Cyclic AMP potentiates Ca\(^{2+}\)-dependent exocytosis in pancreatic duct epithelial cells

Seung-Ryoung Jung, Bertil Hille, Toan D. Nguyen, and Duk-Su Koh

Exocytosis is evoked by intracellular signals, including Ca\(^{2+}\) and protein kinases. We determined how such signals interact to promote exocytosis in exocrine pancreatic duct epithelial cells (PDECs). Exocytosis, detected using carbon-fiber microamperometry, was stimulated by \([\text{Ca}\(^{2+}\)]\) increases induced either through Ca\(^{2+}\) influx using ionomycin or by activation of \(\text{P2Y2}\) or protease-activated receptor 2 receptors. In each case, the exocytosis was strongly potentiated when cyclic AMP (cAMP) was elevated either by activating adenylyl cyclase with forskolin or by activating the endogenous vasoactive intestinal peptide receptor. This potentiation was completely inhibited by H-89 and partially blocked by Rp-8-Br-cAMPS, inhibitors of protein kinase A. Optical monitoring of fluorescently labeled secretory granules showed slow migration toward the plasma membrane during Ca\(^{2+}\) elevations. Neither this Ca\(^{2+}\)-dependent granule movement nor the number of granules found near the plasma membrane were detectably changed by raising cAMP, suggesting that cAMP potentiates Ca\(^{2+}\)-dependent exocytosis at a later stage. A kinetic model was made of the exocytosis stimulated by UTP, trypsin, and Ca\(^{2+}\) ionophores with and without cAMP increase. In the model, without a CAMP rise, receptor activation stimulates exocytosis both by Ca\(^{2+}\) elevation and by the action of another messenger(s). With CAMP elevation the docking/priming step for secretory granules was accelerated, augmenting the releasable granule pool size, and the Ca\(^{2+}\) sensitivity of the final fusion step was increased, augmenting the rate of exocytosis. Presumably both cAMP actions require cAMP-dependent phosphorylation of target proteins. CAMP-dependent potentiation of Ca\(^{2+}\)-induced exocytosis has physiological implications for mucin secretion and, possibly, for membrane protein insertion in the pancreatic duct. In addition, mechanisms underlying this potentiation of slow exocytosis may also exist in other cell systems.

INTRODUCTION

Eukaryotic cells discharge secretory vesicles with the plasma membrane, a process that is often regulated. In neurons and endocrine cells, exocytosis uses intracellular Ca\(^{2+}\) as the final trigger and can be enhanced in different ways by protein kinases. Thus, in pituitary gonadotropes, protein kinase C (PKC) increases the Ca\(^{2+}\) sensitivity of exocytosis (Zhu et al., 2002; Yang et al., 2005), whereas in insulin-secreting \(\beta\) cells and in adrenal chromaffin cells, cyclic AMP (cAMP)–dependent protein kinase (PKA) and PKC augment the size of the readily releasable pool of secretory vesicles (Gillis et al., 1996; Nagy et al., 2004; Wan et al., 2004; Yang and Gillis, 2004).

In exocrine cells, potentiation of Ca\(^{2+}\)-dependent exocytosis by protein kinases has received less attention. Secretion of cellular products from epithelial cells can be stimulated independently by Ca\(^{2+}\) and protein kinases, with varying effectiveness depending on the cell type (Koh et al., 2000; Nakahari et al., 2002; Yoshimura et al., 2002; Jung et al., 2004). Unlike excitable cells, epithelial cells seem to have few of their secretory granules in close proximity to the plasma membrane primed for immediate exocytosis (Oda et al., 1996; Chen et al., 2005), so the signals for persistent exocytosis may mobilize secretory vesicles to the plasma membrane and/or promote priming for release. Therefore, there can be a significant delay between the generation of intracellular exocytotic signals and the elevation of exocytosis. In this paper, using different real-time single-cell measurements, we determined whether two stimuli, elevation of Ca\(^{2+}\), and elevation of cAMP, act synergistically on exocytosis in dog pancreatic duct epithelial cells (PDECs). Such cross talk is prominent and involves phosphorylation.
by PKA. The cross talk can be evoked by stimulating different endogenous G protein–coupled receptors (GPCRs) of the duct epithelial cells.

