Regulation of secretory granule pH in insulin-secreting cells

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The regulation of pH within subcellular compartments is crucial for maintaining macromolecular trafficking from one intracellular compartment to another (20). In the endocytic pathway, receptor-bound ligands and dissolved substances in the extracellular fluid are taken up at the plasma membrane by specialized structures that form “early” endosomes (31). The endosomal lumen rapidly acidifies, inducing the dissociation of endocytosed receptor-ligand complexes. The endosomal contents may be recycled back to the plasma membrane, as in the transferrin system (10), or undergo further degradation in “late” endosomes and lysosomes (31). In the regulated exocytic pathway of hormone secreting cells, the acidic environment of the secretory vesicle regulates the proteolytic processing of prohormones into the mature form of the secreted peptide (2). In the pancreatic β-cell, cleavage of the proinsulin B and C chains by a specific endopeptidase requires an acidic environment within the secretory granule (7). Moreover, it has been suggested that “priming” of insulin containing secretory granules involves further acidification of the granule lumen (4). Conversely, it has been proposed that alkalinization of the secretory granule lumen upon activation of secretion should occur to promote solubility of the stored and condensed insulin, thereby enhancing its release from the granule upon fusion with the plasma membrane (3).

Although it is appreciated that changes in the pH of secretory granules must occur for normal protein processing, the regulation of pH in these compartments has not been studied in detail. Seminal studies monitored accumulation of the fluorescent base acridine orange to evaluate granule pH in isolated β-cells (26). More recently, Barg et al. (4) utilized a lysosensor probe (Molecular Probes, Eugene, OR) to monitor pH within acidic compartments during activation of secretion in isolated mouse β-cells. However, both acridine orange and lysosensor distribute into all acidic compartments, including endosomes. Therefore, any global measure of vesicular pH with these probes must be influenced by the low pH of the endocytic pathway such that changes in their fluorescence may equally reflect changes in endosomal pH and pH within the secretory pathway. Knowledge of the mechanisms by which proteins are sorted within cells has provided an approach to target ion-sensitive fluorescent proteins to specific subcellular compartments. Several probes that have shown great utility include the Ca2+ indicator protein aequorin and the pH-sensitive variants of green fluorescent protein (GFP) (23).

Because GFP is encoded by DNA, targeting the GFP protein product to a specific subcellular site can be achieved with a few strategic genetic manipulations. These properties have allowed GFP to be used to localize fusion proteins within specific subcellular compartments (13, 15, 32) and to investigate vesicular trafficking in the regulated and constitutive secretory pathways.
pathways (12, 28). With the development of red-shifted GFP mutants having enhanced pH sensitivity, it has become possible to monitor pH regulation within a variety of subcellular compartments to which the GFP has been targeted (13, 15). With respect to secretory granules, GFP targeting has been used to study activation-induced changes in synaptic vesicle pH within PC-12 cells (9) and to monitor synaptic vesicle exocytosis in hippocampal neurons (21).

To monitor pH within vesicles of the regulated secretory pathway in endocrine cells, Pouli et al. (28) developed a preproinsulin-GFP construct and expressed it in insulin-secreting INS-1 cells. In most transfected cells, the GFP chimera was localized to the endoplasmic reticulum, whereas in ~12% of cells GFP appeared to target to both the Golgi and punctate secretory granules. No release of GFP was observed even in the presence of maximally stimulating concentrations of glucose (30 mM). We made similar observations with a preproinsulin-targeted construct (33), in which only a small population of insulin-containing secretory granules also contained GFP. In an attempt to improve on the targeting procedure, the NH2-terminal leader sequence of human growth hormone (24) was inserted in frame with the GFP sequence (EGFP; F64L/S65T) such that, upon expression, EGFP was targeted to the regulated secretory pathway in rat insulinoma cells (RIN1046-38 parental). The specific localization of the human growth hormone (hGH)-EGFP fusion protein (hGH-EGFP) was characterized by colocalization with antibodies to insulin. The ability to utilize this targeted probe for analysis of cell secretion and changes in pH within secretory granules was then evaluated.

