Ion channels in secretory granules of the pancreas and their role in exocytosis and release of secretory proteins

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Thévenod, Frank. Ion channels in secretory granules of the pancreas and their role in exocytosis and release of secretory proteins. Am J Physiol Cell Physiol 283: C651–C672, 2002; 10.1152/ajpcell.00600.2001.—Regulated secretion in exocrine and neuroendocrine cells occurs through exocytosis of secretory granules and the subsequent release of stored small molecules and proteins. The introduction of biophysical techniques with high temporal and spatial resolution, and the identification of Ca2+-dependent and -independent “docking” and “fusion” proteins, has greatly enhanced our understanding of exocytosis. The cloning of families of ion channel proteins, including intracellular ion channels, has also revived interest in the role of secretory granule ion channels in exocytotic secretion. Thus secretory granules of pancreatic acinar cell express a CIC-2 Cl− channel, a HCO3−-permeable member of the CLCA Ca2+-dependent anion channel family, and a KCNQ1 K+ channel. Evidence suggests that these channels may facilitate the release of digestive enzymes and/or prevent exocytosed granules from collapsing during “kiss and run” recycling. In pancreatic β-cells, a granular CIC-3 Cl− channel provides a shunt pathway for a vacuolar-type H+-ATPase. Acidification “primes” the granules for Ca2+-dependent exocytosis and release of insulin. In summary, secretory granules are equipped with specific sets of ion channels, which modulate regulated exocytosis and the release of macromolecules. These channels could represent excellent targets for therapeutic interventions to control exocytotic secretion in relevant diseases, such as pancreatitis, cystic fibrosis, or diabetes mellitus.

acini; β-cells; secretion; zymogen granules; sulfonylureas

EXOCRINE AND ENDOCRINE CELLS, such as pancreatic acinar or β-cells, are morphologically characterized by the presence of intracellular membrane-bound vesicles or granules that secrete their content into the extracellular milieu in a regulated manner. A key aspect of regulated secretion concerns exocytosis and release of macromolecular secretory products, for instance, digestive proenzymes (the “zymogens”) in pancreatic acinar cells or insulin in pancreatic β-cells. In this process, secretory granules gather beneath the cell membranes in clusters and lie there waiting until a signal reaching the cell membrane induces the granules to fuse with the plasma membrane (PM) and to discharge their stored macromolecules and/or solute molecules into the cell exterior. The sequence of events preceding and accompanying granule export has been described in great detail by ultrastructural techniques (143). A decisive element of this process is a “fusion-fission” reaction between the membrane of secretory granules and the PM of secretory cells, which may also involve the formation of proteinaceous pores between apposed membranes as the basis for fusion (128).

Up to about 1990, the term “exocytosis” described the export process of membrane-impermeant molecules stored within the secretory granules (6). Exocytosis was viewed as comprising several distinct stages, whose nomenclature had been established on the basis of ultrastructural studies (143). After granule assembly in the Golgi and “translocation” beneath the PM, a
stimulus would lead to the “fusion” or juxtaposition of granules and PM, a process that would require an intracellular signal, such as an increase of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]. This initial fusion occurred between the PM inner leaflet and granule outer leaflet (the “pentalaminar complex”). Finally, the barrier between granule interior and extracellular medium would break (undergoing “fission”) and secretion would ensue.

The ultrastructural studies by Palade (143) led Pollard and coworkers (151) to propose a “chemiosmotic hypothesis of exocytosis,” according to which H\(^{+}\) and Cl\(^{-}\) fluxes through granule channels and transporters were energetically coupled to granule fusion and fission via osmotic swelling of the secretory granules. This model was based on experiments in cell groups and isolated granules. Subsequent studies on isolated cells using techniques with a higher temporal and spatial resolution seem to have disproved the hypothesis forwarded by Pollard (see Refs. 40, 234).

The introduction of electrophysiological and optical methods with high temporal and spatial resolution, the identification of Ca\(^{2+}\)-dependent and -independent proteins participating in the fusion-fission reaction, and the application of kinetic analysis to the process of granular secretion have contributed to a better understanding of regulated vesicular secretion. This allowed the detection of distinct stages of secretion occurring in a time frame of milliseconds. Exocytosis, by definition, now refers to a phase of secretion at which an electrical shunt for the proton flux of anions, particularly of H\(^{+}\), is formed from the condensing vacuole, which buds off the trans face of the Golgi complex. The condensing vacuole is a membrane-bound organelle that contains secretory proteins in dilute form. It then undergoes a packaging process, during which its content is condensed by processes that are still poorly understood. Most secretory granules, including chromaffin and islet granules, gradually acidify through the activity of a V-ATPase (71). Once the granule has matured and has attained its highest density, it serves as a storage depot. The granules of exocrine acinar cells are exclusively found at the apex, which highlights the polarity of these cells (143). In β-cells, granules are stored throughout the cytosol. However, they preferentially accumulate at release sites near PM L-type Ca\(^{2+}\) channels, which indicates some degree of polarity in β-cells as well (36). Ultimately, a stimulus induces secretion by granule fusion with the PM, exocytosis, and discharge of its content into the lumen.

**Chemiosmotic Hypothesis of Exocytosis**

Almost 25 years ago, Pollard and collaborators (146, 151) proposed that anion conductances play a crucial role in Ca\(^{2+}\)-dependent secretion. According to their chemiosmotic hypothesis of exocytosis, an H\(^{+}\)-ATPase expressed in the secretory granule membrane actively pumps protons into the granule. Proton influx is electrically balanced by influx of anions, particularly of Cl\(^{-}\), through a conductive pathway, its main role consisting in providing an electrical shunt for the proton pump that acidifies the granule lumen. The influx of secreting β-cells, which provide compelling evidence that ion flux through granule ion channels is required for secretion to occur. This happens by modulating the final steps of the secretory process, namely, exocytosis and/or the release of macromolecular secretory products.

**BACKGROUND**

**Mechanisms of Regulated Protein Secretion**

Secretory proteins (including precursor proteins such as proenzymes or proinsulin) are synthesized on the rough endoplasmic reticulum (RER) and inserted into the cisternae of the RER in statu nascendi. This is the only time they cross a cellular membrane. thereafter, they are confined within membrane-bound cellular compartments. The secretory proteins are transferred from one compartment to the next by vectorial transport while undergoing several maturation steps, which are initiated in the Golgi network. Processing and sorting occur within the Golgi complex. Whereas the pH in the RER is near neutral (108), the trans-Golgi network has a pH of ~6.0–6.5 (57) because of active H\(^{+}\) pumping into the organelle by an electrogenic proton-translocating ATPase, the vacuolar-type H\(^{+}\)-ATPase (V-ATPase) (71). Acidic pH is required if Golgi-localized enzymes involved in posttranslational modifications, such as sialylation, sulfation, and glycosylation, are to operate efficiently. The secretory granule is formed from the condensing vacuole, which buds off the trans face of the Golgi complex. The condensing vacuole is a membrane-bound organelle that contains secretory proteins in dilute form. It then undergoes a packaging process, during which its content is condensed by processes that are still poorly understood. Most secretory granules, including chromaffin and islet granules, gradually acidify through the activity of a V-ATPase (71). Once the granule has matured and has attained its highest density, it serves as a storage depot. The granules of exocrine acinar cells are exclusively found at the apex, which highlights the polarity of these cells (143). In β-cells, granules are stored throughout the cytosol. However, they preferentially accumulate at release sites near PM L-type Ca\(^{2+}\) channels, which indicates some degree of polarity in β-cells as well (36). Ultimately, a stimulus induces secretion by granule fusion with the PM, exocytosis, and discharge of its content into the lumen.

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osmotically active solutes provides the “physical” energy, via granule swelling, to overcome “repulsive forces” between granule and PM. Thus the increase of osmotic strength inside the granule furnishes the energy for membrane fusion (151).

This model was based on studies in chromaffin cells (146, 151). Reports in pancreatic islets supported Pollard and coworkers’ chemiosmotic hypothesis in so far as they observed a Cl⁻ dependence of glucose-induced insulin release (140, 184). In subsequent work on permeabilized adrenal medullary cells, Knight and Baker (111) found that high concentrations of Cl⁻ could promote release of granular protein in the absence of Ca²⁺, Ca²⁺-dependent release, however, was inhibited by Cl⁻. These data were therefore not consistent with the suggestion of Pollard and coworkers that entry of Cl⁻ into chromaffin granules promotes exocytosis (see Ref. 21). Unfortunately, the discrepancies regarding the role of Cl⁻ in secretion of chromaffin cells have not been followed up and resolved. The fact remains that chromaffin granules are acidified by the action of a V-ATPase (192), which necessitates a parallel conductance to shunt its electrical current for efficient operation and is mediated by either influx of anions or efflux of cations (12).

In exocrine glands, condensing vacuoles of pancreatic and parotid secretory granules were found to be acidic by quantification of the partition of a permeant weak base by immunoelectron microscopy (141). The internal pH of mature granules, however, may or may not be acidic. In one in vitro study, the intragranular pH of isolated pancreaticzymogen granules (ZG) was estimated to be ~6.5 with the pH-sensitive fluorescent dye 2’7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (81). This was confirmed in intact cells with the pH-sensitive fluorescent dyes acridine orange (56, 83, 137), LysoTracker (83), or quinacrine (208). This is also supported by the fact that the 31- and 70-kDa subunits of the V-ATPase are expressed in the membranes of rat pancreatic ZG (167). In contrast, evidence for a V-ATPase activity in secretory granules from exocrine glands is lacking (14, 15). Moreover, other studies in intact cells using the weak base 3-(2,4-dinitroanilino)-3’-amin-N-methylidipropylamine (DAMP) or the pH-sensitive fluorescent dyes acridine orange and LysoTracker red came to the conclusion that the pH of ZG is neutral (141, 185). Consequently, ZG acidification as a prerequisite for a chemiosmotic mechanism of exocytosis is still an unresolved matter.

In 1985, Stanley and Ehrenstein (186) proposed that an initial step in the process of exocytosis of neurosecretory granules is activation of Ca²⁺-dependent K⁺ channels present in granule membranes. In this model, salt influx into the granule is controlled by a K⁺ channel that opens when intracellular [Ca²⁺] is elevated as a result of receptor activation. Electrical coupling to anion pathways results in salt and water influx and osmotic swelling, followed by fusion of the vesicle membrane with the luminal membrane and release of the vesicle contents. This model was used to explain protein secretion in exocrine cells with granules, which may lack an active V-ATPase, such as ZG (79).

