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Topical Review

Signal Transduction and Activation of Acid Secretion in the Parietal Cell

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Introduction

Parietal Cells Secrete HCl by Translocation and Recruitment of the Gastric Proton Pump, H^+,K^+-ATPase

Gastric acid secretion has long fascinated physiologists, cell biologists and biochemists because of many attractive riddles in the field. Pure gastric juice, secreted by the parietal cell, contains highly concentrated hydrochloric acid, with pH as low as 0.8, that is, the established proton gradient is greater than 4 million times that in the blood stream. The origin and mechanism of how this gigantic electrochemical potential can be formed and maintained has been extensively studied. Also the hormonal and neural regulation of acid secretion, especially the role of histamine as a final common mediator, had been focus of much debate (Black, 1993). In this review we will concentrate on the intracellular events that couple the stimulus to activation of the parietal cell.

The parietal cell is considered to have at least three types of activating receptors on its basolateral membrane, i.e., histamine H_2, acetylcholine M_3, and gastrin CCK_B, although much of the action of acetylcholine or gastrin is mediated by the release of endogenous histamine. It is believed that the H_2 receptor couples to Gs to activate adenylate cyclase (ACase) producing adenosine 3′,5′-cyclic monophosphate (cAMP) and subsequent activation of cAMP-dependent protein kinase (PKA), whereas both M_3- and CCK_B-type receptors couple to non-Gs/Gi systems, probably Gq, to activate phospholipase C (PLC) producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, with the former releasing Ca^{2+} from intracellular stores, and the latter activating protein kinase C (PKC). This scheme is consistent with the knowledge obtained from many types of cells. The target of our interest, as unique events in the parietal cell, is focused in this brief review on the downstream reactions forming the PKA-, PKC-, or Ca^{2+}-pathways of activation.

The final step of HCl production is operated by the gastric proton pump, the H^+,K^+-ATPase. This enzyme was first discovered by Forte, Forte & Saltman (1967) as a ouabain-resistant K^+-stimulated phosphatase activity, and later shown as a K^+-stimulated ATPase common to the vertebrate stomach (Forte et al., 1975). Recent progress in molecular biology has revealed that the enzyme consists of a catalytic α-subunit of approximately 100 kDa molecular size and a highly glycosylated β-subunit (Chow & Forte, 1995), and shows striking similarity to another P-type cation pump, the Na^+,K^+-ATPase. The gastric enzyme uses the energy of ATP for the electroneutral countertransport of H^+ for K^+, in contrast to the Na^+,K^+-ATPase which is obviously electrogenic. As shown in Fig. 1, the function of the gastric proton pump is under dual-restriction while the parietal cell is in the resting state, i.e., the enzyme is sequestered within a population of cytoplasmic vesicles called tubulovesicles spatially insulating them from the gastric lumen, and the low permeability of tubulovesicular membranes to KCl limits the turnover of the pump even through there is ample ATP around the enzyme. Activation of acid secretion is achieved by two concomitant functional changes, namely, (i) tubulovesicles fuse with the apical secretory membrane thus recruiting functional pumps to

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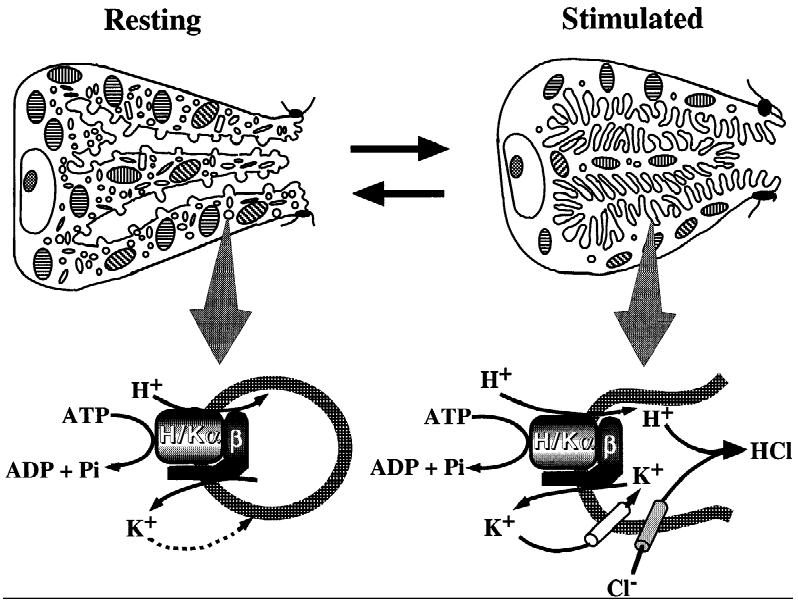

Fig. 1. Schematic representation of parietal cells at rest and under stimulation. In the resting state, H^+,K^+-ATPase is sequestered within cytoplasmic vesicles called tubulovesicles, and the low permeability of tubulovesicular membranes to KCl limits the turnover of the pump even though there is ample ATP around the enzyme (left). Activation of acid secretion is achieved by two concomitant functional changes, namely, tubulovesicles fuse with the apical secretory membrane thus recruiting functional pumps to the expanded microvillar surface and the apical membrane acquires a permeability to KCl (right).

the expanded microvillar surface (Forte, Machen & Forte, 1977) and (ii) the apical membrane acquires a permeability to KCl (Wolosin & Forte, 1984; Forte et al., 1990; Forte & Yao, 1996). In other words, the pump molecule itself is not activated during the activation of acid secretion. Although two potential phosphorylation sites for PKA exist in the α-subunit (Maeda, Ishizaki, & Futai, 1988), and both Tyr^7 and Tyr^{10} are shown to be phosphorylated by an unidentified membrane bound kinase (Togawa et al., 1995), their role in enzymatic activity is unclear at present. It is thus concluded that the intracellular signal transduction pathways should be connected to the machinery leading to the fusion of tubulovesicles with the apical membrane and to the activation of apical K^+ and Cl^- transporters.