MATERIALS AND METHODS

Cell culture and gene transfection. The insulin-secreting rat insulinoma RINr1046-38 cell line was obtained from Dr. Sam Clark (BetaGene, Dallas, TX) and cultured as previously described (6). Briefly, the cells were cultured in RPMI 1640 (Sigma Chemical, St. Louis, MO) supplemented with 5 mM glucose and 5% fetal bovine serum and maintained in a 95% air-5% CO2 humidified atmosphere at 37°C. RIN-38 cells (passage 1–8) were electroporated with plasmid (0.5 mg/ml DNA plasmid per 106 cells). The hGH-EGFP-transfected cells were kept under selection with 0.25 mg/ml active G-418. For secretion experiments, cells were plated at 2 × 104 cells/cm2 into six-well culture dishes, and for imaging experiments the wells contained 25 mm of round glass (no. 1) coverslips.

Construction of hGH-EGFP expression vectors. The plasmid pBJ001 containing the hGH gene (a gift from Dr. Sam Clark (BetaGene, Dallas, TX)) was cut with Tsp509I and BsrBI at bases 376 and 656; GenBank accession no. U57608) and reverse primer 5' TTACTTTGACAGCTGTC- CTTGCCGAGAGTACCC 3' (bases 1403–1370; GenBank accession no. U57608), and the 2.4-kb fragment of the hGH-EGFP construct was subsequently sequenced (ARL Labs, Division of Biotechnology, Tucson, AZ).

EGFP secretion from cell populations. Cells were grown to ~75–80% confluence in six-well plates. For analysis of EGFP secretion, the culture media from individual wells were replaced with 1 ml of Hanks' buffered saline (HBS) without substrates. HBS contained (in mM) 138 NaCl, 0.2 NaHCO3, 0.3 Na2HPO4, 5 KCl, 0.3 KH2PO4, 1.3 CaCl2, 0.4 MgSO4, and 10 HEPES. Each well was rinsed three times with fresh HBS and then incubated in 1 ml of HBS containing 0.05 mM glucose and no other substrates for 1.5 h at 37°C incubator. These media were replaced by either 1 ml of the same medium (low glucose) or medium containing 5 mM glucose with or without potentiators of glucose-stimulated insulin secretion. After another 1.5-h incubation period, the media were again collected; these media samples were centrifuged at 3,000 g for 2 min, to pellet particulates including cells that were released into the media, before analysis of medium EGFP fluorescence. The cells from each six-well chamber were removed by incubation in trypsin (500 U/ml) EDTA (0.02%) buffer, and cell density was determined using a Neubauer hemocytometer. Cells released to the medium (analyzed from the pellets) amounted to <1% of attached cells and did not vary significantly for any experimental condition. Fluorescence of EGFP released to the medium was determined by using a Hitachi F2000 fluorimeter with excitation set at 480 nm and emission at 530 nm. The 1.5-h incubation periods were required to allow for sufficient secretion of EGFP to be measurable in the 1-ml volume. To allow comparison between different experiments, secretion rate data for cells incubated under activated conditions (5 mM glucose) are presented as a percentage of the rate measured for the same cells incubated with low glucose (0.05 mM glucose, control). Cell counts were performed for all control experiments to normalize secretion data for differences in constitutive secretion due to differences in passage number (number of cells expressing EGFP) and cell density between experiments. Absolute secretion rates were estimated by constructing a calibration curve with pure EGFP (Clontech).

Antibodies and immunocytochemistry. Primary and secondary antibodies were guinea pig antiporcine insulin (ICN Biological, Costa Mesa, CA), rabbit anti-GFP (Clontech), goat anti-guinea pig IgG, Texas red, and goat anti-rabbit IgG fluorescein isothiocyanate. Cells grown on no. 1 glass coverslips were fixed in 4% paraformaldehyde at room temperature and then permeabilized for 15 min in 0.5% Triton X-100 in 150 mM NaCl buffered with sodium citrate (15 mM). The coverslips were sequentially exposed to primary and secondary antibodies for 45 min each at room temperature with 10-min washes in antibody-free buffer in between incubations with antibodies (16).