According to the models proposed by Pollard and coworkers (151) and by Stanley and Ehrenstein (186), increasing the extragranular tonicity should inhibit secretion (72). Evidence for this effect of osmolarity was, for instance, provided by Knight and Baker (111) in chromaffin cells whose PM had been permeabilized by dielectric breakdown with intense electric fields but also by Fuller et al. (79) in protein secretion studies using isolated pancreatic acini permeabilized with digitonin.

However, subsequent pioneering studies in beige mouse mast cells with electrophysiological (patch clamp) membrane capacitance measurements (40, 234) demonstrated that fusion actually precedes swelling, which proved that osmotic swelling is not required for fusion. Swelling did occur, but it took place after fusion of granules with the PM (40, 234). Both reports speculated that granule swelling was necessary to stabilize and widen the exocytotic pore and that swelling occurred by movement of extracellular small solutes through the exocytotic pore into the granule matrix (40, 234). These observations were taken as strong evidence to fully dismiss the swelling (= chemiosmotic) hypothesis of exocytosis.

The studies by Zimmerberg et al. (234) and Breck- enridge and Almers (40) were published at about the same time as interest was developing in the cellular and molecular biology of protein factors mediating membrane fusion in yeast. This shifted the focus toward other mechanisms that might explain the membrane fusion process in eukaryotic systems and led to the identification of ubiquitously expressed and obligatory protein components of cellular fusion events. These include soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins, Sec1/Munc18 homologs (SM proteins), and Rab proteins. These three classes of Ca²⁺-dependent and -independent docking and fusion proteins appear to be universally involved in intracellular fusion reactions, and enormous progress has recently been made in the understanding of their role in vesicle fusion. A thorough and detailed discussion of their components and function is not the scope of this review and can be obtained elsewhere (see, for example, Ref. 101).

Chemiosmotic Hypothesis of Exocytosis: An Obsolete Mechanism?

In 1988, Gasser, DiDomenico, and Hopfer (82) proposed that the fluidity of the pancreatic primary secretion is of paramount importance for the discharge of digestive proenzymes from exocytosed secretory granules. They argued that the fluidity of the primary secretion is also determined by the amount of electrolytes and water, which is secreted by acini. Cl⁻ channels located at the apical membrane are essential for secretion of fluid and electrolytes by acinar cells. Because a consequence of granule fusion represents the insertion of components of the granule membrane into...
the granule membrane occurring via anion (e.g., Cl\(^-\)) and cation (K\(^+\)) channels would significantly contribute to an increase of fluidity (82). Salt and water would “flush out” the stored macromolecules into the acinar lumen and provide for an appropriate amount of fluid to be secreted with the proteins. The authors hypothesized that this mechanism might be implicated in the pathophysiological events associated with cystic fibrosis (CF). CF is a fatal genetic disease associated with abnormalities of fluid and electrolyte transport in exocrine epithelia (156). In the exocrine pancreas of CF patients, discharge of digestive enzymes to the gastrointestinal tract is also impaired, resulting in pancreatic insufficiency. These alterations are morphologically characterized by luminal obstructions and dilatations of the secretory acini and ducts followed by atrophy and degeneration of the exocrine parenchyma. As in the sweat gland, airway, and intestine, the pancreas shows a deficiency of cAMP-mediated mechanisms of stimulus-secretion coupling, which is caused by mutations in a Cl\(^-\) permeability mediated by the cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7 according to the new nomenclature) (88, 163). A defect of Cl\(^-\) pathways in the granule membrane would therefore contribute to the increased viscosity of the primary acinar secretion associated with CF. A drawback of this hypothesis, however, is that so far CFTR has not been found to be expressed in ZG. Nevertheless, Hopfer and coworkers (82) originated the notion that exocytosis and the release of macromolecules are separate secretory events and that the discharge of macromolecules may require activation of transport processes in the granule membrane. More recently, similar mechanisms have been suggested to promote secretion of mucins in submandibular glands, airway, and gallbladder epithelia, where CFTR is expressed in secretory granules (Refs. 115, 129, 130, 136; see CFTR Cl\(^-\) Channel).

Thus recent studies have led to a more differentiated reevaluation of the chemiosmotic hypothesis of exocytosis. They indicate that a chemiosmotic process may be operative at particular stages during the sequence of events associated with protein secretion in exocrine cells. Support for the model forwarded by Stanley and Ehrenstein (186) has been obtained from studies on mucin granules, which provide evidence for Ca\(^{2+}\)-activated K\(^+\) channels in the membrane of these granules (136). This study implies that Ca\(^{2+}/K^+\) exchange in the granule matrix may induce disaggregation and swelling of granules, possibly because of K\(^+\) preventing protein condensation and binding of aggregates to granule membranes (51). Further evidence compatible with the importance of granule K\(^+\) channels for secretion as proposed by Stanley and Ehrenstein (186) has been obtained by Hoy et al. (97) in pancreatic α-cells and by Jensen and collaborators (103) in renal juxtaglomerular cells. Data in pancreatic β-cells (22, 24) are more in line with a critical role of granule acidification and concomitant Cl\(^-\) fluxes for the process of exocytosis and insulin secretion, as originally implied by Pollard et al. (151).

This new insight into a critical role of granule ion channels and ion fluxes for exocytosis and release of macromolecular secretory products has been made possible because of the recent availability of very sensitive techniques that allow us to study these complex processes in more detail. There is also increasing functional evidence for the presence of intracellular ion channels (for review, see Refs. 196, 227), which belong to several families of cloned ion channel proteins and also include putative intracellular ion channels.

**EXPERIMENTAL TECHNIQUES TO STUDY EXOCYTOSIS AND RELEASE OF SECRETORY PRODUCTS**

A classic approach to study secretion involves the stimulation of a preparation of isolated cells or cell groups and measurements of the secreted proteins by radioactive metabolic labeling, ELISA, or enzymatic assays. This can also be done after permeabilization of the cells to gain access to the cytosol (79, 111, 226), thus providing some means of controlling intracellular processes and the cytosolic environment. However, these methods require large numbers of cells and offer little temporal resolution.

Three main approaches are currently used to study secretion from single cells: capacitance measurements that trace changes of the cell surface area due to membrane addition by exocytosis of secretory granules; electrochemical detection by oxidation or reduction of released transmitter molecules at the surface of a carbon-fiber electrode placed in close vicinity to the site of release; and optical techniques to monitor release, membrane addition, or pH changes.

**Capacitance Measurements of Cell Surface Area**

Granule fusion with the PM and membrane reuptake during exo-and endocytosis lead to an increase or decrease in the cell surface area and, correspondingly, in the cell’s membrane capacitance \(C_m\) (135). \(C_m\) is proportional to its surface area with a specific capacitance of 10 fF/μm² (135). The electrical continuity of granule and PM can thus be considered as an indicator of exocytosis. The average capacitance of pancreatic acinar and β-cells is ~7 pF. In β-cells, addition of a single granule adds ~2 fF, corresponding to a secretory granule diameter of 250 nm (8); in acinar cells, where a mean ZG diameter of ~700 nm has been determined (27), values vary between ~13 (172) and 20–40 (86) fF, suggesting the possibility of “compound exocytosis.” Patch-clamp measurements of exocytosis rely on these changes in \(C_m\), which can be measured in single cells on a time scale of milliseconds (93). In this method, a high-resistance seal is formed between a patch of the cell surface membrane and a glass-made recording electrode. Electrical contact with the cytosol is then established by rupturing the membrane within the patch. \(C_m\) can be measured by circuit analysis.
(123), and different techniques have recently been reviewed (84). For instance, a common method for measuring changes in \( C_m \) utilizes a sinusoidal current with a phase-sensitive detector or “lock-in amplifier” implemented either in hardware or software (85).

Any membrane that is added by exocytosis is detected as a capacitance increase; endocytosis on the other hand removes membrane from the cell surface and thus reduces the capacitance. This means that one problem associated with \( \Delta C_m \) measurements is that they only report net changes of membrane surface, which do not necessarily reflect reliable estimates of the rate of secretion. In \( \beta \)-cells, this will not influence the measurement of exocytosis because endocytosis proceeds on a relatively slow time scale (66). However, \( C_m \) measurements may not be sufficient to independently resolve these processes in cells in which a close temporal coupling of exocytotic and endocytotic events prevails. This concern is of particular importance in pancreatic acinar cells, which secrete at a slower rate and for longer time periods than do neuroendocrine cells. Recently, optical measurements of membrane turnover have been developed, which use membrane-sensitive fluorescent probes and can provide real-time measurements of secretory dynamics (29, 30). This technique, in combination with \( C_m \) measurements, allows for the independent evaluation of exocytotic and endocytotic activity and reports spatial information about these processes (182). The most widely used probe is FM1–43, a fluorescent amphiphatic seryl dye, which rapidly and reversibly partitions into the outer leaflet of biological membranes and becomes trapped in recycled vesicles on endocytosis. On stimulation, preloaded vesicles release dye into the bathing medium and fluorescence thereby declines (31). A study in rat pancreatic acinar cells selected this approach by monitoring changes of membrane surface area (\( C_m \)) in combination with measurements of the membrane turnover using FM1–43 (86). This study revealed that exocytosis and endocytosis arise within seconds after secretagogue-induced activation and coincide both temporally and spatially (86).

After incorporation of the granule membrane into the apical cell membrane, any granule conductance should contribute to the whole cell conductance (\( G_m \)). Studies have aimed at answering the question of whether fusion of individual granules with the PM inserts granule channels into the cell membrane. These studies combined capacitance with single-channel measurements in the whole cell configuration and attempted to correlate temporal changes in \( C_m \) and \( G_m \) induced by secretory stimuli. Studies carried out in rodent pancreatic acinar cells have yielded controversial results, either claiming a contribution of granular ion channels to increases in \( G_m \) after secretory stimuli (126) or not (172). However, the hypothesis tested by Schmid and Schulz (172) and Maruyama et al. (126) that granule exocytosis (\( \Delta C_m \)) and opening of putative granule ion channels (\( \Delta G_m \)) should temporally overlap is not valid. The studies by Zimmerberg et al. (234) and Breckenridge and Almers (40) demonstrated that granule swelling (and thus release of secretory products) lags significantly behind granule exocytosis. Similarly, significant delays between \( \Delta C_m \) and discharge of secreted macromolecules were recently reported in mast cells (225) and pancreatic \( \beta \)-cells (23). If the assumption that activation of granule ion channels promotes the release of secreted macromolecules is correct, then an increase of \( G_m \) (induced by opening of granule ion channels) will occur after a change in \( C_m \). This change of \( G_m \) may be difficult to resolve within the large increase of whole cell \( G_m \) (reflecting activities of \( Cl^- \) channels in the PM). One additional problem with the negative study (172) was also the choice of a nonphysiological stimulus [guanosine 5'-O-(3-thiotriphosphate); GTP-\( \gamma \)S], when a physiological Ca\textsuperscript{2+}-dependent agonist, such as acetylcholine (ACh) or cholecystokinin (CCK), would have been appropriate.