Activation of PKA is the Main Pathway in the Secretory Cascade

There is a consensus that histamine, the main stimulant for the acid secretory cascade, stimulates H_2 receptors to activate Gs coupled with adenylate cyclase leading to the production of cAMP to PKA. Although some receptors other than H_2 have been shown to couple with Gs in the parietal cell (Yokotani et al., 1994; Ota et al., 1989; Schepp et al., 1992), they may have a minimal physiological role. There are two types of PKA: type I and II with different regulatory subunits, RI and RII, respectively, and it has been postulated that the former is predominantly cytosolic, whereas the latter associates with the cytoskeleton or membranes. Chew (1985) first reported that type I kinase was selectively activated by histamine-stimulation of the parietal cell, whereas subsequent study revealed that the dephosphorylation of RII, which led to the release of catalytic subunits, occurred during stimulation by the cAMP-pathway suggesting that PKA type II may also have a regulatory role (Goldenring et al., 1992). At least in the frequently used rabbit parietal cell model, PKA appears to be the primary activating factor since maximal stimulation of isolated glands or parietal cells, as monitored by ^{14}C-aminopyrine accumulation, was achieved when the intracellular content of cAMP was fully increased (Chew, 1983b). There is also an important background from the experimental and clinical literature that H_2-receptor antagonists are much more powerful inhibitors of gastric acid secretion in vivo than muscarinic- or gastrin-antagonists (Hirschowitz et al., 1995).

It is well known that the activation of H_2 receptors also elicits an increase of intracellular Ca^{2+}, at least in rabbit parietal cells (Chew, 1986; Muallem et al., 1986; Negulescu, Reenstra, & Machen, 1989). In the canine parietal cell, activation of H_2 receptors failed to elicit a Ca^{2+} response in contrast to cholinergic or gastrinergic stimuli (DelValle et al., 1992*a*). It is reasonable to propose that histamine is a full agonist in the rabbit parietal cell because it elicits two signals at once, i.e., elevation of cAMP and Ca^{2+}; whereas, in the canine parietal cell histamine and carbachol produce equivalent stimulatory responses because they operate through exclusive cAMP or Ca^{2+} pathways. This of course begs the questions as to how histamine is connected to Ca^{2+} in the rabbit cell and disconnected from Ca^{2+} in the canine cell. As exogenously added dibutyryl cAMP (dbcAMP: a cell permeant cAMP analogue) failed to increase Ca^{2+}, it has been

postulated that histamine also could activate PLC to produce IP_3, especially in the highly purified rabbit parietal cell (Chew & Brown, 1987*a*), suggesting that the H_2 receptor couples to PLC as well as to ACase (DelValle et al., 1992*b*). However, this hypothesis cannot explain why phosphodiesterase inhibitors or forskolin induce increments of Ca^{2+} in the parietal cell (Chew, 1986; Chew & Brown, 1987*a*). An alternative explanation might assume putative PKA-activated Ca^{2+} channels and compartmentalization of the components. We recently observed that histamine and forskolin elicited H89-sensitive, $[Ca^{2+}]_o$-dependent, increase of $[Ca^{2+}]_i$ in rabbit parietal cells. Although dbcAMP failed to reproduce this phenomenon, the more powerful analogue, Sp-adenosine 3′,5′-cyclic monophosphothioate produced a late onset but large and long lasting Ca^{2+} influx (Urushidani, Muto, & Nagao, 1996). These data could be interpreted by a compartmentalization model in which the direct (forskolin) or indirect (H_2) activation of ACase could effectively raise the local concentration of cAMP in the vicinity of the putative Ca^{2+} influx channel to induce full phosphorylation and opening. In contrast, exogenously added cAMP analogues would only produce a uniform but slight increase in the level of cAMP throughout the cell. However, this issue seems to be more complex since a recent report suggests that the canine H_2 receptor, which does not induce a Ca^{2+}-response, activated PLC via an unknown GTP-dependent mechanism (Wang, Gantz, & DelValle, 1996).

Ca^{2+} Works to Potentiate the cAMP/PKA Pathway

There has been only limited information on the mechanism by which intracellular messengers elicit the final physiological action. Especially for Ca^{2+}, definitive targets have not yet been elucidated, although it is said to be essential for many types of secretory events. Accumulating data suggest that the Ca^{2+} pathway itself cannot provide full activation of the secretory machinery, but that it works as a potentiating factor for the cAMP pathway (Soll, 1982; Negulescu, Reenstra, & Machen, 1989; Chiba et al., 1989; Li, Cabero & Mardh, 1995). It is well known that stimulation of M_3-receptors elicits a large Ca^{2+} response with little stimulation of acid secretion, at least in the rabbit parietal cell (Negulescu, Reenstra, & Machen, 1989). Activation of CCK_B-receptors also produces a calcium response in both rabbit and canine cells (Chew, 1986; DelValle et al., 1992*a*), although evidence for a direct effect on acid secretion is again quite weak (Soll, 1982; Chew & Hersey, 1982). Stimulation of M_3 receptors usually elicits a single, biphasic response of $[Ca]_i$, i.e., an initial transient rise due to the release from intracellular stores and a sustained plateau supported by Ca^{2+}-influx. Whereas, stimulation of CCK_B receptors with low levels of agonist elicited a repetitive transient rise (oscillation) in $[Ca]_i$ (DelValle et al., 1992*a*). CCK-induced $[Ca^{2+}]_i$-oscillations have also been observed in the Chinese hamster ovary cells transfected with cloned human CCKB-receptor (Akagi, Nagao & Urushidani, 1997). It seems therefore that the ability to produce $[Ca^{2+}]_i$-oscillation is inherent to the CCK_B receptor as also reported for the CCK_A receptor (Yule et al., 1993), however, histamine at the maximal dose and carbachol at a threshold dose both elicited $[Ca^{2+}]_i$ oscillations in cultured rabbit parietal cells (Ljungström & Chew, 1991). The mechanism as well as the physiological meaning of $[Ca^{2+}]_i$ oscillation is considered to be an interesting target for future study.