Digital imaging microscope and optics. An Olympus IMT-2 microscope equipped for epifluorescence was used to image live cells and immunochemically labeled samples. The excitation path included a 200-W mercury lamp coupled with a 10-nm band-pass excitation filter centered at 480 nm and a long-pass dichroic mirror transmitting wavelengths of 500 nm and longer. EGFP fluorescence was imaged through a 30-nm band-pass emission filter centered at 525 nm. For all live-cell EGFP imaging experiments, coverslips containing
subconfluent cells were placed into a perfusion chamber on the microscope containing HBS held at 37°C. To image Texas red fluorescence from immunochemically stained cell preparations, a 10-nm band-pass excitation filter centered at 570 nm and an appropriate dichroic mirror coupled with a 10-nm band-pass emission filter centered at 610 nm were employed (all mirrors from Chroma, Brattleboro, VT). Fluorescence images were captured with a liquid cooled charge-coupled device (CCD) camera using a Technochrome 512 × 512-pixel imaging chip (Photometrics, Tucson, AZ). The light emitted from cell samples was collected by an Olympus S Plan Apo ×60 oil-immersion objective (NA 1.4), and a ×6.7 imaging eyepiece was used to focus the light emerging from the microscope onto the CCD chip. Photometrics Imaging Software was used to acquire and store images.

**Simultaneous measurement of cytosolic and secretory granule pH.** To measure emission intensity from vesicular EGFP and cytosolic carboxyseminapthorhodofluor (SNARF-1) simultaneously, we utilized a spectral imaging microscope system as previously described (18). Briefly, a 10-nm band-pass filter centered at 490 nm coupled with a dichroic mirror passing light above 505 nm (Chroma) was used to direct monochromatic excitation light to the sample. The emitted light from the sample was focused onto a ×6.7 eyepiece onto a high-resolution diffraction grating (300 grooves/mm; Aries 250/IS spectrograph; Chromex, Albuquerque, NM). First-order emission spectra (500–700 nm) were focused onto the chip of the CCD camera. For these experiments, coverslips containing subconfluent cells were placed into HBS in the 37°C chamber on the microscope stage. A group of cells expressing high levels of EGFP were selected, and then the medium was replaced with HBS containing 5–15 μM SNARF-1 acetoxyester (AM). When the peak intensity at 570 nm (SNARF-1 acidic peak) reached approximately equal intensity to that of EGFP, the coverslips were washed for >20 min in HBS before initiation of an experiment. SNARF-1-AM (Molecular Probes) was solubilized in anhydrous dimethyl sulfoxide (Aldrich, Milwaukee, WI) and stored desiccated at ~80°C.

**In situ calibrations.** In situ pH calibration of hGH-EGFP was performed as described previously (15, 19). The calibrations were initiated after stable resting intensity values (530-nm emission) from hGH-EGFP-expressing cells were obtained. The cells were then equilibrated in pH 5.5 calibration buffer. The calibration buffer contained (in mM) 110 KCl, 20 NaCl, 0.5 CaCl2, 0.5 MgCl2, 10 bicine, 10 pipazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), and the K+∕H+ ionophore nigericin (2 μM) and K+ ionophore valinomycin (5 μM) to allow equilibration of intracellular and extracellular pH. For most calibrations, pH was increased stepwise from a pH of 4 or 5 to a pH of ~8.0 in six to eight steps. The cells were allowed to equilibrate at each pH change for 3 min before data acquisition. Alternatively, the cells were equilibrated in pH calibration buffer at a pH of ~8.0 and sequentially exposed to increasing [H+]i. Because EGFP fluorescence intensity is sensitive to pH but no spectral shift in emission frequency is observed, a method for normalizing the signal is required to compare the signal response between individual cells where total EGFP concentration might differ. To standardize the effect of pH on hGH-EGFP in each cell, the fluorescence intensity of hGH-EGFP (I530) measured throughout the experiment was normalized to the maximal hGH-EGFP intensity observed at a pH >8.0 (I530max). For all experiments, this value (I530max) was determined at the termination of the experiment by incubating cells with ionophores (nigericin and valinomycin) at high pH (~8.0). For the calibrations, the hGH-EGFP I530∕I530max values were plotted against pH. Data were fit to a four-parameter sigmoidal relation by using SigmaPlot to estimate K4 values.