**Amperometric Detection of Released Molecules**

Electrochemical detection is based on the oxidation or reduction of released endogenous or preloaded molecules (46, 223). In the case of preloading of molecules, the tracer (an easily oxidized molecule such as serotonin) is taken up into the granules. It is subsequently cosecreted with stored macromolecules (e.g., insulin), which are not sufficiently “electroactive,” i.e., they are oxidized so slowly at the electrode that they are not detectable and therefore not useful to be used as tracer molecules (for more recent technical developments and for measurements of macromolecule release on a millisecond time scale, see, however, Refs. 98, 222). A positively charged carbon fiber electrode placed next to the cell will then detect exocytotic events as current spikes due to oxidation of the tracer molecule by the electrode. The oxidation current is a direct measure of release and is not susceptible to interference from endocytosis. The cell is not subject to whole cell dialysis and “washout” of diffusible cytoplasmic constituents. The amperometric method was previously applied to measurements of secretion in pancreatic \( \beta \)-cells (36, 183). However, the use of serotonin to assess insulin secretion was recently questioned (232). Moreover, this technique may reasonably reflect the time course of release of endogenous small molecules, but not necessarily the kinetics of insulin secretion (23). This method is also not generally applicable to secretory cells, because it requires an inside-to-outside pH gradient across the granule membrane and a granule uptake system for the tracer molecule, a vesicular monoamine transporter (VMAT) that exchanges two protons per substrate molecule (145). Amperometry has been applied to detection of endogenous catecholamine from single adrenal chromaffin cells (46, 121, 223), dopamine from single pheochromocytoma (PC12) cells (44), and indoleamine from single mast cells (7). It has even been successfully applied to measure secretion in cultured pancreatic epithelial duct cells (112), but it cannot be applied to exocrine acinar cells, whose granules may lack an acidic pH and/or VMAT.
Although both capacitance and electrochemical techniques allow measurements of single-vesicle fusion to be performed with a time resolution of milliseconds, these techniques suffer from two major drawbacks. Their spatial resolution is low (as with whole cell $C_m$ measurements or large-diameter carbon fiber electrodes with 5- to 8-$\mu$m tip diameter). Moreover, they do not provide information on the steps before membrane fusion or after endocytic uptake of membrane, because they only measure membrane addition or release, respectively.

**Evanescent Wave Microscopy**

The technique of evanescent wave or total internal reflection fluorescence microscopy (TIR-FM) offers a compromise between electron microscopy, which offers only “snapshots” of the exocytotic process, and $C_m$ and amperometric techniques in that good spatial and temporal resolution can be achieved.

TIR is based on Snell’s law of optics: If light traveling in a dense medium (e.g., a glass coverslip with a high refractive index, $n_2$) hits a less dense medium (e.g., an aqueous medium of lower refractive index, $n_1$), beyond a certain “critical angle,” $\theta_c$, the light will undergo TIR. This critical angle depends on the relative refractive indexes of the two media. If the $n_2$-to-$n_1$ ratio is very small, the critical angle is shallow ($\theta_c = 24.6^\circ$) and TIR is readily achieved.

In practice, cells are grown on glass coverslips or transparent materials of high refractive index, and a beam of light, usually from a laser, is optically coupled into the coverslip by a prism or the objective itself. If light approaches the aqueous medium at $>\theta_c$, it totally reflects into the glass. However, at angles $>\theta_c$, some of the energy slightly penetrates the aqueous medium as an “evanescent wave,” propagating parallel to the interface, which can be derived from Maxwell’s equations on the behavior of electromagnetic fields at a dielectric interface (18, 20). An important property of the evanescent wave is that the intensity falls off exponentially away from the coverslip. The “penetration depth” (the distance where the intensity $I$ has decreased to $I/e$, where $I$ is intensity at distance $o$ and $e$ is natural logarithm) depends on the incidence angle, wavelength, and polarization of light, as well as the refractive index of the coverslip and the medium. Penetration depths of <100 nm are achieved without difficulty. Thus only fluorophores near the coverslip are excited. TIR-FM illuminates vertical slices with the dimensions of a thin electron microscopy section (<100 nm) as opposed to slices of ~500–800 nm for one- and two-photon confocal systems, respectively. This thin optical sectioning means that the signal-to-noise ratio is much better than with confocal images and cellular photodamage and photobleaching are minimal.

TIR-FM is a complementary approach that can be combined with other microscopy techniques, such as brightfield, epifluorescence, confocal, or atomic force microscopy (AFM) (see Atomic Force Microscopy). TIR-FM applications in cell biology have been expanded to studies of secretion (94, 104, 119, 139, 165, 188, 189, 210) by the recent advent of green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* for more specific staining of secretory organelles and its cyan, yellow, and red derivatives (CFP, YFP, and RFP, respectively). When linked to granular proteins and expressed in cells, it retains its fluorescence properties and can therefore be used as a granule marker (105, 119, 153). Like acidotropic dyes, e.g., acridine orange or dyes of the LysoTracker LysoSensor families, it is released with the granular contents after membrane fusion such that secretion is observed as a decrease in fluorescence. Recently, pH-dependent GFP mutants have become available (131). Further improvement of TIR-FM has also been accomplished by the recent introduction of new objective lenses and condensers (19).

**Atomic Force Microscopy**

In AFM, a fine silicon or silicon nitride tip scans the surface of the sample. Any deflection of the tip due to surface topography is recorded. The AFM provides three-dimensional data of biological samples with a lateral or $x,y$-resolution in the range of 50–100 nm and with a height or $z$-resolution in the range of ~1 nm! One advantage of AFM as opposed to conventional electron microscopy is that it allows the study of the morphology of living cells or organelles under physiological conditions in real time (175). AFM has been used to study membrane dynamics, such as exo- or endocytosis on living cells, or the swelling behavior of isolated secretory granules. In rat pancreatic acinar cells, discrete areas of transient exocytosis have been detected when imaged by AFM (176). By probing the outer surface of acinar cells large, craterlike areas or “pits” (diameter 0.5–2 $\mu$m) were only found at the apical surface, where regulated secretion took place. Inside the pits, “depressions” of ~150-nm diameter were identified, which presumably represent the docking and fusion sites of ZG that are ready to fuse with the PM (corresponding to the “readily releasable pool” (RRP) of secretory vesicles in neuroendocrine cells). After the cells were stimulated, the diameter of the depressions increased within 5 min and returned to the initial size after a further 20 min. The increase and decrease of the diameter of the depressions correlated well with the amount and kinetics of amylase secretion (176). Hence, exocytotic secretion appeared to be very slow (with a time frame of minutes) compared with neuroendocrine cells. The reversible changes of the diameter of the depressions suggested that the ZG transiently fuse with the PM. They then release their content into the extracellular space via a fusion pore before the “empty” granules are retrieved intact from the PM (the kiss and run or “transient fusion” mechanism) (Refs. 5, 13, 41; see also Refs. 86, 172). This differs from a mechanism of endocytosis occurring by a separate process after complete incorporation of the secretory granule membrane into the PM (“full fusion”).
Jena et al. (102) asked how the ZG morphology associated with kiss and run recycling could be maintained during secretion. They argued that ZG diameter should decrease during amylase secretion and therefore, as a consequence of Laplace’s law, ZG surface tension should increase. An increase of ZG surface tension should further enhance enzyme release, which should therefore decrease ZG dimensions until total collapse of the granule. However, full fusion does not occur according to their observations (176). To clarify this issue, they used AMF in combination with confocal microscopy to study the three-dimensional dynamics of isolated ZG during stimulation (102). Exposure of ZG to GTP, but not GDP, increased their height by 15–25% as measured by AFM. A comparable increase in diameter was determined by confocal microscopy. Similar effects were observed with NaF. An active mastoparan analog (Mas7) known to stimulate G-proteins increased ZG GTPase activity and also induced swelling. Mas7, NaF, and GTP increased vesicle swelling to a similar extent, suggesting that ZG-associated heterotrimeric GTP-binding proteins (Gαq, based on immunoblots of ZG membrane fractions) participate in regulating ZG size. Moreover, the effects of Mas7, NaF, and GTP on ZG size occurred in the presence of KCl, but not when KCl was replaced by cyclamide, suggesting that swelling of ZG may be mediated by ion flux through K+ and Cl− channels in the granule membrane (102). Interestingly, 100 μM Ca2+ or 200 μM EGTA had no effect on ZG size [see Expression of a Ca2+-activated and bicarbonate-permeable anion channel (CLCA)]. On the basis of these AFM studies in pancreatic acinar cells and isolated ZG, Jena and coworkers (102) proposed that K+ and Cl− channels in the granule membrane are required to induce granule swelling during secretion to prevent ZG collapse. Ion fluxes through K+ and Cl− channels in the granule membrane and osmotic swelling thus appear to maintain granule integrity and morphology as a prerequisite for kiss and run recycling (102, 175, 176).

**CLONED MAMMALIAN INTRACELLULAR ION CHANNELS**

**CIC Cl− Channels**

The gene family of CIC Cl− channels is highly conserved in evolution and includes at least nine different members in mammals (218). In the latter, CIC proteins show differential distribution as well as different functions, including transepithelial salt transport, electrical excitability, cell volume regulation, and charge compensation necessary for the acidification of intracellular organelles (218). On the basis of homology, these channels have been grouped into three different classes. Channels of the first class (CIC-0,1,2, Ka, Kb) are thought to be predominantly PM Cl− channels, whereas members of the two other classes (CIC-3,4,5 and CIC-6,7) mainly perform their function in intracellular membranes. However, as shown below, experimental evidence with tissue from control and CIC knockout animals indicates that this distinction may be an oversimplification. In addition, other factors, including regulatory subunits, may also determine whether these ion channels are preferentially targeted to the PM or to the membrane of intracellular organelles.

**CIC-2.** A whole cell patch-clamp study on Cl− channel activity in pig pancreatic acinar cells provided the first evidence that a CIC channel might function as an intracellular Cl− channel (42). The Cl− current was activated by strong hyperpolarization and cell swelling, which are biophysical properties reminiscent of CIC-2. This anion current also shared the Cl− > I− selectivity sequence with other CIC proteins. Immunohistochemical localization with a CIC-2 antibody revealed expression in both apical PM and ZG, suggesting that it may operate as a Cl− efflux pathway in pancreatic acinar cells and remain functional in the PM after exocytosis, thus contributing to primary salt secretion. Subsequently, expression of CIC-2 in both rat pancreatic plasma and ZG membranes has been confirmed by immunoblotting (unpublished observations). Functional studies in isolated ZG have recently identified a largely Ca2+-independent Cl− conductance in isolated ZG that could represent CIC-2 and may contribute to granule swelling [see Expression of a Ca2+-activated and bicarbonate-permeable anion channel (CLCA)].