There could be two possible modes of action for Ca^{2+}, i.e., Ca^{2+} by itself and via the activation of calmodulin. There are at least seven calmodulin-dependent protein kinases, i.e., myosin light chain kinase (MLCK), phosphorylase kinase, and calmodulin kinases I to V (CaMKI-V). Pharmacological data have suggested that CaMKII is involved in the activation process via the M_3-receptor, since the CaMKII inhibitor KN62 preferentially inhibited carbachol-stimulated, but not histamine-simulated, aminopyrine accumulation in rabbit parietal cells (Tsunoda et al., 1992). We recently reported (Urushidani et al., 1997) the potential involvement of MLCK in the activation of acid secretion especially in the translocation of tubulovesicles which will be discussed later. Among the many potential targets other than calmodulin, calpain, a Ca^{2+}-dependent neutral protease, might be involved in parietal cell function (Yao, Thibodeau, & Forte, 1993), especially through its preference to cytoskeletal components including ezrin, which will be discussed later. Another cytoskeleton-related protein, gelsolin, is known to sever filamentous actin (F-actin) in a Ca^{2+}-dependent manner. A recent report has suggested the possible involvement of gelsolin in parietal cell function, especially in reorganization of the cytoskeleton during stimulation of the cell (Urushidani, Muto & Nagao, 1997). Beside the proteins with EF-hand structure, there is a protein family, the annexins, which show a common property of Ca^{2+}-dependent binding to phospholipids (*for review*, Creutz, 1992). The most productive speculation is that annexins are involved in the fusion process of the tubulovesicles as in the case of chromaffin cells where annexin II (Regnouf et al., 1996), or VII (Caohuy, Srivastava & Pollard, 1996) mediate vesicular fusion in a Ca^{2+}- and phosphorylation-dependent manner. Despite their predicted significance in the common secretory machinery, studies on the annexins in parietal cell biology are few (Urushidani & Nagao, 1997). Another Ca^{2+} and phospholipid-dependent enzyme, PKC, will be discussed in the next section.

PKC Has Both an Inhibitory and Facilitatory Role in Acid Secretion

There have been several controversial reports concerning the physiological role of PKC in the activation of acid secretion. Early studies reported that activation of PKC by phorbol esters exclusively showed inhibition of acid secretion (Muallem et al., 1986). In other studies, however, it was found that phorbol ester weakly stimulated acid secretion by itself, like carbachol (Chew, Zhou & Parente, 1997; Brown & Chew, 1986; Chiba et al., 1989), and it potentiated the stimulatory effect of dbcAMP (Brown & Chew, 1986). The bulk of evidence seems to favor the view that activation of PKC is inhibitory to stimulated secretion before PKA activation, e.g., activation of PKC by phorbol esters attenuated the responses to receptor-mediated agonists as well as to forskolin (Brown & Chew, 1986; Muallem et al., 1986), but not to dbcAMP (Brown & Chew, 1986; Nandi, Crockett, & Levine, 1994). On the other hand, acid secretion was augmented when PKC was inhibited, either directly by drugs such as H7 (Nandi, Crockett, & Levine, 1994; Urushidani & Nagao, 1996) and Ro31-8220 (Chew, Zhou & Parente, 1997), or indirectly by nonsteroidal antiinflammatory drugs (Nandi, Crockett, & Levine, 1994). Although there could be some involvement of somatostatin release from D cells (Chiba et al., 1987), receptor downregulation by PKC activation (Chiba et al., 1989) seems to be the most feasible mechanism for inhibition of the receptor-mediated route, and this has been supported by recent works describing that phosphorylation by PKC triggers receptor desensitization and down regulation (*for review*, Chuang et al., 1996). However, the fact that phorbol esters inhibit forskolin-induced acid secretion suggests there may be an additional site of action beyond adenylate cyclase. The regulation of adenylate cyclase activity by phosphorylation is complex (Sunahara, Dessauer, & Gilman, 1996). For example, phosphorylation of adenylate cyclase by PKC has been reported to be either inhibitory or stimulatory depending upon the subtype of the enzyme. It is also possible that the site of PKC action is beyond cAMP, since the cAMP-stimulated response was augmented by H7, an inhibitor of PKC (Nandi, Crockett, & Levine, 1994) and phorbol ester was found to inhibit the secretion stimulated by a phosphoprotein phosphatase inhibitor (Urushidani & Nagao, 1996). As the subtype of PKC in the parietal cell has recently been established (Chew, Zhou & Parente, 1997), the actual site of action of PKC will likely be elucidated in the near future.

Inhibitory Receptors Serve as Feedback Regulators of Acid Secretion

Receptors involving direct inhibition of acid secretion include somatostatin, prostaglandins (PGs), epidermal growth factor (EGF)/transforming growth factor (TGFα). Other endogenous antisecretory factors are considered to elicit their effects, directly or indirectly, on sites other than parietal cell, e.g., blood flow or alkaline secretion. Although it was shown that the activation of adenosine A1 receptors on the parietal cell led to an inhibition of acid secretion (Gerber & Payne, 1988), possibly via Gi, the physiological meaning of the receptor is presently unclear.

Somatostatin is considered to be one of the most important endogenous modulators of gastric acid secretion. Most of the inhibitory activity of somatostatin can be attributed to its wide inhibitory profile on the neuroendocrine system, although direct inhibition on the parietal cell also occurs. In an early report, somatostatin was shown to inhibit acid secretion stimulated by histamine, but not by dbcAMP, cholera toxin, or carbachol in rabbit gastric glands and parietal cells (Chew, 1983*a*). Inhibition of histamine-stimulated secretion by somatostatin was attenuated by pertussis toxin (Park, Chiba, & Yamada, 1987; Schepp et al., 1992), leading to the postulate that inhibition by somatostatin is on ACase via Gi. However, some additional mechanism might be necessary to interpret the results that somatostatin also inhibits dbcAMP-, carbachol- and gastrin-induced acid secretion in canine parietal cells (Park, Chiba, & Yamada, 1987), and this might be explained by multiple signals dependent on the receptor subtypes, 5 of which have been cloned and analyzed so far (Reisine, 1995).

