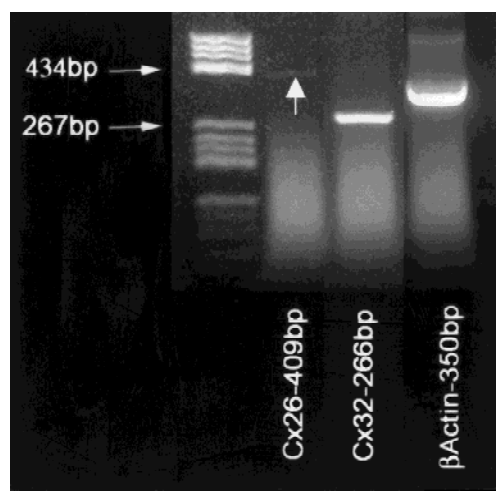

Fig. 3. RT-PCR against Cx26 and Cx32. Note that connexin26 was only weakly expressed at 409 bp (arrow), whereas Cx32 showed a strong band at 266 bp. A control was run against βactin (right column).

Microinjection Studies

Lucifer Yellow (LY; molecular weight 443) was not transferred from the microinjected parietal cell to the neighboring parietal cell in the resting state. Following histamine or gastrin stimulation we observed a decrease in fluorescence in the injected cell and a rise in fluorescence in the neighboring cell until equilibrium was reached (Fig. 5). This change in fluorescence typically occurred within 2 minutes of addition of the agent to the bath solution. Pretreatment with the gap junctional inhibitor 18α-glycyrrhetinic acid (6 experiments) prevented a transfer of fluorescent signal from cell to cell after either histamine or gastrin stimulation. This histamine- and gastrin-stimulated increase in Lucifer Yellow transfer was observed in all 15 experiments.

Discussion

Acid secretion is a multi-factorial process that is regulated through a variety of different pathways and is affected by the conglomeration of several regulatory signals. The central regulation involves direct neural input, whereas peripheral regulation involves endocrine, paracrine, and indirect neural pathways.

The regulation of acid secretion at the parietal cell involves at least three stages, i) regulation of basolateral membrane receptors for both stimulatory and inhibitory