Cytoplasmic pH was evaluated by loading cells with the pH-sensitive fluorophore SNARF-1. The SNARF-1 fluorescence emission spectrum is sensitive to pH, with protons eliciting a shift in peak fluorescence from 640 to 570 nm. To analyze the SNARF-1 and EGFP signal responses simultaneously, spectral imaging microscopy was used to monitor fluorescence emission intensity from 500 through 700 nm. Cytosolic pH was calculated by fitting the ratio of fluorescence intensities (1) measured at the ion-sensitive wavelengths of SNARF-1 (570 and 640 nm) into the following equation: pH = pK + log(R − Rmin∕Rmax − R), where R = I(640nm)∕I(570nm), Rmin is the fully protonated form of SNARF-1, Rmax is the fully deprotonated form of SNARF-1, and pK is the apparent K4 of SNARF-1 for protons (18, 19). Conversely, fluorescence emission of SNARF-1 at 600 nm is relatively insensitive to pH, providing a measure of pH-independent (artificial) changes in fluorescence. By taking advantage of this property, the intensity of fluorescence at 530 nm (EGFP) was compared with that at 600 nm to correct for pH-independent changes in fluorescence. This approach for the normalization of a “single emission wavelength probe” has been previously validated (18). The I530∕I600 ratio was compared with the I530∕I600 ratio to evaluate the reliability of the “single wavelength” EGFP signal normalization approach during in situ calibrations. SNARF-1 also has a lower affinity for protons (pK4 ~7.5), providing a good comparison to the EGFP signal response.

**Cell permeabilization with digitonin.** To gain access to the cytosolic space, the cell plasma membrane was permeabilized by incubation with 0.1% digitonin in buffer that mimics cytosolic ionic contents. The digitonin was then removed, and the cells were incubated in digitonin-free cytosolic buffer. The cytosolic buffer contained (in mM/l) 110 KCl, 0.5 MgCl2, 20 HEPES, and 20 NaCl. After stabilization of the fluorescence signal, Mg2+∕ATP in a small volume of HEPES-buffered solution (pH 9) was added to provide a final ATP concentration of 5 mM, with medium pH and ionic strength not appreciably altered. Addition of digitonin causes a significant increase in nonspecific fluorescence, making calculation of pH values using the standard calibration curve impossible. Therefore, data from these experiments are presented as I530∕I530max values.

**Statistical analysis.** Data are presented as means ± SE unless otherwise noted. Statistical differences between group means were determined with the use of a two-tailed unpaired Students t-test. A value of P < 0.05 was taken as indicative of a statistically significant difference between group means.

**RESULTS**

**Expression of targeted EGFP and its subcellular distribution.** Transfection of the hGH-EGFP construct was accomplished by electroporation of the rat insulinoma cell line RIN1046-38 with the hGH-EGFP plasmid. Cells that survived selection with G-418 were propagated, and clonal lines were imaged for EGFP fluorescence. A range of distributions of EGFP were observed for different clones, with most exhibiting punctate fluorescence. However, most cells in the population express...
EGFP that is localized to punctate structures with diameters of <1 μm. To determine the subcellular origin of the fluorescence, an image of live cells exhibiting high levels of punctate EGFP was acquired. Cells were then fixed with paraformaldehyde on the microscope stage, and another fluorescent image of the same cells was captured. There was little or no change when comparing the distribution of EGFP fluorescence in live or fixed preparations (data not shown). Fixed cells were labeled with rabbit anti-EGFP antibody, followed with anti-rabbit Texas red. The anti-EGFP (TR) antibody coincided with punctate EGFP fluorescence, confirming that the fluorescence originates from EGFP, rather than cellular autofluorescence (not shown). Co-localization of hGH-EGFP with insulin-specific antibodies was investigated to determine whether the hGH-EGFP was properly targeted to secretory vesicles. A typical image pair is shown in Fig. 2. The image in Fig. 2B represents EGFP fluorescence, and Fig. 2A represents anti-insulin. Clearly, EGFP is colocalized with insulin within secretory granules in these cells, as it is in most cells in this population. Although the absolute level of EGFP in the cytosol cannot be determined relative to the compartmentalized EGFP in these images, the cytosolic component must be relatively small on the basis of the imaging results.