In the case of PGs there appears to be a consensus that the inhibitory action, mainly PGE_2 (Seidler, Beinborn, & Sewing, 1989), is specific for histamine-stimulated secretion by inhibiting ACase via Gi (Chen, et al., 1988; Schepp et al., 1992). Among the 4 cloned PG-receptors, only EP3 is known to couple with Gi (Coleman, Smith, & Narumiya, 1994). It is therefore reasonable to postulate EP3 as the parietal cell subtype, and its mRNA was recently demonstrated to be dominant in murine parietal cells by in situ hybridization (Sugimoto et al., 1997).

For EGF/TGFα receptors, the situation is more complicated. It has been postulated that EGF and its homologue TGFα play important roles as endogenous modulators of gastric acid secretion as well as in the mitotic regulation of gastric epithelial cells (*for review*, Barnard et al., 1995). EGF/TGFα appears to modulate parietal cell functions in an autocrine-like manner, since it has been shown that mRNA for both TGFα and its receptor colocalize within the parietal cell (Beauchamp et al., 1989). Setting aside the observation that chronic treatment with EGF enhances acid production of cultured parietal cells (Chew, Nakamura, & Petropoulos, 1994), stimulation of EGF receptor has been consistently shown to inhibit histamine-stimulated acid secretion through Gi (Atwell & Hanson, 1988; Lewis et al., 1990; Chew, Na-

kamura, & Petropoulos, 1994). However, this does not explain the inhibitory action of EGF on carbachol-stimulated secretion, which might be mediated by the activation of tyrosine kinases (Tsunoda, Modlin, & Goldenring, 1993; Chew, Nakamura, & Petropoulos, 1994). A recent report (Wang et al., 1996) suggested that activation of PKC, possibly PKCα and β1 isoforms, by EGF was the main pathway of the inhibition. Therefore, this argument is to be joined with the above mentioned, controversial PKC story, in which PKC was postulated to be an inhibitory signal. If EGF is able to activate PKC via tyrosine kinase, the most feasible pathway is via activation of PLCγ producing IP_3 and diacylglycerol (Reynolds et al., 1993). However, this pathway generates messages quite similar to those mediated by the M_3 receptor (i.e., activation of PLCβ producing IP_3 and diacylglycerol), thus, EGF would be an expected agonist as effective as carbachol. Interestingly, EGF alone was shown to cause a transient stimulation of acid secretion as carbachol did (Lewis et al., 1990), while it did not affect the concentration of intracellular Ca^{2+} either in the resting parietal cell or those stimulated by carbachol (Lewis et al., 1990, Wang et al., 1996). Although the situation is chaotic at present, analysis of EGF effects appears to be an attractive area for continued work since it might provide some insight into the crosstalk within the parietal cell among PKA, PKC, tyrosine kinases and phosphatases (Tsunoda, Modlin, & Goldenring, 1993; Chew, Nakamura, & Petropoulos, 1994), and MAP-kinase (Nakamura et al., 1996) pathways.

From a different point of view, inhibitory signals could be mediated by the action of phosphoprotein phosphatases as the activation of acid secretion is ultimately mediated by serine/threonine kinases. Although it was suggested that phosphoprotein phosphatase I has a regulatory role in parietal cell activation (Goldenring et al., 1992; Urushidani & Nagao, 1996), there is no known mediator or direct activator of phosphatase, except for an early report suggesting that somatostain may be a phosphatase activator (Reyl & Lewin, 1982).

Pharmacological Agents May Have Nonspecific Effects on Proton Pump and Leak

A large part of the argument supporting any signal transduction pathway is dependent on pharmacological studies using various inhibitors. Interpretation of these results is totally dependent on the specificity of the agent to its targeted component in the pathway. In the case of gastric acid secretion there are additional pitfalls in the possibility that the inhibitor might alter the measured proton gradient via some action as a protonophore, or by a direct effect on the proton pump. It was reported that KN-93, a CaMKII inhibitor, was a potent antisecretory activity in vitro due to its protonophoretic action and not due to the inhibition of kinase (Mamiya et al., 1993). We recently found that U73343, a negative control of PLC inhibitor, U73122, was also a protonophore (Muto, Nagao, & Urushidani, 1997). Screening of various drugs has revealed some protonophoretic action of the following agents near the respective ED50 for inhibiting the putative target: ML7 and ML9, MLCK inhibitors; ONO-RS-082, a PLA2 inhibitor; and H89, a PKA inhibitor. Furthermore, the following showed some inhibitory action on H^+,K^+-ATPase: neomycin, a PLC inhibitor; and ONO-RS-082, a PLA2 inhibitor (Sugita, Nagao, & Urushidani, *preparation*). This apparent widespread susceptibility to nonspecific effects demands that investigators be circumspect in using and interpreting results with inhibitors.

The Search for Substrates of Parietal Cell Kinases

Activation of PKA, PKC, or calmodulin-dependent kinase(s) inevitably transduces its signal via the phosphorylation of some endogenous components, thus it is important to identify the substrate(s) for each kinase. Table 1 summarizes the candidate substrates for the kinases categorized into PKA and Ca^{2+}/PKC. Most of the proteins in the table were identified on the basis of increased labeling of a spot or band on an electrophorogram from stimulated parietal cells labeled with radioactive phosphate, although a few cases, e.g., the Cl^- channel, were identified by functional analysis. Recently, a convenient and effective model using α-toxin permeabilized gastric glands was developed (Thibodeau, Yao & Forte, 1994). As this is the only permeabilized model that is directly responsive to second messenger-mediated stimulation, it should be a useful tool for detecting putative substrates for kinases (Yao et al., 1996). Most of the candidate substrates for the Ca^{2+}/PKC pathway are phosphoproteins with no specified function, whereas many of those for the PKA pathway have been correlated with physiological functions. There has been some recent progress on the nature of KCl transporters involved in activating the acid secretory process. A chloride channel of 99 kDa, whose conductance was increased by PKA, has been cloned from a parietal cell cDNA library (Malinowska et al., 1995). Also, an 18 kDa substrate for PKA has reportedly endowed potassium conductance to isolated gastric microsomes (Sack, 1993), although this protein has not yet been purified or further characterized. For the stimulation-associated membrane transformations between cytoplasmic tubulovesicles and apical plasma membrane, the most feasible and likely candidates are cytoskeletal proteins and docking/fusing proteins. Among them, ezrin, a putative membrane-cytoskeletal linker phosphoprotein, is an attractive candidate as a downstream effector.