However, it is puzzling that a recent study on murine CIC-2 channel disruption did not describe any expected phenotype from its postulated ubiquitous physiological role as a channel involved in volume regulation, which is activated by cell swelling (I_{Cl-swell}) (37). The study with CIC-2-deficient mice did, however, uncover a critical role of CIC-2 in the control of the ionic environment of male germ cells and retinal photoreceptors (37).

**CIC-3.** Several studies have suggested that CIC-3 may be the major candidate for the volume-regulated Cl− channel, I_{Cl-swell} (60, 61), but this has not remained uncontested (122). In contrast, a recent study provides compelling evidence that this channel plays a critical role in acidifying synaptic vesicles. Stobrawa et al. (190) reported a phenotype associated with targeted inactivation of the murine clcn3 gene. The mice exhibited postnatal growth retardation, blindness secondary to progressive retinal degeneration, and behavioral abnormalities associated with hippocampal degeneration. Further investigations revealed that CIC-3 is present on synaptic vesicles and participates in their acidification. This occurs through active proton pumping by an electrogenic V-type H+ pump. In the absence of a conductive pathway, the generation of an inside-positive potential difference across the vesicle membrane will limit the degree of acidification that can be achieved by the pump. The dissipation of this voltage by an electrical shunt allows for higher rates of proton transport. Indeed, biophysical studies have revealed Cl− conductances in many intracellular organelles, including the endoplasmic reticulum (ER), Golgi, vesicles of the exocytotic and endocytotic pathways, lysosomes, and synaptic vesicles (3, 196, 227). Their acidification serves various purposes, such as modulation of enzy-
motic activities, processing of prohormones, differential sorting of receptors and ligands, endocytosis, and other vesicle trafficking. The electrochemical proton gradient also provides the driving force for the transport of other substances across the vesicle membrane as in the concentrative uptake of neurotransmitters into synaptic vesicles, e.g., of glutamate (127).

Synaptic vesicles purified from CIC-3-null mice exhibited lower rates of in vitro ATP-dependent acidification and diminished glutamate uptake (190). The phenotype of the knockout mice and the observation that the rates of vesicular acidification were only partially reduced indicate that CIC-3 is not the only Cl\(^{-}\) channel of synaptic vesicles or that different vesicle populations are associated with distinct shunt pathways. Although these experiments provide strong evidence for a biological role of CIC-3 in synaptic function, it should be noted that CIC-3 is not exclusively found in synaptic vesicles. It also colocalizes with markers for late endo-/lysosomal compartments in neuronal tissues (190) and is also expressed in secretory granules of islet \(\beta\)-cells (Ref. 22; see Expression of a CIC-3 Cl\(^{-}\) channel) but not in ZG (unpublished observation).

**CIC-5.** The Cl\(^{-}\) channel CIC-5 shares the Cl\(^{-}\) > I\(^{-}\) selectivity with other CIC channels. Mutations encountered in Dent’s disease, a rare familial disorder, reduce or abolish Cl\(^{-}\) currents associated with the functional CIC-5 cDNA in the *Xenopus laevis* oocyte expression system. This disease is associated with low-molecular-weight proteinuria and hypercalciuria; thus CIC-5 may mediate renal protein endocytosis. Indeed, CIC-5 knockout mice exhibiting a targeted disruption of the murine *clcn5* gene showed impaired proximal tubule protein absorption and reduced receptor-mediated and fluid-phase endocytosis in proximal tubular cells (150, 220). After budding from the PM, endocytic vesicles are progressively acidified on their way to the lysosomes, and CIC-5 may provide a rate-limiting anion conductance for efficient endosomal acidification in the proximal tubule (150, 220). However, CIC-5 expression in *X. laevis* oocytes or mammalian cells elicits strongly outwardly rectifying Cl\(^{-}\) currents that can be detected only in a voltage range more positive than +20 mV (75). However, such an inside-positive membrane potential is highly unlikely in intracellular compartments. Moreover, CIC-5 expressed in *X. laevis* oocytes or mammalian cells is inhibited by extracytosolic acidic pH (174). These properties cast doubt on CIC-5 being solely responsible for Cl\(^{-}\) conductance in renal endosomes because Cl\(^{-}\) movement into the acidifying compartment would be greatly limited. This suggests that endosomal anion conductance requires additional transport pathways and/or that CIC-5 displays different properties in situ because of the presence of regulatory subunits.

CIC-5 is also expressed in intracellular vesicles and colocalized with the V-type H\(^{+}\) pump in acid-secreting \(\alpha\)-intercalated cells of the renal cortical collecting duct (59, 92). In these cells, the H\(^{+}\) pump is mainly found in intracellular vesicles, which are inserted into or retrieved from the PM by exo- or endocytosis to adjust and maintain acid-base homeostasis. Thus CIC-5 may also mediate concomitant Cl\(^{-}\) fluxes during renal acid secretion, although the strong outward rectification of the channel may limit ion movements. In essence, these data suggest that CIC-5 is associated with vesicle trafficking along the endo- and exocytotic pathways in the kidney and possibly other epithelial tissues.

**CIC-7.** Osteoclastic bone resorption requires extracellular proton accumulation (198). Osteoclasts are multinucleated cells formed by the fusion of mononuclear hematopoietic stem cells belonging to the phagocyte series. When attached to bone, osteoclasts create lacunae, into which protons and acid hydrolases are translocated to digest mineralized bone matrix. Acid secretion is mediated by the fusion of internal vesicles containing V-type H\(^{+}\) pumps into the osteoclast surface membranes adjacent to the bone surface, creating the “ruffled border” where bone resorption occurs. The extracellular acidic lacunae require fluxes of counterions, e.g., of Cl\(^{-}\) through Cl\(^{-}\) channels for charge compensation. A recent report by Kornak et al. (113) defined the role of the CIC-7 channel protein in osteoclast-mediated bone resorption by studying *clcn7*-deficient mice. Inactivation of CIC-7 caused a severe phenotype with osteopetrosis (bone petrification) and retinal degeneration. The defects of skeletal morphogenesis could all be explained by impaired osteoclastic bone resorption. Osteoclasts in CIC-7-deficient mice exhibited poorly developed ruffled borders and did not form resorption lacunae. Immunohistochemical staining for CIC-7 in normal osteoclasts demonstrated intracellular staining of late endo-/lysosomal compartments as well as localization along ruffled border membranes similar to the distribution of the V-type H\(^{+}\)-ATPase, which is consistent with a distribution of CIC-7 in subplasmalemmal secretory vesicles and in the PM. Further functional studies demonstrated a defect in extracellular acidification by osteoclasts at the PM, but no deficient late endo-/lysosomal or lysosomal acidification was observed (113). This indicates that CIC-7 is not solely responsible for the acidification of late endosomes and may also involve other anion channels, such as p62 (171) (see **CLIC/p64 Cl\(^{-}\) Channels**). Interestingly, a recent in situ hybridization study showed that CIC-6 and CIC-7 are strongly expressed in mouse pancreatic acinar cells but not in islets (107), suggesting that these channel proteins may also operate as intracellular anion channels in acinar cells, but their intracellular localization and physiological function is currently unknown.

**CLIC/p64 Cl\(^{-}\) Channels**

The bovine Cl\(^{-}\) channel protein p64 was originally isolated from kidney cortical vesicles and tracheal apical membranes. Biochemical and electrophysiological studies led to its assignment as a Cl\(^{-}\) channel protein (65, 116, 117, 161). Immunocytochemical studies showed that p64 is localized in PM and intracellular membrane vesicles (161). However, expression of bovine p64 in *X. laevis* oocytes resulted in the incorpora-
The hypothesis has been tested directly for CLIC1, which they could perform both functions. The endogenous ion channels (either directly or indirectly), or be channel-forming proteins, they could activate in general alternative ways in which CLIC proteins could be absent from mock-transfected cells (62). There are several intracellular anion channel activity that is transported. Cells overexpressing recombinant CLIC4 contain intracellular anion channel activity that is potential generated by proton ATPases (116, 161). Confirmation of certain intracellular organelles by the acidic residues led to the hypothesis that p64 facilitates partitioning of p64 into microsomes but not into PM. These observations led to the hypothesis that p64 facilitates acidification of certain intracellular organelles by providing an electrical shunt to dissipate the electrical potential generated by proton ATPases (116, 161). Consistent with this view, the ruffled border of the osteoclast PM expresses a closely related Cl– channel, p62, which could play a role similar to that of CIC-7 in bone resorption by regulating acid transport (171).

Recently, several related human genes that share homology with the COOH-terminal half of bovine p64 have been cloned. These genes constitute a protein family called CLIC (Cl– intracellular channel). The CLIC/p64 superfamily has grown to include p64 itself, the p64-like protein paracomin (138) and five CLIC proteins. The first CLIC member to be identified, CLIC1/NCC27, was originally detected in cell nuclei (214) and was subsequently shown to be enriched in the brush border of proximal tubule cells (211). So far there is no evidence that CLIC2, CLIC3, and CLIC5 are expressed in secretory vesicles (28, 95, 154). CLIC4/huH1 was recently identified (64) as the human homolog of a rat brain protein termed p64H1 (47, 62). CLIC4 mRNA is widely expressed in neuronal and nonneuronal tissues. Although rat brain p64H1, which encodes for a 28.6-kDa protein, colocalized with markers for the ER of transiently transfected rat hippocampal HT-4 cells (62), in another study it was localized to the large dense-core vesicles of rat hippocampal neurons (47).

Indirect immunofluorescence, cell fractionation, and immunoblotting studies have localized native and recombinant CLIC4 proteins both to the cytosol and to intracellular membranes. A similar, very unusual, dual localization is characteristic of many CLIC and p64-related proteins (47, 64, 70, 138, 154, 160, 214). For example, human CLIC4 is enriched in the apical region of proximal tubule cells but partly colocalizes with caveolae in a pancreatic cell line (64). In addition, the mouse homolog of CLIC4, called mc3s5/mtCLIC, was recently localized to the mitochondria and cytoplasm of keratinocytes (70). Such a protein distribution could reflect a shuttling of CLIC4 between the cytoplasm and different endomembrane systems (including secretory vesicles, mitochondrial membranes, and caveolae) and an involvement of the protein in widespread cell biological processes such as membrane trafficking or vesicle transport.