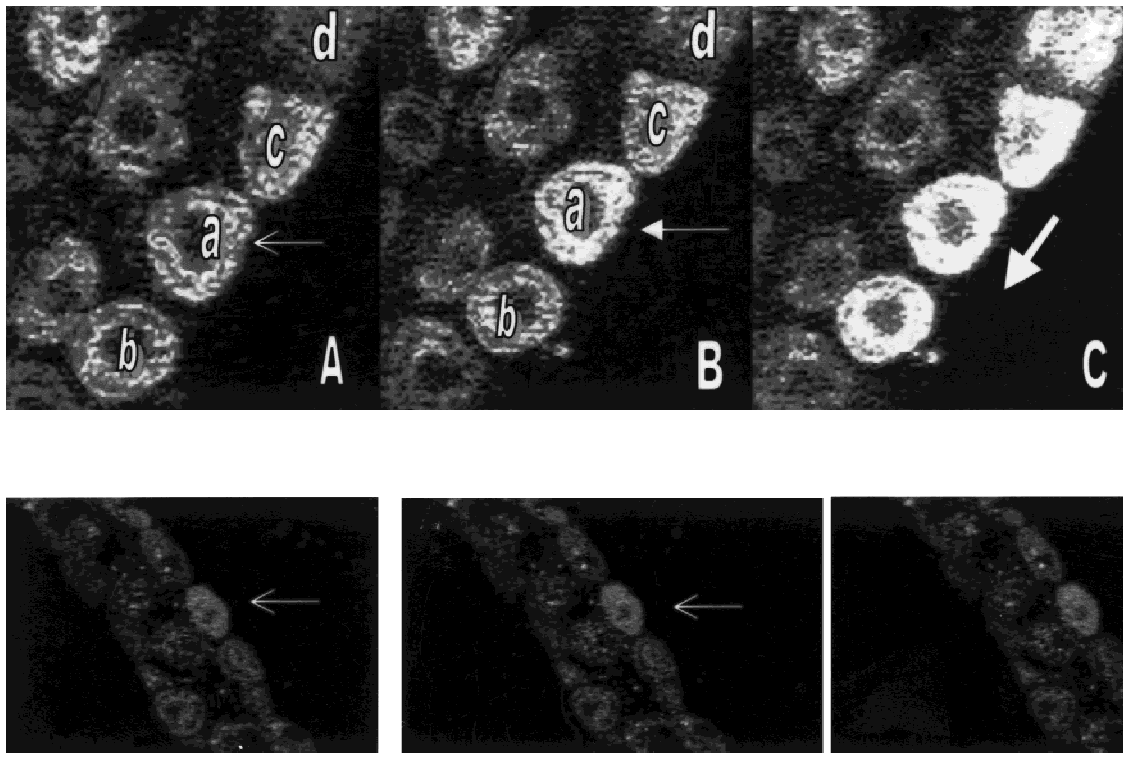

Fig. 4. (*Upper panel*) Demonstration of intracellular calcium increases under confocal microscopy in neighboring parietal cells after incubation of a gastric gland with the calcium-sensitive fluorescent dye flu-3 AM and focal stimulation of one parietal cell (*A*, arrow) with gastrin (5×10^{-7} M) via a micropipette. (*B*) Calcium increase in one stimulated cell (arrow). (*C*) Signal transfer to the neighboring parietal cells (b, c, and subsequently to d) via gap junctional proteins. The arrow indicates the flow direction of the bathing solution. (*Lower panel*) No intracellular calcium rise occurred in neighboring parietal cells after incubation of the gland in 18α-glycyrrhetinic acid, a gap junction inhibitor, and subsequent focal stimulation of a parietal cell (arrow) with gastrin (5×10^{-7} M).

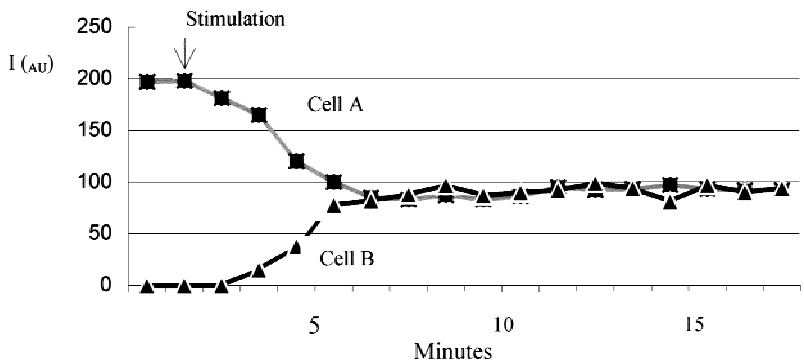

Fig. 5. Signal intensity of two neighboring parietal cells. Cell A was injected with Lucifer Yellow. After histamine stimulation (arrow), the signal in cell A decreased, but increased in the neighboring parietal cell B. The intensity increase in the neighboring cells (8 experiments with 20 cells measured) was significant ($p < 0.0001$; 95% confidence interval; two sample *t*-test). I_{AU}, intensity in arbitrary units.

ligands, ii) cytoplasmatic events secondary to ligand-receptor interactions with the cell membrane, and iii) regulation of the H^+,K^+-ATPase itself (Sachs, 1997). All of these processes require a synchronized activation of all the parietal cells along the length of the gastric gland to produce a coordinated production of protons at the apical surface of the gland. Conversely it is as critical to have a regulated and coordinated downregulation of the acid response when the digestive cycle is complete. Both of these pathways could rely on the use of the gap junctional complexes to allow for a rapid transfer of message from cell to cell and also allowing for a rapid deactivation following closure of the gap junctional complexes.

As part of the regulatory cascade of acid secretion we found that following hormonal stimulation of the gland there was not only a rapid release of histamine from the ECL cell, but also a rapid transient rise in intracellular calcium in the parietal cells (Geibel et al., 1995). When we examined this response with the con-

focal microscope at high resolution we were able to observe a phasic increase in Ca^{2+} in adjacent cells following stimulation with gastrin. This cyclical calcium pattern could potentially occur by the transfer of second messengers such as IP_3, 3′,5′-cyclic phosphate, or inositol 1,4,5-trisphosphate (Kam et al., 1998) from cell to cell via gap junctions resulting in the sequential rise in intracellular Ca^{2+}. Alternatively, it could reflect a direct exchange of calcium between cells. This increase in Ca^{2+} could also be the signal for activation or deactivation of the parietal cells in a synchronized manner. A similar use of gap junctions for calcium increase has been described in astrocytes (Giaume & Venance, 1998).

Evidence for the existence of connexins in the gastric glands comes from our immunofluorescence, RT-PCR and Western Blot analysis of the glands. Our data show that two connexins are expressed in the gastric gland. Cx32, which appears to be distributed along the plasma membrane, and Cx26, which is mainly found in the cytoplasmic compartment. There are two explanations that could account for this distribution: i) Cx26 could be involved in the microtubule and microfilament assembly and the trafficking of the vesicles with the pumps to the membrane, while Cx32 is the cell-to-cell connective element and pathway or, ii) Cx26 could be the unassembled complex that then traffics to the membrane to cause the assembly of active Cx32 at the cell-cell interface. This latter type of response has previously been demonstrated where newly synthesized connexins are transported from the endoplasmatic reticulum to the Golgi apparatus, and later to the junctional membrane where they accumulate (Spray, 1998). Thus, cytoplasmatic staining of newly synthesized connexins as found here would be expected in addition to membrane staining. Western blots and RT-PCR confirmed our hypothesis concerning the expression of Cx26 and Cx32 in the gastric gland.