Table. Candidates of substrates for kinases in the parietal cell

PKA			
M.W.	Reference	Identification	Function
130K	Cuppoletti & Malinowska, 1988	Vinculin	Regulation of cytoskeleton?
120K	Urushidani, Hanzel, & Forte, 1987	Not identified	Kinase?
99K	Malinowska et al., 1995	ClC family	PKA-activated Cl channel
80K	Urushidani, Hanzel, & Forte, 1987	Ezrin	Membrane-cytoskeletal linker, AKAP
52K	Goldenring, et al., 1992	PKA-RII	Dephosphorylation leads to stimulation
40K	Chew & Brown, 1987b	Not identified	PKA substrate?
30K	Oddsdottir et al., 1988	Not identified	Identical to 27K?
27K	Chew & Brown, 1987b	Not identified	Identical to 30K?
18K	Sack, 1993	Kayletin	K^+-transporter?
Ca^{2+}/PKC			
M.W.	References	Identification	Function
100K	Oddsdottir, et al., 1987 Mayer et al., 1994	Not identified	CaMKIII substrate
80K	Chew, Zhou, & Parente, 1997	Ezrin	(PKC inhibitor sensitive)
66K	Brown & Chew, 1989	Not identified	PKC substrate, cytoskeletal localization
50K	Mayer et al., 1994	CaMKII α subunit	
40K	Urushidani & Nagao, 1997	SuccinylCoA synthetase?	
36K	Brown & Chew, 1989	Not identified	PKC substrate
28K	Parente et al., 1996	CSPP28	(Ubiquitous distribution)

Ezrin Serves as a Membrane-Cytoskeletal Linker in the Apical Pole of Parietal Cells

Ezrin was identified as an 80 kDa apical membrane protein whose phosphorylation was increased when parietal cells were stimulated via the PKA pathway (Urushidani, Hanzel, & Forte, 1986; 1989). Subsequently (Hanzel et al., 1991), the protein was found to be identical to ezrin, the microvillar core protein (Bretscher, 1983), which was also found to be a substrate for tyrosine kinase in A431 cells stimulated by EGF (Gould et al., 1986).

From the early stages of its identification, the physiological role of ezrin has been postulated to be related to the cytoskeleton. In the parietal cell, it was recovered in the cytoskeletal fraction by biochemical procedures (Urushidani, Hanzel & Forte, 1989) and found to be colocalized with F-actin by immunocytochemistry (Hanzel et al., 1989; 1991). Ezrin was also richly represented in a variety of microvilli and in the villial membrane rufflings of cultured cells (Gould et al., 1986; Pakkanen et al., 1987). Based on cDNA sequence homology ezrin is classified as a member of the ezrin/radixin/moesin/merlin (ERM) family of proteins, which all belong to the much larger band 4.1 superfamily of cytoskeletal-membrane linker proteins (Gould et al., 1989; Sato et al., 1992). This was further confirmed by recent expression studies. Algrain et al. (1993) showed that full length ezrin expressed in CV-1 cells colocalized with F-actin with partial solubility to detergent; whereas, expressed N-terminal ezrin behaved as membrane protein and expressed C-terminal ezrin colocalized with F-actin with perfect resistance to detergent. Furthermore, Turunen, Wahlstrom and Vaheri (1994) showed that the actin binding domain existed within the C-terminal 34 amino acids.

Many investigators had observed that purified ezrin scarcely binds to F-actin in vitro, contradictory to the observations in vivo (Bretscher, 1983). Our own early studies found only weak binding capacity of ezrin to F-actin prepared from skeletal muscle, undermining the conclusion that ezrin was an actin-binding protein. More recent observations by Yao, Cheng, & Forte (1996) and by Shuster and Hermann (1995) demonstrated that the binding of ezrin to F-actin is actin-isotype-specific, that is, the affinity is much higher for cytoplasmic β-actin rather the α-skeletal muscle isoform. Interestingly, the phosphorylation of ezrin does not seem to be essential for binding to actin, but one study suggests that phosphorylation may augment the binding to the cytoskeleton (Hanzel, et al., 1991). This could explain how bundles of microfilaments within microvilli might be stabilized by phospho-ezrin and why the binding complex could not be formed in the early experiments using α-actin. An apparent contradiction to this ezrin-actin isotype specificity is that the C-terminal portion of bacterially expressed ezrin effectively binds to α-actin, whereas full length ezrin does not (Turunen, Wahlstrom, & Vaheri, 1994). It is conceivable that the N-terminus conveys structural and isotypic specificity to the C-terminal binding site. However, based on the interactive tendencies

they observed for the C-terminal and N-terminal domains of ezrin and its family members, Gary and Bretscher (1995) have proposed an alternative explanation. They argue that ezrin readily forms head to tail homodimers preventing (or regulating) access of the actin-binding (C-terminal) or membrane associating (N-terminal) sites, and further that ezrin monomer exists in a form in which the C-terminal interactive domain is masked by a folding that partially obscures the N-terminal interactive domain. Given these ''stick'' tendencies the key, of course, is how can ezrin be regulated to effect the appropriate cytoskeletal and membrane interactions in vivo. Phosphorylation is one possible modification to expose binding sites. Purified gastric ezrin contains multiple phospho-isoforms, even in the resting state, and phosphorylation is enhanced with stimulation (Urushidani, Hanzel & Forte, 1989; Yao et al., 1995; Chen, Cohn, & Mandel, 1995). An alternative explanation comes from Shuster & Herman (1995) who proposed that the preferential binding of ezrin to β-actin filaments is promoted by an intermediary protein, possibly a 73 kDa peptide they saw in the binding assay. Furthermore, they observed that the cleavage of ezrin by calpain abolishes the actin binding ability. It is clear that the mechanisms of ezrin-actin interaction and its regulation are complex and more information is necessary for a complete picture.