Various electrophysiological studies have shown that several members of this gene family, including bovine p64 (65, 116, 117), CLIC1 (214), CLIC3 (154), and rat brain p64H1/CLIC4 (62), play a role in Cl– transport. Cells overexpressing recombinant CLIC4 contain intracellular anion channel activity that is absent from mock-transfected cells (62). There are several alternative ways in which CLIC proteins could be linked to intracellular ion channel activity. They could be channel-forming proteins, they could activate endogenous ion channels (either directly or indirectly), or they could perform both functions. The “ion channel” hypothesis has been tested directly for CLIC1, which forms channels after being incorporated, as a pure recombinant protein, into planar bilayers (212). Although it has yet to be shown that this channel activity in vitro corresponds to an endogenous ion channel, it is clearly possible that CLIC1 and other CLIC proteins might have a direct role as intracellular ion channels. An alternative hypothesis is that some or all of the CLIC proteins are anion channel regulators, rather than (or as well as) ion channels. Several CLIC proteins are also expressed in endomembranes, which are not associated with acidic compartments (62, 64, 211, 214). This suggests that the role of CLIC proteins may not be restricted to its operation as a shunt pathway for electrogenic V-ATPases. However, as long as knockout animals for the various CLIC proteins are not available, the exact function of these proteins remains difficult to establish.

**CFTR Cl– Channel**

The cloning of the gene encoding CFTR (ABCC7 according to the novel nomenclature) in 1989 (163) allowed the study of the molecular pathophysiology of CF, and information on the genetics, tissue distribution, and function of CFTR has been acquired subsequently. Studies of CFTR expression in a variety of cell types, including mammalian cells (9, 10), and functional reconstitution of CFTR protein in planar lipid bilayers (26) have demonstrated that CFTR can function as a cAMP-regulated Cl– selective anion channel, which is localized at the apical membrane of Cl– secretory epithelial cells. However, an incomplete understanding of the molecular mechanisms by which alterations in an apical membrane Cl– conductance could give rise to the various clinical manifestations of CF has prompted the suggestion that CFTR may also play a role in the normal function of intracellular compartments.

Studies using isolated tissue from control and CF submandibular salivary glands (129) showed that β-adrenergic agonists were able to stimulate the release of mucin and amylase from control tissue, but the secretory responses of CF tissues were reduced by ~60%. No differences were found between the total mucin pools of control and CF tissues. Similar observations were made in CFTR knockout mice (132). In these studies, release of [14C]glucosamine-labeled mucins from mouse submandibular glands was monitored in response to activation of the cAMP-mediated second messenger cascade. Although a large, sustained release of mucin was observed in control mouse tissue, the stimulated release of mucin from CFTR knockout mouse tissue was significantly reduced (132).

The regulation of mucin secretion by CFTR has also been assessed in airway epithelial cells. Merger et al. (130) studied the release of mucin after incorporation of [14C]glucosamine into mucins. Stimulation of immortalized normal human airway epithelial cells, with either isoproterenol or forskolin, led to a 40% increase in mucin release. In contrast, stimulation of immortalized CF airway cells resulted only in a 1–3% increase.
in mucin release. The defective cAMP-mediated regulation of mucin secretion was restored after adenovirus-mediated gene transfer of wild-type CFTR to these cells. Granule exocytosis and mucin release in airway epithelial cells were also monitored by release of previously internalized fluorescein isothiocyanate (FITC)-dextran, a fluid-phase endocytosis marker, and by increases in \( C_m \) measured with whole cell patch clamp (179). Treatment of non-CF airway cells with a membrane-permeant cAMP analog resulted in a significant increase in \( C_m \), consistent with net incorporation of membrane into the cell surface. Simultaneous release of FITC-dextran in response to the cAMP analog suggested that the cAMP-dependent increase in \( C_m \) is partly caused by fusion and incorporation of exocytotic vesicles into the PM. Under identical experimental conditions, stimulation with the cAMP analog had no effect on either \( C_m \) or discharge of internalized marker in airway cells derived from a CF patient (179).

Mucin secretion has also been studied in gallbladder epithelium, a model system for mucus secretion by columnar epithelial cells (115). Mucin granules released mucus by merocrine secretion in mouse gallbladder epithelium when examined by transmission electron microscopy. Immunofluorescence microscopic studies revealed intracellular colocalization of mucins and CFTR (115). The data in submandibular, airway, and gallbladder epithelia strongly suggest that CFTR Cl\(^{-}\) channels are present in mucin granule membranes and support a model according to which CFTR-mediated influx of Cl\(^{-}\) into the granule enhances secretion by modulating fusion, exocytosis, and/or release of mucus.

**KCNQ1 (K\(_{\text{LQT1}}\) K\(^{+}\) Channel**

\( K_{\text{LQT1}} \) (KCNQ1) is a very low-conductance (<1.5 pS) voltage-gated K\(^{+}\) channel distributed widely in epithelial and nonepithelial tissues (for review, see Refs. 32, 164). KCNQ1 was found to be mutated in the hereditary cardiac disease long QT syndrome 1 (219). In the heart and inner ear, KCNQ1 coassembles with a \( \beta \)-subunit KCNE1 (IsK, minK) (197) to form the \( I_{\text{Ks}} \) K\(^{+}\) current (25, 169). Besides its expression in the heart and inner ear, KCNQ1 is also abundant in small and large intestine, pancreas, and stomach (for review, see Ref. 32). Its specific biophysical and pharmacological properties are determined by the regulatory \( \beta \)-subunits IsK (KCNE1), MiRP1 (KCNE2), and MiRP2 (KCNE3), which are expressed in a tissue-specific manner to form the native K\(^{+}\) channel (1, 25, 169, 177, 197). In the heart and inner ear KCNQ1 interacts with KCNE1 to conduct the slowly activating low-conductance K\(^{+}\) channel current \( I_{\text{Ks}} \) (25, 169). Both KCNQ1 and KCNE1 are expressed in rodent pancreatic acinar cells (58, 114, 197). In the colon KCNQ1 coassembles with KCNE3 to conduct a constitutively active K\(^{+}\) current (32, 221). KCNQ1 may be associated with other yet unknown regulatory subunits, which could account for activation of the K\(^{+}\) current by cAMP or Ca\(^{2+}\) (32). \( I_{\text{Ks}} \) are selectively blocked by the chromanol 293B, which binds to KCNQ1 (191, 228). Interactions with as yet unknown regulatory subunits may determine the properties of KCNQ1 in other epithelial tissues, in which KCNQ1-mediated K\(^{+}\) current is not inhibited by chromanols. In Cl\(^{-}\) secretory epithelia, such as the colon and pancreas, this Ca\(^{2+}\)-activated K\(^{+}\) current provides the driving force for Cl\(^{-}\) exit and is located in the basolateral membrane (109, 114). Recently, KCNQ1 has been identified as a K\(^{+}\) channel located in intracellular tubulovesicles and apical membrane of parietal cells, where it colocalizes with the H\(^{+}\)-K\(^{+}\)-ATPase (87). Inhibition of KCNQ1 current by the chro- manol 293B abolished acid secretion. The \( \beta \)-subunits KCNE2 and KCNE3 were expressed in stomach; KCNE1, however, was not. This suggested that KCNQ1 is the pore-forming subunit of the K\(^{+}\) channel responsible for sustained HCl secretion (87). Thus in parietal cells KCNQ1 also appears to have a dual subcellular distribution by trafficking between intracellular tubulovesicles and the apical PM.

**SECRETORY GRANULE ION CHANNELS AND REGULATED SECRETION**

**Pancreatic Zymogen Granules**

**Background.** Secretion by the exocrine pancreas is carried out by two morphologically and functionally distinct epithelia, the acini and ducts. Acinar cells secrete a plasmalike primary fluid together with digestive enzymes, which are stored as proenzymes in ZG at the apex of the cells. The primary fluid is modified by the downstream duct cells, which generate the HCO\(_3\)-rich pancreatic juice.

In acinar cells, secretion of the near neutral primary fluid by acinar cells occurs by Na\(^{+}\)-coupled secondary active Cl\(^{-}\) transport (180). The consensus model for exocrine acinar fluid secretion is based on uptake of Cl\(^{-}\) through the basolateral membrane. The cellular Na\(^{+}\) gradient generated by the basolateral Na\(^{+}\)-K\(^{+}\)-ATPase energizes a basolateral Na\(^{+}\)/H\(^{+}\) exchanger. This process raises the cytosolic pH (pHi) and promotes formation of HCO\(_3\) by hydration of CO\(_2\), catalyzed by carbonic anhydrase. The HCO\(_3\) so formed exits to the interstitial space via a Cl\(^{-}\)/HCO\(_3\) exchanger in the basolateral membrane, which is activated by alkaline pH\(_{i}\). In the rat pancreas, a recently identified electrogenic Na\(^{+}\)-HCO\(_3\) cotransporter (NBC) in the basolat- eral membrane may additionally fuel the Cl\(^{-}\)/HCO\(_3\) exchanger (134, 206). Alternatively, in salivary glands a Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (NKCC1) may be involved in Cl\(^{-}\) uptake into acinar cells (69). By these means, Cl\(^{-}\) accumulates into the cell above its electrochemical equilibrium concentration.

Pancreatic acinar cells are able to switch the primary fluid formation process on and off. This is accomplished by cytosolic Ca\(^{2+}\) signals elicited by neurotransmitter (e.g., ACh) or hormone (e.g., CCK) interaction with receptor sites on the outside of the acinar cell membrane (224). Ca\(^{2+}\) is released from inositol trisphosphate (IP\(_3\))-sensitive intracellular stores in the ER and/or the secretory granules (148,
The evidence that IP₃ receptors may be expressed on granules comes from studies on ZG, which release Ca²⁺ in the presence of IP₃ or cADP ribose (83). This is supported by studies showing that IP₃ releases Ca²⁺ from chromaffin (229, 230), mucin (136), mast (155) and possibly islet (33) cell granules. Quite importantly in the context of this review, IP₃-induced Ca²⁺ release in mucin granules and the ER is made possible by functional linkage with a Ca²⁺-activated K⁺ channel, resulting in a highly cooperative Ca²⁺/K⁺ exchange process (136). This model was later confirmed in secretory granules of mast cells (155). In essence, the presence of IP₃-sensitive Ca²⁺ channels on ZG of pancreatic acinar cells and their involvement as an IP₃-sensitive, acidic Ca²⁺ pool in Ca²⁺ signaling is likely (Refs. 83, 208; see, however, Ref. 231).

The cytosolic Ca²⁺ signals activate two classes of ion channels, permeable to K⁺ or Cl⁻, respectively (147). On secretagogue stimulation, Cl⁻ exits into the acinar lumen via a Cl⁻-activated Cl⁻ channels in the luminal membrane (144, 233), whose molecular identity is still unknown. Ca²⁺-activated K⁺ channels (possibly involving KCNQ1) are present in the basolateral membrane of rat pancreatic acinar cells (109, 114), which allow K⁺ to leave the cell at the basolateral cell side and provide the electrical driving force for continuous Cl⁻ secretion. The lumen negativity induced by the flux of Cl⁻ attracts Na⁺ through paracellular pathways, and water follows osmotically.