**Effect of cell activation on EGFP secretion from RIN-3M21 cells.** Release of EGFP from cells into the culture medium was analyzed under nonstimulatory conditions (0.05 mM glucose) and compared with EGFP release in the presence of stimulatory glucose (5 mM) and in the presence of a potentiator of the secretory response (3-isobutyl-1-methylxanthine, IBMX). Over the course of a 1.5-h incubation period, nonstimulated constitutive secretion was significant. On the basis of calibration curves constructed from purified EGFP, the rate of secretion was ~0.5 (±0.1) nmol EGFP·10⁶ cells⁻¹·h⁻¹ (n = 16). The effects of activators and potentiators of insulin secretion on EGFP release are shown in Table 1. When incubated in the presence of stimulatory levels of glucose, secretion more than doubled (222 ± 29%, n = 12). This rate of EGFP secretion is about one-third the rate of glucose-stimulated insulin secretion measured in the parental line (6). Potentiators of glucose-induced insulin secretion enhanced the release of EGFP. IBMX, which elevates cAMP and potentiates insulin secretion, increased EGFP secretion nearly threefold (Table 1). This effect of IBMX was reduced to levels observed in the presence of glucose alone when the cells were preincubated for 1 h with 10 μM of the protein kinase inhibitor 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride (H-7; Ref. 1).

**In situ pH calibration of hGH-EGFP.** The fluorescence emission of secretory granule-targeted hGH-EGFP was analyzed as the cells were sequentially incubated in buffers of varying pH containing nigericin and valinomycin to abolish pH gradients (19). As expected, the fluorescence of hGH-EGFP in secretory vesicles was most sensitive to changes in pH below 7.0 (Fig. 3). To correct for differences in absolute EGFP fluorescence between individual cells, the signal measured at each pH was normalized to the maximal signal response elicited at pH ~8.0 in the presence of nigericin and valinomycin. This was the approach used to calculate pH for all experiments. The fluorescence of
Table 1. Secretion of EGFP from RIN-3M21 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>%Change From Control Rate</th>
</tr>
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<tbody>
<tr>
<td>Glucose (5 mM)</td>
<td>12</td>
<td>+222 ± 29</td>
</tr>
<tr>
<td>Glucose (5 mM), IBMX (10 μM)</td>
<td>17</td>
<td>+305 ± 35</td>
</tr>
<tr>
<td>Glucose (5 mM), IBMX (10 μM), H-7</td>
<td>3</td>
<td>+269 ± 7</td>
</tr>
<tr>
<td>Glucose (5 mM), H-7 (10 μM)</td>
<td>3</td>
<td>+162 ± 13</td>
</tr>
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</table>

Values are means ± SE; n = no. of wells analyzed. The constitutive secretory rate in the presence of 0.05 mM glucose was 0.5 (±0.1) nmol EGFP·10⁶ cells⁻¹·h⁻¹ (n = 16), which amounted to the secretion of ~2.3 (±0.1)% of total cellular green fluorescent protein (GFP) in 1 h (n = 4). All changes are significantly different from the control rate. Where indicated, H-7 was present 1 h before initiation of the experiment and was then maintained throughout the entire experimental time course. EGFP, enhanced GFP; IBMX, 3-isobutyl-1-methylxanthine.

hGH-EGFP analyzed in situ at 37°C has an apparent pKa of 6.24, which is similar to published values for EGFP in vitro and in situ [pKa = 6.15 (15); pKa = 5.98 (13); both measured at 22°C].

The sensitivity of the EGFP protein in situ was compared with that of the ratiometric H⁺-sensitive fluoroprobe SNARF-1, which was loaded into the cell cytosol by incubation with its AM form. In calibration experiments where SNARF-1 was present in the cell cytosol, the EGFP signal (530 nm) was normalized to both the I₅₃₀max and the signal measured at the iso-emission point for SNARF-1 (600 nm), as described previously (18). When normalized to the SNARF-1 iso-emissive signal, a similar value of EGFP affinity for H⁺ was obtained (pKa = 6.19), confirming the validity of the internal (I₅₃₀/I₅₃₀max) normalization approach. In the presence of a nonstimulatory concentration of glucose (0.05 mM), the resting cytosolic pH estimated from SNARF-1 measurements was 7.39 ± 0.09 (n = 17).