The membrane binding domain of ezrin is in the N-terminal region. Niggli et al. (1995) showed that the N-terminal portion, 1-309, interacted with liposomes, and that the interaction was most prominent to phosphatidylinositol 4,5-bisphosphate (PIP_2). It was reported that the binding of ERM proteins to CD44, a putative binding partner, was strengthened by PIP_2, and a potential involvement of the small GTP-binding protein, Rho, in this system was suggested (Hirano et al., 1996). In analogy with other cytoskeletal proteins, it is possible that membrane recruitment in the parietal cell is regulated with phosphoinositide turnover through the linking of ezrin between the cytoskeleton and plasma membrane.

Ezrin has recently been reported to have activity as an A-kinase anchoring protein (AKAP). Using gels overlaid with RII regulatory subunit, Dransfield, Bradford, & Goldenring (1995) found two parietal cell proteins, 78 kDa and 120 kDa, with AKAP activity. Subsequently, the 78 kDa protein was found to be ezrin (Dransfield et al., 1997), and they postulated a putative RII binding site in ezrin, in the region of α-helix between 417 and 433, which is conserved in radixin and moesin. These authors also showed that RII was redistributed from cytosol to canalicular membrane when the parietal cell was stimulated. This is an interesting example of compartmentalization in which an important substrate binds its corresponding kinase on the cytoskeleton; the exact role for specific ezrin/RII association remains to be determined.

Also of interest is the recent finding of the potential involvement of MLCK in the role of ezrin. A new antiulcer drug, ME3407, with inhibitory activities on MLCK and PKA, was found to strongly inhibit the stimulation-associated translocation of tubulovesicles containing H^+,K^+-ATPase and to alter the distribution of ezrin (Urushidani et al., 1997). Possible involvement of MLCK was further supported by the observation that wortmannin, in the concentration range inhibitory to MLCK, produced results similar to ME3407 with respect to the effects on translocation of tubulovesicles and distribution of ezrin (Urushidani et al., 1997). Because of its strict substrate specificity, it is reasonable to suppose that inhibition of MLCK interferes with actomyosin interaction, e.g., cytoskeletal-membrane locomotion. Myosin I has been suggested as a motor for vesicles derived from the Golgi apparatus moving toward the apical membrane in chick intestinal cell (Fath & Brugess, 1993). As myosin-I has been identified in rabbit parietal cells (Yao & Forte, 1996), the role for myosin motors in the acid secretory process is an interesting issue.

Phosphorylation of Ezrin is Correlated with Gastric Acid Secretion

Although the precise mechanism is still unknown, it appears that ezrin interacts with the cytoskeleton in a phosphorylation-dependent manner. For example, dephosphorylation of ezrin occurs during the destruction of microvilli induced by ischaemia in renal cells (Chen, Cohn, & Mandel, 1995). In the parietal cell, striking morphological changes, such as depicted in Fig. 1, and the phosphorylation of ezrin are both correlated with the stimulation of acid secretion via the PKA activation pathway. Recent work with inhibitors of phosphoprotein phosphatase serve to emphasize the functional importance of these correlations.

When rabbit gastric glands were treated with calyculin A, an inhibitor of protein phosphatase I and II, acid secretion was strongly stimulated, whereas okadaic acid, an inhibitor of phosphatase II, did not show such an effect (Urushidani & Nagao, 1996). It was thus suggested that phosphorylation by PKA might occur constitutively and that the resting state in the parietal cell is maintained by the dephosphorylation of ezrin via protein phosphatase I. For parietal cells maximally stimulated with histamine plus isobutylmethylxanthine the phosphorylation of immunoprecipitated ezrin was 3 times higher than that from resting cells, whereas for glands stimulated with calyculin A ezrin phosphorylation was increased more than 10 times, although the index of acid secretion was much less than those stimulated by histamine plus isobutylmethylxanthine. While there is an apparent lack of correlation between the extent of acid secretion and ezrin phosphorylation, the net effect of

calyculin A seems to be a model for the extreme phosphorylation of the protein. Close examination of the material that co-immunoprecipitated with anti-ezrin from the detergent-soluble fraction of calyculin A-treated glands revealed several proteins coprecipitating with ezrin, including β-actin, α-actinin, and α and β-fodrin (Urushidani et al., *in preparation*). Phosphorylation occurred exclusively on ezrin and β-fodrin. α-Actinin was found to bind PIP_2 as reported for fibroblasts (Fukami et al., 1994). In contrast to the above-mentioned study on liposomes (Niggli et al., 1995), ezrin itself did not bind PIP_2 in the immunoprecipitated fraction. Ankyrin, which was reported to be bound to tubulovesicles (Smith et al., 1993), was not found in the ezrin-immunoprecipitate. These results suggest that a detergent-resistant complex of cytoskeletal components was formed when ezrin or β-fodrin was phosphorylated.

These results imply several interesting and suggestive features. Ezrin contains a proline-rich domain whose function is presently unknown (Gould et al., 1989). Recent work suggests that SH3 domains exist in various kinds of proteins including cytoskeletal proteins and that they interact with proline-rich domains (Ren et al., 1993). Among the components of the above mentioned complex, α-fodrin actually possesses an SH3 domain. An attractive, though tentative, hypothesis is that the phosphorylation of ezrin reveals not only an actin binding domain but also a proline rich domain to interact with fodrin. Furthermore, in analogy with other cell systems (Fukami et al., 1994) where bound PIP_2 regulates the bundling effect of α-actinin on F-actin, PIP_2 and PLC might serve in the regulation and reorganization of the cytoskeleton in the parietal cell.