An increase of cytosolic [Ca²⁺] evoked by secretagogues also affects Ca²⁺-sensitive docking and fusion proteins (101), which interact at different stages of the exocytotic process to promote ZG fusion with the PM and the release of the granule contents into the lumen. However, in conjunction with exocytosis, components of the granule membrane also get inserted into the apical PM. Thus this process may also involve insertion of Ca²⁺-regulated ion channels expressed in the membrane of ZG into the apical PM, which could also contribute to Ca²⁺-dependent secretion of primary fluid by acinar cells. Experiments carried out on digitonin-permeabilized rat pancreatic acini (79) showed that enzyme secretion evoked by the Ca²⁺-dependent secretagogues ACh and CCK was critically dependent on the presence of Cl⁻ and K⁺ in the cytosol. Furthermore, secretion could be abolished by application of Cl⁻ and K⁺ channel blockers (79). These results led to the hypothesis that granules actively participate in secretion of primary fluid and enzymes and that some of the physiological targets in the cascade of Ca²⁺-dependent events leading to secretion of primary fluid and enzymes are anion and cation channels in the ZG membrane (79, 82).

To test this hypothesis, a rapid technique for isolation of pure and stable secretory granules from exocrine pancreas and parotid gland was developed (55). Ion transport pathways in the granular membrane were subsequently identified with a quantitative in vitro assay involving rapid osmotic swelling and end point measurements of granular osmotic lysis to measure macroscopic ion fluxes (54, 200, 202). De Lisle and Hopfer (54) described a Cl⁻ conductance, which appeared to be regulated by hormone and second messengers. In ZG isolated from the pancreas of rats pretreated with secretin or CCK, a high Cl⁻ permeability was measured, whereas ZG of rats pretreated with an adrenergic or muscarinic antagonist possessed a low permeability to Cl⁻ (82). Similar results were obtained by Fuller et al. (78), who stimulated suspensions of pancreatic acinar cells by CCK, carbachol, or cAMP plus phorbol ester and found a high Cl⁻ permeability in ZG isolated from stimulated acini. They suggested that this effect was mediated by phosphorylation of transporters or transporter-associated proteins via cAMP-dependent protein kinase (PKA) and Ca²⁺-phospholipid-dependent protein kinase (PKC)-induced protein phosphorylation. These observations were consistent with the hypothesis that activation of regulated ion conductive pathways in ZG membranes plays a significant role in modulating secretion triggered by secretagogues (79, 82). Subsequently, cation conductive pathways have been also identified in ZG isolated from rat pancreatic tissue (200, 202).

As a first step toward the molecular identification of the ion channel proteins associated with these ZG conductive pathways, a pharmacological “footprinting” was carried out. Inhibitors of K⁺ and Cl⁻ channels, such as Ba²⁺ or 4,4′-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), inhibited K⁺ and Cl⁻ conductive pathways, respectively (54, 202). Compounds with different physicochemical properties, such as glyburide (glibenclamide; Glib), quinidine, or ATP and nonhydrolyzable ATP analogs blocked K⁺ conductance and increased Cl⁻ conductance (200–203). Furthermore, evidence was obtained that the effect of these compounds results from their binding to a 65-kDa multidrug resistance P-glycoprotein (ABC1B; mdr1) gene product, which regulates Cl⁻ and K⁺ conductances (Refs. 38, 202, 204, 207; Fig. 1).

The first evidence for the expression of a cloned Cl⁻ channel in ZG was provided by Carew and Thorn (42). They recorded ClC-2-like Cl⁻ currents in pig pancreatic acinar cells by the whole cell patch-clamp technique. Immunohistochemical localization with a ClC-2 antibody revealed expression in both apical PM and ZG. This suggested that the channel remains functional in the PM after exocytosis and that it may function as a Cl⁻ efflux pathway in pancreatic acinar cells. Subsequently, the expression of ClC-2 in both rat pancreatic PM and ZG membranes has been confirmed by immunoblotting (unpublished observations).

Expression of a Ca²⁺-activated and HCO₃⁻-permeable anion channel (CLCA). A member of the CLCA channel family could be a potential candidate for a ZG anion conductive pathway, which is directly activated by micromolar concentrations of Ca²⁺. CLCA proteins currently include at least 10 isoforms from different species (76, 77). These putative anion channels are gated by Ca²⁺ and mostly expressed in the PM of epithelial tissues. When heterologously expressed in HEK 293 cells, all isoforms investigated (hCLCA1, hCLCA2, mCLCA1) are associated with the appear-
Fig. 1. Several candidate ion channel proteins have been identified, which are expressed in the membrane of pancreatic acinar zymogen granules (ZG). A Ca$^{2+}$-activated anion channel (CLCA) is permeated by HCO$_3^-$ and blocked by dithiothreitol (DTT) and H$_2$-DIDS. Activation of this channel may promote enzyme release by increasing the dissolution of the condensed granule contents. Luminal alkalinization may enhance exocytotic membrane retrieval. A DIDS-sensitive ClC-2 Cl$^{-}$ channel may promote enzyme release by increasing the dissolution of the condensed granule contents. A nonselective monovalent cation conductance, whose molecular identity is unknown, is blocked by DIDS or the reducing agent dithiothreitol (DTT) (50). So far there had been no evidence to suggest an intracellular localization of CLCA channels, but using in situ hybridization techniques, Gruber et al. (90) demonstrated the expression of CLCA mRNA in mouse pancreatic acini.

With primers derived from the sequence of mouse CLCA1, a RT-PCR product was generated with rat pancreatic mRNA, which exhibited 81%, 77%, and 57% amino acid similarity to the three mouse isoforms mCLCA1, -2 and -3 (mgob-5), respectively (201). Immunofluorescence light microscopy with an antibody against a 38-kDa subunit of bovine CLCA1 (50) showed labeling of acinar cells in the granular area, which is consistent with the data obtained by Gruber et al. (90) in the mouse pancreas by in situ hybridization. This observation was confirmed by preembedding electron microscopy immunogold labeling of ZG membranes. The pattern of immunoreactive bands obtained in ZG membranes by SDS-PAGE and immunoblotting suggests that rat pancreatic CLCA undergoes posttranslational modification to form a heterodimer consisting of a 90-kDa and a 30- to 40-kDa subunit, as proposed for other members of this CLCA protein family (89, 91). Functional studies in isolated rat pancreatic ZG showed a complex pattern of modulation by Ca$^{2+}$. The [Ca$^{2+}$] required for activation of the anion conductive pathway with I$^-$/HCO$_3^-$ as permeant anions was in the range of 2.5–50 μM, whereas [Ca$^{2+}$] > 100 μM was inhibitory. Previous studies using bovine CLCA1 reconstituted into planar lipid bilayers showed that this channel protein is sensitive to phosphorylation by Ca$^{2+}$/calmodulin protein kinase II (CaMK II). CaMK II-dependent phosphorylation shifts the Ca$^{2+}$ sensitivity of the channel to submicromolar [Ca$^{2+}$] and is associated with a decrease of channel opening at higher [Ca$^{2+}$] (80). The experiments in ZG suggest a similar biphasic effect of Ca$^{2+}$ for the conductive pathway permeated by HCO$_3^-$ or I$^-$. The effect of CaMK II has not yet been tested. Inhibitors of the CLCA channels, H$_2$-DIDS and DTT, inhibited Ca$^{2+}$-activated I$^-$/HCO$_3^-$ conductances, whereas in the absence of Ca$^{2+}$ both compounds were without effect (201). In contrast, the [Ca$^{2+}$] required for activation of the ZG anion conductance with Cl$^-$ as major anion exceeded 100 μM.

These results indicate that two different anion conductive pathways are present in rat pancreatic ZG, which differ with regard to both Ca$^{2+}$ activation and
anion selectivity (see Fig. 1). They further suggest that the mechanism responsible for Ca\(^{2+}\)-dependent activation of ZG Cl\(^{-}\) conductance is different from that for the conductive pathway permeated by HCO\(_3\)\(^{-}\) (or \(\mathrm{OH}^{-}\)). A CLCA protein could account for the Ca\(^{2+}\)-activated HCO\(_3\) conductance of pancreatic ZG membranes and contribute to secretagogue-stimulated secretion of salt and digestive enzymes by pancreatic acini. It is interesting to note that the physiological anion that permeates the Ca\(^{2+}\)-sensitive anion conductance is not Cl\(^{-}\), but HCO\(_3\)\(^{-}\). HCO\(_3\)\(^{-}\) is a relevant anion in the process of disaggregation and decondensation of the stored digestive enzymes that could promote the release ofzymogens during the exocytotic process (45, 157). Moreover, alkalization of the pancreatic acinar lumen enhances retrieval of exocytic membranes by cleavage of glycosyl phosphatidylinositol (GPI)-anchored proteins (73, 74). This leads to speculations suggesting that changes in luminal HCO\(_3\)\(^{-}\) concentration mediated by activation of a ZG HCO\(_3\)\(^{-}\)-permeable and Ca\(^{2+}\)-sensitive anion channel could affect acinar membrane dynamics by enhancing apical endocytosis. The second anion conductance is a poorly Ca\(^{2+}\)-sensitive Cl\(^{-}\)-selective pathway, which is activated by secretagogues, ATP and nonhydrolyzable ATP analogs, quinidine, and Glib and inhibited by DIDS (54, 78, 200, 203). It may be associated with the CIC-2 channel protein and could play a role in granule swelling (42).

**Expression of chromanol-sensitive K\(^{+}\) channel KCNQ1.** Two monovalent cation conductive pathways contribute to K\(^{+}\) fluxes into ZG. A nonselective monovalent cation conductive pathway is equally permeant to K\(^{+}\) and Na\(^{+}\) and blocked by flufenamic acid (200). The K\(^{+}\)-selective conductive pathway is blocked by ATP and Glib (38, 200, 202) and thus appears to be similar to ATP-sensitive K\(^{+}\) (K\(\text{ATP}^\text{−}\)) channels (2, 100, 209). However, Kir6.2, the pore-forming subunit of K\(\text{ATP}^\text{−}\) channels, is not expressed in rat pancreatic acini (194). KCNQ1 (K\(\text{LQT}^\text{I}\)) may be a candidate for the ZG K\(^{+}\) channel. In situ hybridization studies have shown that KCNQ1 and KCNE1 are expressed in rodent pancreatic acini (58, 114, 197). Moreover, KCNQ1, which is the apical K\(^{+}\) channel required for active K\(^{+}\)/H\(^{+}\) exchange and stimulated HCl secretion by gastric parietal cells, has also been detected in intracellular organelles of parietal cells, the tubulovesicles (87). In epithelial tissues, KCNQ1 K\(^{+}\) channels are activated by cAMP or Ca\(^{2+}\) and selectively inhibited by the chromanol 293B (32, 191, 228).