**Effect of glucose on secretory granule pH measured in single cells.** Approximately 40% of individual RIN-3M21 cells within the cell population are insensitive to glucose, i.e., no change in pH is observed (passages 14–17). On the other hand, in ~58% of all cells (n = 236), elevation of glucose from low levels (0.05 mM) to stimulatory levels (5 mM) initiated a decrease in single-cell EGFP fluorescence, which is consistent with release of the EGFP protein from the cells, i.e., secretion and loss of cellular EGFP (Fig. 4). However, in cells in which stimulatory levels of glucose were replaced with equimolar mannitol, a recovery of EGFP fluorescence toward baseline was observed. As seen in Fig. 4, pH does not fully recover to control levels after the washout of activating glucose. When analyzed as a decrease in total EGFP signal per cell, a decrease of 4% (±0.02, n = 16) is calculated. This decrease in signal, measured from individual cells, is similar in magnitude to the amount of total cellular EGFP content released after cell activation (2–5% of total cell EGFP/h based on cell population measurements). Because only a small amount of EGFP is released and EGFP fluorescence is sensitive to changes in pH below 7.5 (Fig. 3), the ability to reverse the glucose-induced decrease in EGFP fluorescence indicates that changes in single-cell EGFP fluorescence intensity are primarily due to the pH sensitivity of the EGFP within the secretory pathway. Consistent with this proposition, resting vesicular pH is significantly more acidic in cells incubated in 5 mM glucose (6.06 ± 0.05; n = 42) than in cells incubated with nonstimulatory glucose (6.22 ± 0.03; n = 62). Because imaging data demonstrate localization of EGFP within insulin secretory granules (Fig. 2), these changes in EGFP fluorescence at the single-cell level are indicative of changes in pH within

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**Fig. 3.** In situ calibration of EGFP and cytosolic carboxysemimithorodofluor (SNARF-1) in hGH-EGFP-expressing cells. hGH-EGFP-expressing cells were loaded with SNARF-1 acetomethoxys-ter (AM) and then treated with valinomycin and nigericin in media of varying pH. EGFP signal was normalized to its fluorescence intensity at a pH at which the signal response was maximal (pH ~7.8). SNARF-1 ratio indicates the relation between intensities measured at the SNARF-1 basic (640 nm) and acidic (570 nm) peaks. Data represent the responses averaged from 6 cells. Calibration experiments were carried out at 37°C in the presence of nonstimulatory glucose to mimic experimental conditions. Values are means ± SE.

**Fig. 4.** Effect of glucose on EGFP fluorescence in single hGH-EGFP-expressing cells. Glucose elicits an acidification of the secretory granule lumen in ~60% of cells within the population (see text). Average responses of 16 cells that responded to glucose are shown. Cells were equilibrated for at least 1 h in stage medium in the presence of nonstimulatory levels of glucose (0.05 mM). After a stable pH was measured, glucose was elevated to 5 mM. To wash out glucose without altering osmolarity, the wash medium contained 0.05 mM glucose plus 5 mM mannitol. Values are means ± SE. Glu, glucose. HBSS, Hanks’ buffered saline solution.
immature insulin secretory granules that are not released immediately upon cell stimulation.

The low pH of specific subcellular compartments is generated and maintained by the activity of the V-type H\textsuperscript{+}-ATPase. To directly assess the role of active proton transport in vesicle acidification, cells were permeabilized by treatment with 0.1% digitonin for 2 min at 37°C, and all ATPase-driven ion gradients were allowed to dissipate due to the loss of ATP. After detergent treatment, a gradual alkalinization of the secretory granule lumen was observed (Fig. 5). After stabilization of the fluorescence signal, Mg\textsuperscript{2+}-ATP was added to provide a final ATP concentration of 5 mM with medium pH and ionic strength not appreciably altered. Upon addition of Mg\textsuperscript{2+}-ATP, the EGFP-containing compartments rapidly acidified. Subsequent addition of bafilomycin, an inhibitor of the V-type H\textsuperscript{+}-ATPase, caused alkalinization to near the original steady-state value. To determine whether the dynamic range of the probe had been exceeded, NH\textsubscript{4}Cl was added to transiently sequester remaining free H\textsuperscript{+} in the EGFP containing compartments. As shown in Fig. 5, NH\textsubscript{4}Cl elicited a rapid alkalinization, suggesting that the full dynamic range of the EGFP was not reached after bafilomycin treatment. To determine whether H\textsuperscript{+}-ATPase is involved in the glucose-mediated response, cells were incubated with bafilomycin before treatment with glucose. In intact cells incubated with low glucose, bafilomycin elicits alkalinization of the secretory granule lumen. In the presence of bafilomycin, subsequent elevation of glucose to 5 mM is without effect on granule pH, although the response of the EGFP was not yet saturated, as demonstrated by the further increase in EGFP fluorescence after nigericin treatment (Fig. 6).