In A-431 cells stimulated by EGF, ezrin was phosphorylated at Tyr^{145} and Tyr^{353} (Kreig & Hunter, 1992) in association with membrane surface remodeling (Bretscher, 1989). Bretscher's observation (1989) that the EGF stimulation of A-431 cells also induced ezrin-phosphorylation on Ser residues reminds us of the above mentioned effect of EGF to activate PKC. Several sequons in ezrin have been pointed out as potential phosphorylation sites for PKC (Gould, 1989), and the possible phosphorylation of parietal cell ezrin by PKC was recently reported (Chew, Zhou & Parente, Jr., 1997). On the other hand, no detailed information is currently available on the PKA-phosphorylation sites of ezrin, which is considered to be the principal regulatory pathway and mode of ezrin phosphorylation in the parietal cell. Thus our perception of the role of ezrin in parietal cell activation is still evolving.

Vesicular Docking and Fusion Proteins are Involved in Membrane Traffic

The highly selective mechanisms for the recruitment of transport proteins by exocytic fusion and retrieval by endocytosis represent an exciting area of current research. A growing body of evidence supports the notion that the general machinery governing the docking/fusion of membranes is highly conserved (Sollner et al., 1993; Rothman & Warren, 1994). In neurons, the so-called SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptor) hypothesis holds that vesicle targeting is mediated by the unique pairing between membrane proteins on the transport vesicle (known as v-SNARES) and the proteins on the target membrane (known as t-SNARES). The resulting SNARE complex provides the binding site for soluble cytoplasmic factors to catalyze subsequent vesicle-plasma membrane fusion events (Bennett & Scheller, 1994). Several of the specific proteins and their isoforms that function in synaptic vesicle docking/fusing reactions have been identified in parietal cells (Peng et al., 1997). These include syntaxin isoforms 1 through 4, VAMP (vesicle-associated membrane protein, also known as synaptobrevin) and SNAP-25 (synaptosome-associated protein of 25 kDa), all of which are especially enriched in parietal cells, compared to other gastric epithelial cells. Detection of syntaxin 1 and SNAP-25 in parietal cells was unusual since these proteins had been found only in neural or endocrine cells, where they have been localized to the plasma membrane as t-SNARES. Subcellular fractionation revealed that syntaxin 3 and VAMP copurified with H^+,K^+-ATPase rich tubulovesicles, thus fitting the general definition as v-SNARES; whereas, syntaxin 1 and SNAP-25 were found in membranes other than tubulovesicles. It is tempting to speculate that syntaxin 1 and SNAP-25 reside at the apical plasma membrane as docking targets for the VAMP-laden tubulovesicles. The unusual abundance of syntaxin 3 on tubulovesicles is of interest. It will be important to establish whether syntaxin 3 serves an exclusive role as a v-SNARE, namely, tubulovesicles target to the apical membrane (Mercier et al., 1989) or whether it acts as a t-SNARE to promote homotypic fusion of tubulovesicles constructing an interconnected tubular network. Whether the docking and fusion of tubulovesicles occurs exclusively with the apical membrane (heterotypic fusion) or whether there is a potentiation of recruitment in the form of tubulovesicle-tubulovesicle interaction (homotypic fusion) remains to be established. However, one preliminary report notes that Ca^{2+}-triggered homotypic fusion of isolated tubulovesicles in vitro was dependent upon a small GTP-binding protein (G-protein; possibly Rab2) and phospholipase A_2 activity (Pereyra & Machen, 1994), and Ogata & Yamasaki (1993) have observed tubulovesicular interconnections in their deep-etched preparations of parietal cells in the early phase of activation.

Members of small G-proteins have been implicated as regulators of trafficking, targeting and fusion of membrane vesicles (*for review*, Wagner & Williams, 1994),