In rat pancreatic acini, 293B (10 \(\mu\) M) completely inhibited K\(^{+}\) conductance, whereas the nonselective and Cl\(^{-}\) conductances were not affected by up to 100 \(\mu\) M of the drug (205). Although 293B is not absolutely specific for KCNQ1, higher concentrations of the drugs are required to inhibit other ion channels (120, 191, 221, 228). 293B also blocked CCK-octapeptide-stimulated amylase secretion from permeabilized rat pancreatic acini in a concentration-dependent manner (EC\(_{50}\text{≈}10\ \mu\text{M}\)), whereas basal amylase release was not changed. Inhibition of enzyme secretion, however, was only observed when 293B was combined with the inhibitor of the nonselective cation conductance, flufenamic acid. Both drugs individually applied had no effect (205). This suggests that both nonselective and K\(^{+}\)-selective conductance pathways can mediate secretagogue-induced enzyme secretion. With a specific antibody against the cytosolic NH\(_2\)-terminus of KCNQ1 (87), a protein of \(\sim80\) kDa was detected in ZG membranes and PM of rat pancreas, which is consistent with its predicted \(M_\text{r}\) based on a protein of 669 amino acids (32).

**Regulatory proteins of ZG ion conductive pathways.** The regulation of ZG K\(^{+}\) and Cl\(^{-}\) conductance pathways by ATP and Glib is reminiscent of members of the ATP-binding cassette (ABC) superfamily of transporters, which are either ion channels or channel regulators (4, 178). Specific antibodies (JSB1, C219) against cytosolic domains of the ABC transporter multidrug resistance P-glycoprotein (MDR1/PGY1/ABCB1) blocked ZG Cl\(^{-}\) conductance and bound to a 65-kDa protein of ZG membranes (200, 207). Subsequently, immunoblots were performed and K\(^{+}\) and Cl\(^{-}\) conductive pathways were characterized in isolated ZG from control and mdr1 knockout mice (204). The experiments provided evidence that this protein is a mdr1 gene product—possibly a spliced or truncated variant—that inhibits ZG K\(^{+}\) and activates Cl\(^{-}\) conductance (Ref. 204; see also Ref. 216 for the role of MDR1 as a regulatory protein). A 65-kDa \[^{3}H\]Glib binding protein (dissociation constant (\(K_\text{d}\)) \(\approx 6\ \mu\text{M}\) ) has also been identified in ZG membranes (38). However, it is not known whether the 65-kDa mdr1-like gene product and the 65-kDa \[^{3}H\]Glib receptor of ZG membranes are identical. A high-affinity receptor for a derivative of dihydropyridine (34) has also been purified, which selectively regulates the K\(^{+}\) conductance in ZG (39). This protein is identical to ZG-16p, a recently cloned ZG protein with no known function (49). Interestingly, ZG-16p is enriched in rafts, cholesterol/glycosphingolipid-rich membrane microdomains, and the disruption of the latter by cholesterol depletion enhances stimulated amylase secretion (173). This observation is compatible with the hypothesis that ZG-16p operates as an inhibitor of the ZG K\(^{+}\) channel and that this channel plays a critical role in controlling regulated secretion (39). It also suggests that ZG ion channels and their regulators may be clustered in lipid rafts (see, for example, Ref. 125).

**ZG K\(^{+}\) conductance and pancreatitis.** Studies in goblet cells indicated that opening of Ca\(^{2+}\)-sensitive K\(^{+}\) channels of secretory granules causes K\(^{+}\) influx, which by exchanging with Ca\(^{2+}\) bound to the granule polyionic matrix results in decondensation and disaggregation of the matrix and granule swelling (136). This concept could be relevant to the pathophysiology of certain forms of hyperstimulatory acute pancreatitis with abnormally elevated cytosolic [Ca\(^{2+}\)] (158). Abnormal Ca\(^{2+}\) signals may enhance fusion of granules with acidic lysosomes, resulting in vacuole formation and activation of trypsinogen (159, 187). Concurrent Ca\(^{2+}\)-dependent opening of ZG K\(^{+}\) channels and K\(^{+}\) influx before fusion with the PM, intracellular swell-
ing, and lysis of ZG in situ could fuel acinar autodigestion by increasing intracellular release of digestive proenzymes.

The membrane of ZG appears to be equipped with two cation and two anion conductance pathways, which contribute to \( K^+ \) and \( Cl^- \) fluxes into the granules and promote release of digestive enzymes (Fig. 1). This apparent “functional redundancy” of ion channels could provide an explanation for why mdr1 knockout mice do not show any pathophysiological abnormality of digestive function (170). A functional redundancy of ion channels in secretory granules appears to be a more widespread phenomenon. Thus knockout (−/−) studies of ClC-3, a Cl− channel that promotes acidification of synaptic vesicles (190), show that ClC-3 (−/−) synaptic vesicles still acidify, though at a lower rate, suggesting the contribution of other Cl− channels to vesicular acidification. Furthermore, late endosomes and lysosomes of osteoclasts from ClC-7 (−/−) mice show normal acidification rates, although the Cl− channel ClC-7 is expressed in these organelles (113). Similarly, we found (205) that CCK-stimulated enzyme secretion in permeabilized rat pancreatic acini is abolished only if the inhibitor of nonselective cation conductance flufenamate is applied together with the KCNQ1 K+ channel inhibitor 293B. This observation suggests a functional overlap of both monovalent cation conductive pathways with regard to stimulated enzyme secretion (see above).

Biochemical and molecular evidence for the presence of ion channel proteins in the membrane of ZG has just begun to emerge (Fig. 1). Together with functional data on pancreatic acini and isolated ZG, this lends support to the hypothesis that, after exocytosis elicited by physiological stimuli, regulated ion channels in ZG control the release of stored digestive enzymes. Functional studies on ion conductive pathways in isolated ZG are based on an in vitro assay involving rapid osmotic swelling and end point measurements of granular osmotic lysis (54, 202). They are confirmed by recent reports, which used AFM to study ZG exocytosis in single pancreatic acinar cells and osmotic swelling of isolated ZG (Refs. 102, 176; see Atomic Force Microscopy). These studies have enhanced the understanding of the physiological significance of ZG ion channels for the exocytotic process and release of digestive enzymes. They suggest that granule swelling takes place after exocytosis to prevent granule collapse and ensure kiss and run recycling of secretory granules (175). This concept is fully compatible with the hypothesis that opening of secretory granule ion channels occurs after granule exocytosis and that salt, \( HCO_3^- \), and water flux into the granule matrix evokes swelling and discharge of the matrix from the granule cavity and regulates granule membrane retrieval (Fig. 1).

Pancreatic β-Cell Granules

**Background.** Insulin is produced in the β-cells of the islet of Langerhans, where it is stored in secretory granules until its release into the bloodstream by regulated exocytosis. β-Cells are electrically active and use this property to sense elevated blood levels of metabolic fuels and to couple them to insulin release. Nutrient-stimulated secretion involves two arms of signal transduction. One arm is dependent on modulation of the \( K_{ATP} \) channel in the PM by glucose metabolites. The other arm involves intracellular metabolism of long-chain acyl-CoA in the tricarboxylic acid cycle and generation of excess metabolic products, which promote exocytosis and release of insulin through as yet unknown mechanisms (48, 53).

The transduction pathway linking glucose metabolism to changes of the electrical activity of β-cells to induce insulin secretion is relatively well understood. A consensus model suggests that glucose is taken up by the cells via the glucose carrier GLUT-2 and metabolized to ATP in mitochondria. The increase in ATP-to-ADP ratio leads to closure of \( K_{ATP} \) channels in the PM. Because these channels are responsible for maintaining the resting membrane potential, closure of \( K_{ATP} \) channels leads to a depolarization of the β-cell and to activation of voltage-gated \( L \)-type \( Ca^{2+} \)-channels, which further accentuate the depolarization and initiate action potentials. Influx of \( Ca^{2+} \) through these channels leads to an increase of cytoplasmic \([Ca^{2+}]_c\) \), which then stimulates exocytosis of the insulin-containing granules and the release of insulin (16, 118).

The mechanisms underlying the final steps of exocytosis and insulin release are less well established. Unlike ZG of pancreatic acinar cells, the packaged secretory granules gradually acidify to allow further processing of insulin (99). The granules are stored throughout the cytosol and are eventually translocated to release sites near the PM. The fusion of the granules with the PM is triggered by \( Ca^{2+} \) and controlled by \( Ca^{2+} \)-dependent and -independent docking and fusion proteins, which have recently been identified in pancreatic β-cells and include the SNARE proteins (101, 118). Kinetic modeling of exocytosis has shown that only a small fraction of the granules of a β-cell (−1–5%, also referred to as RRP) are in a “primed” state, i.e., an ATP-, \( Ca^{2+} \)- and temperature-dependent step (68, 162) that makes them immediately available for exocytosis on \( Ca^{2+} \) influx. The remaining granules form a “reserve” pool. This classification into two distinct pools correlates with biphasic kinetics of glucose-stimulated insulin secretion. An early, rapid, and transient component (first-phase secretion) is believed to represent RRP release, and a second slower and sustained component is supposed to reflect a time- and ATP-dependent replenishment of RRP by mobilization of granules from the reserve pool (166). Type 2 diabetes or non-insulin-dependent diabetes mellitus (NIDDM) is associated with disturbances of this release pattern already at early stages of the disease, displaying a characteristic lack of first-phase secretion in response to an elevation of the glucose concentration (152).