Further confirmatory evidence for active functional gap junctions comes from our calcium data in which we show that parietal cells communicate with each other during gastrin or histamine stimulation. We have been able to demonstrate a transient increase in calcium that occurs following gastrin or histamine stimulation, and that, following activation of one cell, there appears to be a sequential rise in neighboring cells. This wave-like pattern is abolished when glands have been pretreated with a gap junctional inhibitor. However, it remains unclear whether calcium itself, or second messengers such as IP_3, cAMP or other Ca^{2+}-releasing second messengers move through the gap junctions. Each of these components remains a viable alternative, and the only limiting factor is that the molecular weight of the messenger must not exceed 1–1.4 kDa (Loewenstein, 1981; Bruzzone et al., 1996; Giaume & Venance, 1998; Kam et al., 1998). The increase of $[Ca^{2+}]_i$ in the neighboring cell may occur either through mobilization of Ca^{2+} from intracellular stores, and/or through membrane permeability to extracellular Ca^{2+}. The latter mechanism has recently been demonstrated in myocytes where a $[Ca^{2+}]_o$-dependent signal transfer of Na^+ trough gap junctions induced a Ca^{2+} entry by the reverse mode of the Na^+/Ca^{2+} exchanger (Ruiz-Meana et al., 1999). It is also possible that the divalent cation receptor on the parietal cell may play a role in the gap junctional activation as stimulation causes increased $[Ca^{2+}]_i$ levels (Cheng, Qureshi & Geibel, 1999). As a result, elevation of $[Ca^{2+}]_i$ in parietal cells ultimately results in acid secretion.

Conductance of gap junctions was further investigated with the cell-impermeant marker Lucifer Yellow (LY) injected into single parietal cells in intact single gastric glands to further investigate cell-to-cell communication. Because of Lucifer Yellow's high water solubility (~8%), its negative charge, and its moderately large molecular size no passive diffusion takes place across nonjunctional cell membranes. Gap junctional coupling was not observed in the nonstimulated state, whereas histamine, and/or gastrin stimulation, caused LY to move within 4 minutes from the microinjected cell into the neighboring parietal cell. When injected glands were pretreated with the gap junctional communication inhibitor 18α-glycyrrhetinic acid, no dye transfer occurred. The increased conductance of the gap junctional channels is attributable to elevated levels of cAMP and protein kinase A induced by histamine binding to the parietal cell (Burghardt et al., 1995; Chanson, White & Garber, 1996), although other cAMP-independent mechanisms such as elevation of intracellular pH by activation of the Na^+/H^+ exchanger can also facilitate gap junctional conductance (Ueda et al., 1994). Short-term exposure of rat myocytes and hepatocytes to PKA activators has previously been shown to cause an increase of already established cell coupling (De Mello, 1991; Godwin et al., 1993). Furthermore, exposure of cells to agents that increase intracellular cAMP levels led to a significant increase of cell coupling, as shown in a human colonic T84 cell line (Chanson et al., 1996). These observations indicate that gap junctional coupling is modulated in a cAMP-dependent manner.

Our investigations reveal that parietal cells are functionally connected via gap junctions. They use this pathway to provide calcium-wave propagation and intracellular message transfer, which indicates their active involvement in the regulatory process of gastric acid secretion. The inhibition of gap junctions leads to a reduction in response to hormonal stimulation, and reduced cell-to-cell communication. As gastrin and/or histamine can induce calcium waves, we find that the gap junctions open in a similar time-dependent manner. The gap junctions appear to be in a closed state during the non-acid secretory phase of the cell cycle. However, it remains

unclear i) which molecules pass through the channel pore, and ii) whether intracellular Ca^{2+} release, and/or influx of extracellular Ca^{2+}, or other pathways cause the elevation of Ca^{2+} signal in the neighboring parietal cells.

In this study, we have found conclusive evidence that the mammalian gastric gland contains the gap junctional proteins Cx26 and Cx32. Furthermore, these proteins appear to be localized mainly to the parietal cells by immunofluorescence. The appearance and localization of connexins demonstrate a new pathway for cell-cell communication in the gastric gland and provides a rapid way for a coordinated acid secretory event following hormonal stimulation.

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