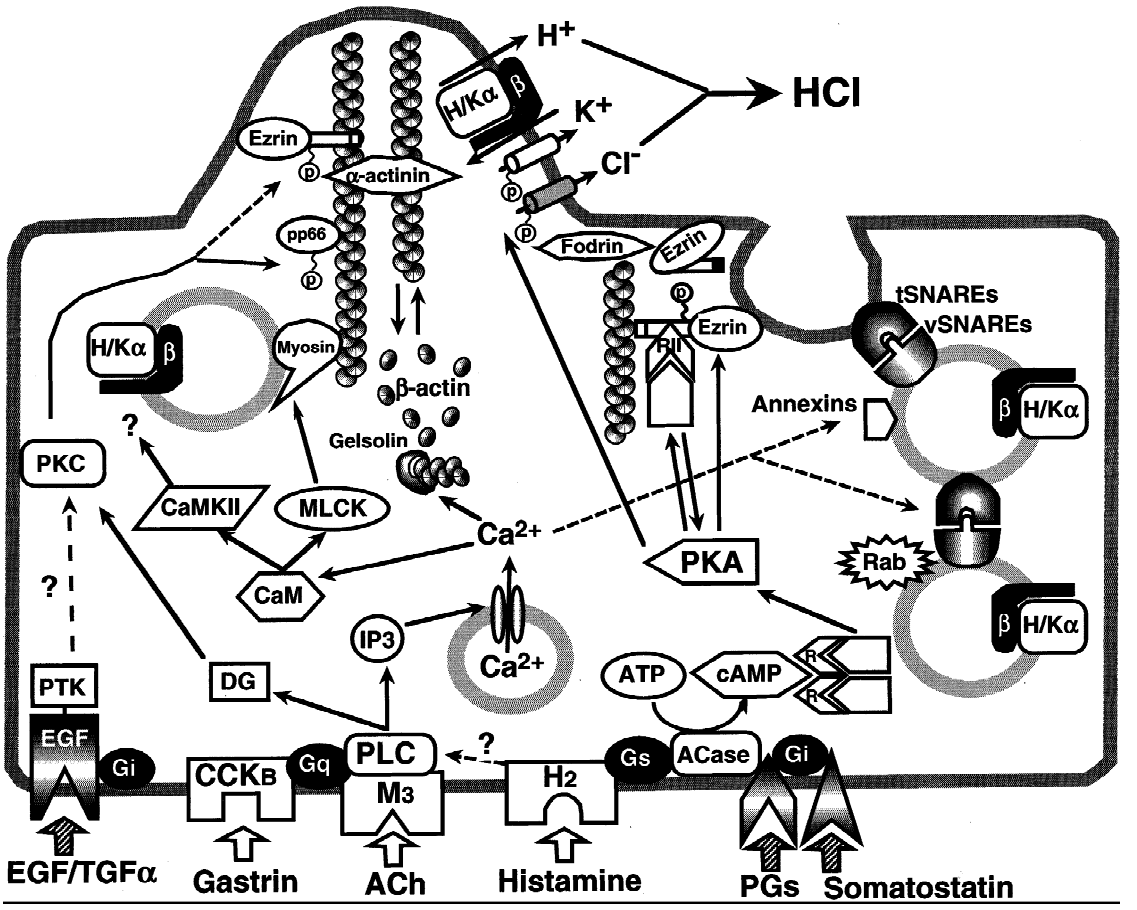

Fig. 2. Model of receptor-mediated pathways and effector activation in gastric parietal cells. Receptors for histamine (H2), acetylcholine (ACh, M3), gastrin (CCKB) and EGF/TGFα (EGF) operate at the basolateral membrane through heterotrimeric G proteins, either stimulatory (Gs or Gq) or inhibitory (Gi). The major path for parietal cell stimulation is via H2-receptor-mediated adenylate cyclase (ACase) and elevation of cAMP to liberate active protein kinase A (PKA) from regulatory subunits (R). PKA phosphorylates key effector proteins, e.g., ezrin, apical Cl^--channels or putative K^+-channels. Activation of PKA also occurs on its substrate ezrin, since these proteins are bound via type II regulatory subunits (RII). Ca^{2+} is clearly an auxiliary second messenger being liberated from intracellular stores by IP3, which in turn is the result M3- and CCKB-coupled activation of phospholipase (PLC). The resulting diglycerides activate protein kinase C (PKC) which may have both inhibitory and excitatory roles. Roles are proposed for elevated Ca^{2+} in several regulatory paths, including (i) activation of calmodulin-dependent (CaM) kinases that could promote vesicular motor activity, (ii) actin filament turnover, or (iii) to promote fusigenic interactions via annexin and SNARE proteins. As H^+,K^+-ATPase-rich (H/K) tubulovesicles migrate to and fuse with the apical membrane, surface remodeling occurs in the form of elongated microvilli that are coordinated by the β-actin-rich microfilaments and the actin-binding and actin-membrane linker proteins.

and among them, Rab family members have been identified in parietal cells apparently co-localizing with the H^+,K^+-ATPase (Tang et al., 1992; Goldenring et al., 1993, 1994). Both Rab2 and Rab11 were found to co-purify with H^+,K^+-ATPase-rich tubulovesicles in resting cells, and they were redistributed to the apical plasma membrane, along with the H^+,K^+-ATPase, when the cells were stimulated to secrete acid. Considering the predicted significance of the cytoskeleton in parietal cell function, another small G-protein, Rho, is also an interesting target, since it has been shown to be involved in cytoskeletal reorganization in various cell types (Takai et al., 1995; Hirano et al., 1996). Rho has been a suggested participant in CCK_B/gastrin receptor mediated stress fiber formation in the transfected fibroblasts (Taniguchi et al., 1996), however, there is scant information available about the role of Rho in native parietal cells. Our own experience with the botulinum toxin C3, an inhibitor of Rho, have produced conflicting results. In a few experiments we have observed that C3 inhibited cAMP-mediated aminpyrine uptake by isolated gastric glands, but in many other tests no such inhibition was seen. Whether this variability represents a problem in the accessibility of inhibitor, or some other artifact, remains to be seen. In general, GTPγS, a nonhydrolyzable GTP analogue, stimulates secretion of various types of permeabilized cells (Wagner & Williams, 1994). However, Miller & Hersey (1996) showed completely opposite re-

sults using α-toxin permeabilized gastric glands, i.e., GTPγS blocked parietal cell secretion beyond the heterotrimeric G-proteins, whereas it stimulated pepsinogen secretion by the chief cell in the same glands. This might suggest that the parietal cell has a dominant population of small G-protein mediating inhibitory signals which may be minor in other exocrine cell types, inasmuch as the effect of GTPγS is specific for small G-proteins in the system. One potential target is the ADP-ribosylation factor (ARF)-coat protein system, whereby GTPγS would inhibit membrane traffic from cis to medial Golgi cisternae because the dissociation of the coat proteins from the vesicles requires GTP hydrolysis by ARF (Serafini et al., 1991).

Projecting Future Work

The recent progress in molecular biology has revealed the complete structures of H_2-, M_3-, and CCK_B-receptors, as well as α- and β-subunits of H^+,K^+-ATPase, namely, the entrance and exit of the parietal cell are now visible. However, the interior of the cell is still dark, although we have been turning on and off many possible switches to decipher the wiring diagram. It appears that many of the necessary parts are now in our hands as depicted in Fig. 2. Biochemical and immunocytochemical data have identified some of the potential characters; the challenge ahead is to define the physiological role of the cytoskeletal components, docking proteins and small G-proteins in mediating membrane turnover during the secretory cycle. The parietal cell model with all of its exaggerated responses may be a particularly useful system in which to probe for the distribution and nature of complexation of the component proteins in various secretory states.

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