Sulfonylureas are used in the treatment of NIDDM (96). Their principal effect is to stimulate insulin secretion from the β-cells (17) by their ability to inhibit the \( K_{ATP} \) channels in the β-cell PM (209), which by-
passes the physiological regulation of the channels by cytosolic concentrations of ATP and ADP. The inhibition of K_{ATP} channels by sulfonylureas results in membrane depolarization, Ca^{2+} influx, and insulin secretion as long as the drug is present (16). It was recently demonstrated that the K_{ATP} channel is composed of two components: a sulfonylurea receptor (SUR) (2), which like MDR1 (ABCB1) and CFTR (ABCC7) belongs to the superfamily of ABC transporters (55, 124), and an inward-rectifier K^{+} channel protein (Kir6.2) (100). The binding of sulfonylureas to the β-cell isoform of SUR, SUR1 (ABCC8), leads to the closure of the K_{ATP} channel. It has been known for quite a while that >90% of the high-affinity sulfonylurea binding sites in the β-cell are intracellular and appear to colocalize with the insulin-containing secretory granules (43, 142, 195). The role and molecular identity of these intracellular sulfonylurea binding sites are not known, but it has been reported that sulfonylureas also potentiate insulin secretion by a direct effect on the exocytotic machinery (67). This stimulation requires low extracellular glucose concentrations, depends on PKC, and is observed at therapeutic concentrations of sulfonylureas. This is very similar to pancreatic ZG membranes, in which ATP- and sulfonylurea-sensitive K^{+} and Cl^{-} conductive pathways have been identified (200, 202, 204); it has therefore been suspected that such channels could also be present in β-cell granules and could participate in sulfonylurea-induced stimulation of secretion.

A 65-kDa mdr1-like sulfonylurea-binding protein that regulates exocytosis. To elucidate the mechanisms underlying the direct stimulatory effect of sulfonylureas on the exocytotic machinery, Barg et al. (24) measured exocytosis under voltage clamp conditions, which circumvents sulfonylurea-mediated depolarization of the cell. Ca^{2+} and test substances were dialyzed into the intracellular space through the recording electrode. Exocytosis and discharge of granule contents were determined by combining measurements of C_{m} and amperometric detection of released serotonin. Stimulation of exocytosis by the sulfonylurea tobutamide was observed slightly above the resting [Ca^{2+}] or by a stepwise increase in [Ca^{2+}] caused by photorelease of caged Ca^{2+}. Pharmacologically, the exocytotic mechanism affected by tobutamide was very similar to the β-cell PM K_{ATP} channels, because it was inhibited by diazoxide but not by pinacidil, which activates cardiac/vascular K_{ATP} channels by binding to the SUR2A/B (ABCC9) sulfonylurea receptor (181). K_{ATP} channels are physiologically activated by increasing the ADP-to-ATP ratio and thus reduce cellular depolarization and insulin secretion (63, 106, 133). ADP applied through the recording pipette also inhibited Ca^{2+}-dependent exocytosis, and this could be reversed by coapplication of tobutamide. Together, the data indicated the involvement of a SUR1 (ABCC8)-like regulatory mechanism in β-cell exocytosis. Evidence for a mechanism similar to that in ZG, where a 65-kDa mdr1 gene product confers the modulation by sulfonylureas (38, 202), was obtained in islet β-cells as well. First, antibodies against cytosolic domains of ABCB1 (mdr1) (JSB-1 and C219) detected a protein of 65 kDa in β-cell granule membrane fractions. In addition, ultraviolet cross-linking revealed binding of the sulfonylurea Glib to a protein of ~65 kDa in the granule fraction (Ref. 24; unpublished observations). Also, intracellular application of the ABCB1 antibody JSB-1 abolished the stimulatory action of sulfonylureas on exocytosis. Finally, a blocker of ABCB1, tamoxifen (110), prevented the stimulation of exocytosis induced by tobutamide. Together, the data suggested that sulfonylureas bind to a granule membrane protein of 65 kDa, which may be an ABCB1 (mdr1) gene product. This leads to the activation of the exocytotic machinery, possibly via opening of ion channels in the granule membrane (see Fig. 2).

Expression of a CIC-3 Cl^{-} channel. In analogy to K_{ATP} channels, it is conceivable that the 65-kDa β-cell granule sulfonylurea-binding protein represents a regulatory subunit of a K^{+} channel, and evidence for this has been obtained in pancreatic α-cells (97). However, this is not likely in β-cells, because Ca^{2+}-dependent exocytosis in the presence or absence of tobutamide

![Diagram of exocytosis mechanism](image-url)

Fig. 2. Islet granules gain secretion competence by intragranular acidification, which depends on the simultaneous operation of a bafilomycin-sensitive V-type H^{+}-ATPase and a CIC-3 Cl^{-} channel, which is blocked by DIDS and an antibody directed against a COOH-terminal domain of CIC-3 (anti hCIC-3C). By analogy to the regulation of the ATP-sensitive K^{+} (K_{ATP}) channel in the plasma membrane, CIC-3 and a regulatory mdr1-like 65-kDa protein may form an ion channel complex in the granular membrane. ATP enhances granular Cl^{-} uptake, acidification, and “priming” by binding to the regulatory 65-kDa protein, whereas ADP attenuates the same chain of events. Tobutamide interacts with the 65-kDa protein to increase Cl^{-} uptake, thereby stimulating exocytosis, and prevents ADP binding by counteracting the inhibitory effects of ADP on priming. The mdr1 antibody JSB-1, tamoxifen, and diazoxide prevent tobutamide-stimulated exocytosis by binding to the mdr1-like 65-kDa protein. An IP_{3}-sensitive Ca^{2+} channel is possibly localized in the membrane of islet granules, which could thus operate as an intracellular Ca^{2+} store involved in Ca^{2+} signaling. Apart from the IP_{3}-sensitive Ca^{2+} channel, the model is derived from data published in References 22 and 24.
was unaffected by replacing intracellular K+ with Cs+ but strongly inhibited by the Cl− channel blocker DIDS (22) or by replacing intracellular Cl− with glutamate. This is different from data obtained in chromaffin cells, in which Ca2+-dependent secretion occurs in the presence of glutamate but is inhibited by Cl− (111). The data therefore suggested that intracellular Cl− fluxes are involved in exocytosis in β-cells. Barg and coworkers (22) found that two antibodies against the Cl− channel CIC-3 labeled β-cell granules. Immunoblot analysis of β-cell homogenates and a granule fraction of the INS-1 cell line revealed an immunoreactive band with a molecular mass of ~90 kDa, as expected for the nonglycosylated form of CIC-3. When a functional antibody against the COOH-terminal cytosolic domain of CIC-3 was dialyzed into the intracellular space through the patch-clamp electrode, exocytosis was strongly inhibited. This interaction with the exocytotic machinery was specific, because a nonfunctional antibody against the NH2-terminal domain of CIC-3 was dialyzed into the intracellular space without effect (22). Barg et al. (22) then investigated whether stimulation of β-cell exocytosis by Cl− influx depends on osmotic forces as a consequence of net accumulation of electrolytes, water uptake, and increased hydrostatic pressure inside the granules to promote membrane fusion (72, 151). When the cytosol was made hyperosmotic to reverse the osmotic gradient for water between cytosol and granule content, exocytosis was not different from isosmotic controls. Instead, the authors hypothesized that acidification by electrogenic H+ pumping into the granule might require a shunt conductance to prevent the buildup of an electrical potential, which would be provided by a CIC-3 Cl− channel. Indeed, exocytosis was also prevented by bafilomycin A, which irreversibly blocks V-ATPases, or by addition of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), which equilibrates pH gradients across membranes. These effects could not be overcome by tolbutamide (22).

Granule acidification can be measured with the fluorescent acidotropic probe LysoSensor green, which accumulates in acidic compartments (mostly granules in β-cells) as the result of protonation. The protonation relieves the fluorescence quenching of the dye such that it is almost nonfluorescent at neutral pH and exhibits a pH-dependent increase in fluorescence at acidic pH. After intracellular dialysis with 3 mM MgATP and 1.5 μM Ca2+ through the patch pipette, there was a net granular acidification. Acidification was prevented by inclusion of either 100 μM DIDS or 5 mM MgADP in the pipette solution. Interestingly, inclusion of tolbutamide alone or in addition to MgADP enhanced granule acidification, thereby mimicking the effects of the compound on exocytosis (22). There was a strong decrease of fluorescence (i.e., an increase of pH) when the protonophore CCCP was introduced into the cytosol. Because CCCP-mediated H+ efflux is electrogenic, dissipation of the pH gradient across the granule membrane will not occur unless a counterion conductance in the granule membrane allows for charge compensation. If CIC-3 Cl− channel activity is required for pH equilibration, then channel inhibition should also affect CCCP-mediated H+ efflux. Accordingly, when the functional antibody against CIC-3 was applied to the pipette solution, the increase of pH elicited by CCCP was prevented. This effect was not observed with the nonfunctional CIC-3 antibody. The authors therefore concluded that electrogenic Cl− transport through CIC-3 provides the counterions required for H+ flux across the granule membrane and thereby modulates the exocytotic capacity of the β-cell (22).

To determine at what stage exocytosis was modulated by granular Cl− and H+ fluxes, the kinetics of exocytosis elicited by trains of voltage-clamp depolarization were analyzed to provide an estimate of the rates of processes before exocytosis. Pancreatic β-cells were maximally stimulated, and the size of the RRP and the rate of its turnover were estimated by capacitance measurements. Experiments using DIDS, CCCP, and CIC-3 antibodies, as described above, indicated that Cl− fluxes mediated by CIC-3 are involved in the priming reaction of insulin granules for Ca2+-elicited exocytosis (22). Granule acidification driven by the H+ pump and/or maintenance of a pH gradient, however, appeared to be necessary for the priming reaction as well as for exocytosis of the RRP. A low granule pH seemed to be required for granules that are ready for exocytosis. Conversely, dissipation of a pH gradient might induce granule “depriming” and prevent exocytosis. How would granular acidification affect the final steps of exocytosis? The authors speculated that a low granular pH could influence the exocytotic machinery by inducing conformational changes in SNARE proteins, rendering them more fusogenic (193) and referred to recent studies that demonstrated that vacuole acidification is involved in the pairing of SNARE proteins in yeast (213). This may differ from pancreatic acinar cells, in which an acidic ZG pH is not requisite for exocytosis and protein secretion (56). A possible model for the regulation of granular pH and exocytosis summarizes the data obtained by Barg et al. (22, 24) (Fig. 2).

PERSPECTIVES

Further studies combining appropriate highly sensitive techniques with specific molecular tools, such as genetically modified animal and cellular models, will be necessary to define with certainty the role of secretory granule ion channels and their regulatory proteins for exocytosis and release of secretory products. From a postgenomic perspective, however, the identification of secretory granule ion channels and the characterization of their physiological significance could gain importance for the development of therapeutic drugs con-
trolling secretory events. This is particularly relevant because secretory granules, similar to other intracellular organelles, seem to be equipped with specific sets of ion channels, which represent ideal targets for the development of drugs with high tissue and/or cell specificity and a selective mode of action. In contrast, docking and fusion proteins involved in exocytosis are ubiquitously expressed and regulate exocytosis by more general mechanisms. They are therefore less well suited as targets of drug action. The use of putative therapeutic drugs as activators or inhibitors of secretory granule ion channels could significantly contribute to the improvement of pathophysiological disease conditions, such as NIDDM, acute pancreatitis, or CF.

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