**Regulation of secretory granule pH by protein kinase A.** A primary mechanism for potentiating glucose-activated insulin secretion is a concomitant elevation in cAMP (29). Little is known regarding the influence of cAMP and the subsequent activation of protein kinase A (PKA) at the level of the secretory granule. As described above, glucose-induced secretion of insulin, and in these cells EGFP secretion, is potentiated by treatment with IBMX, which allows cAMP levels to accumulate by inhibiting phosphodiesterase activity. IBMX also elicited a significant alkalinization of the vesicle lumen in both the presence (Fig. 7) and absence of stimulatory levels of glucose (Fig. 8). This alkalinization was reversed upon removal of the IBMX (Fig. 7). Forskolin directly activates adenylate cyclase, thereby substantially elevating cAMP levels. Similar to findings with IBMX, forskolin elicited an alkalinization of secretory granules independent of activation by glu-
ion-sensitive fluorescent probes that can be targeted to specific organelles (15, 23, 34). Here we describe the targeting of a pH-sensitive form of GFP (EGFP-F64L/S65T) to the secretory pathway of insulin-secreting cells (Fig. 2). At the population level, this targeting provides a measure of cell secretory activity by monitoring appearance of EGFP fluorescence in the culture medium. Gene knockin or knockout in insulin-secreting cell lines has provided a powerful approach to study the mechanisms by which these β-cells sense changes in glucose concentration (5, 11). The ability to determine how alterations in expression of specific components of the glucose-sensing mechanism modify secretion, by simply monitoring release of a fluorescent tag from a cell population, may facilitate such studies. On the other hand, little total signal is lost after activation of secretion (2–5% of total EGFP/h). Therefore, most EGFP must be within immature secretory granules that are not immediately released upon stimulation. Thus, at the single-cell level, observed changes in EGFP fluorescence that occur on the order of minutes are primarily indicative of pH responses within the targeted compartments, rather than a direct measure of cell activation or secretion.

EGFP in the secretory compartments can be reasonably calibrated (Fig. 3), although the pK_a appears to be elevated by ~0.1–0.4 pH units relative to that monitored free in solution (13, 27). The reason for the difference between in situ and in vitro calibration curves is not obvious. However, the milieu within the granules themselves must be quite different from that used for in vitro calibration, particularly with respect to protein (insulin) and ion concentrations and fixed charge characteristics (14), which may explain the shift in sensitivity to the right. On the other hand, in the previously reported in vitro and in situ calibrations, where EGFP was targeted to a range of subcellular compartments, a range of affinities for protons (~5.8–6.17) was also observed (13, 15, and 27). Moreover, these previous calibrations were carried out between 20 and 22°C, whereas our calibrations were carried out at 37°C. Thus a variety of factors could explain the differences in absolute calibration of EGFP. Because of this uncertainty in probe calibration, absolute pH values must be viewed with some care. Nevertheless, the relative changes in EGFP fluorescence in response to physiological perturbations in our studies were both robust and reproducible.

Another issue relevant to the interpretation of the measured absolute pH values is that the signal is averaged over many vesicles that are likely to exhibit a range of resting pH and unique responses relative to their general state of maturation (2, 20). Because the probe is specifically targeted to the regulated secretory pathway, the signal responses are not associated with changes in pH occurring in other subcellular organelles, which is a difficulty when using many vital dyes (4, 26). Moreover, the imaging results indicate that the contribution of signal from probe retained in the Golgi, endoplasmic reticulum, or cytosol is small (Figs. 1 and 2). Thus the targeted EGFP provides an
excellent measure of changes in pH specifically within the total population of insulin-containing secretory granules.

Agents that activate or potentiate insulin secretion were found to regulate secretory granule pH. Elevating glucose to stimulatory concentrations elicited an acidification of granule pH. Elevations in glucose also have been observed to induce changes in ion transport (Ca²⁺, K⁺) in β-cells (30) and other cell types (17, 18, and references therein), suggesting a general effect of glucose on cellular ion transport. However, the acidification in response to increased glucose in the RIN-3M21 cells was not a general phenomenon because it did not occur in a significant number of EGFP-expressing cells within the population. These cells have likely lost some component of the required glucose-sensing apparatus. In glucose-responsive cells, the observed decrease in secretory granule pH may be best explained by an increased H⁺ transport into secretory granules or decreased H⁻ leak (34) after activation of glycolysis. This acidification was reversible and dependent on an active V-type H⁺-ATPase (Figs. 4–6). However, it was difficult to evaluate the underlying mechanism due to the absence of response to glucose in a large number of cells. Therefore, we addressed this issue under conditions where changes in secretory granule pH were consistently observed (e.g., forskolin, see below). Clearly, the mechanism by which increased glucose metabolism regulates secretory granule pH, and the role this plays in the glucose-induced secretory response in β-cells, warrants further investigation.

A potentially important observation in our studies was the consistent alkalinization of secretory granules elicited by activators of PKA. Increases in cAMP are related to enhanced glucose-stimulated insulin release from β-cells (22, 29) and β-cell lines (8). Here we show that factors known to work through cAMP-mediated pathways not only potentiate EGFP release from RIN-3M21 cells (Table 1) but also consistently alkalinize the lumen of secretory granules whether or not stimulatory glucose was present (Figs. 7 and 8). Mechanistically, this alkalinization could be caused by an inhibition of the V-type H⁺ pump, an increase in H⁺ leak, or a decrease in the conductance of a counter-ion such as Cl⁻. Previous studies using clatherin-coated vesicles (primarily endosomes) indicated that Cl⁻ channel activity is required in parallel with proton pumping by the V-type H⁺-ATPase to maintain electroneutrality and thereby allow protons to accumulate in the vesicle lumen (35). In addition, PKA was shown to activate this channel activity in reconstituted membranes (25). However, this finding predicts that activation of PKA would lead to further acidification of the secretory granules. Moreover, we show that inhibiting Cl⁻ channels with DIDS did not significantly inhibit the forskolin-induced alkalinization (Fig. 8) or influence resting granule pH. Therefore, a role for Cl⁻ conductance in regulating secretory granule pH is unlikely to be found in these β-cells. Consistent with our observations of PKA-induced alkalinization of secretory granules, Zen et al. (36) demonstrated that PKA activation inhibits the normal acidification of endosomal compartments in 3T3 Swiss fibroblasts. Although the molecular mechanism by which PKA modulates secretory granule pH remains to be fully elucidated, there may be an important physiological role for PKA in regulating secretory granule pH.

Because insulin is stored as an array within the granule lumen, it has been proposed that insulin must be “decondensed” from its stored form in order for optimal release to occur (3). Moreover, alkalinization of the granule environment is known to facilitate insulin decondensation (3). The decondensation has been thought to occur after the secretory granules fuse with the plasma membrane, exposing the stored insulin to the extracellular fluid that is near neutral pH. However, alkalinization of the granule lumen before insertion into the membrane may be an important step in priming insulin for release (3). Thus our findings support the notion that elevation of vesicular pH occurs in response to potentiators and may shed light on a mechanism through which cAMP and activation of PKA may act in potentiating glucose-induced insulin release.

In summary, the use of the hGH signal sequence for targeting the fluorescent pH indicator EGFP to the secretory pathway in insulin-secreting cells was a clear improvement on previous efforts to target these compartments. At the cell population level, activation of secretion from the population of cells can be monitored by measuring the appearance of EGFP in the culture medium. However, at the single-cell level, changes in EGFP fluorescence are related to changes in pH within the targeted compartments. The regulation of secretory granule pH by activators of PKA is consistent with a role for secretory granule alkalinization in promoting insulin decondensation before granule insertion into the plasma membrane and, thereby, in the potentiation of insulin release.

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