**MATERIALS AND METHODS**

**Chemicals and solutions**

UTP (100 mM) and vasoactive intestinal peptide (VIP; 500 µM) were dissolved as stocks in a saline solution containing: 137.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM Hepes (pH adjusted to 7.3 with NaOH). The trypsin (500 µM) stock was made with distilled water, whereas stocks of ionomycin (1 mM), forskolin (FSK; 1 or 20 mM), thapsigargin (5 mM), and H-89 (10 mM) were dissolved in DMSO. All stock solutions were stored at −20°C except UTP, which was made fresh just before use. VIP was purchased from Bachem. UTP, ionomycin, H-89, and Rp-8-Br-cAMPS were obtained from Calbiochem. Other chemicals were purchased from Sigma-Aldrich. All experiments were performed at room temperature (22–24°C).

**Cell culture**

Nontransformed PDECs were taken from frozen stock that had been derived in 1995 from the main pancreatic duct of dog (Oda et al., 1996). They were cultured on Transwell (Corning Costar) inserts coated with Vitrogen (Inamed Biomaterials) over a feeder layer of human gallbladder myofibroblasts, as previously described (Nguyen et al., 2001; Jung et al., 2006, 2009). For most single-cell experiments except the total internal reflection fluorescence (TIRF) experiments, PDECs were cultured on small Vitrogen-coated glass chips (5×5 mm) coated with Vitrogen, were plated on small glass chips (5×5 mm) coated with Vitrogen, and, 2.9 µM, respectively (Koh et al., 2000; Jung et al., 2004, 2006, 2009). After return to a dopamine-free saline solution, exocytosis was measured as vesicular release of the loaded dopamine. Dopamine oxidation at the tip of the carbon fiber microelectrode (11 µm) polarized to +400 mV generated a spike-like pulse of electric current for each exocytotic event. The amperometric currents were filtered at 0.1 kHz and sampled at 0.5 kHz with an EPC 9 patch-clamp amplifier (HEKA Elektronik).

**Monitoring of granule movements in PDEC**

As described previously (Jung et al., 2009), granule mobility in the cytoplasm was measured from the position of fluorescent granules in wide-field fluorescence images taken every 1.2 s. PDECs, grown on small glass chips (5×5 mm) coated with Vitrogen, were loaded with FM 1–43 (4–8 µM) dye for 6–12 h in conditioned culture medium. Cells were imaged at 535 nm using an epifluorescence microscope fitted with a 100× oil/1.3 N.A. lens (model TE2000; Nikon; excitation at 488 nm with a monochromator; TILL Photonics). Optical magnification (0.1 µm per pixel) was determined with 8-µm beads.

The mobility of granules adjacent to the plasma membrane was measured in a different way. We used prism-based TIRF microscopy, which monitors only objects that lie within several hundred nanometers of the coverslip (Oheim, 2001; Jung et al., 2009). For TIRF experiments, PDECs were grown on 12-mm round glass coverslips coated with poly-l-lysine instead of a Vitrogen layer. The evanescent wave of a 473-nm laser (Extreme Lasers; Seabrook) illuminated FM 1–43-labeled granules near the plasma membrane and adjacent to the glass surface; TIRF images were recorded at 535 nm every 2 s (Jung et al., 2009). To reduce photobleaching, laser light was applied only during TIRF image acquisition. Experiments with ionomycin used Ca²⁺-free solution containing the following: 0.1 mM EGTA, 137.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 10 mM glucose, and 10 mM Hepes (pH adjusted to 7.3 with NaOH).

**Data analysis**

Amperometric spikes were identified semi-automatically using Igor Pro (WaveMetrics). The rate of exocytosis was defined as the number of spikes per 10 s (30 s for the experiments with ionomycin). Cell-to-cell variation was reduced through normalization, setting the baseline value before agonist treatment to 1.0 (“normalized rate of exocytosis”). Relative exocytosis was defined as the mean normalized rate of exocytosis throughout the first 3 min after addition of UTP or trypsin or 2 mM Ca²⁺ in the presence of ionomycin until we mentioned.
Granule mobility was analyzed using an autotracing function based on the threshold algorithm of Metamorph (Universal Imaging) that locates a granule on the x–y plane as previously described (Jung et al., 2009). The speed of granule movement (distance traveled/Δt) was calculated for each image frame, where Δt is the time interval at which images were taken (Δt = 1.2 s for cytoplasmic granules and 2 s for TIRF) and averaged from two adjacent points for Fig. 7. Mean square displacement (MSD) for the nth time interval was calculated using this equation (Qian et al., 1991; Steyer and Almers, 1999):

\[
\text{MSD}(n\Delta t) = \frac{1}{(N-n)} \sum_{j=1}^{N-n} \left\{ \left[ x(j\Delta t + n\Delta t) - x(j\Delta t) \right]^2 + \left[ y(j\Delta t + n\Delta t) - y(j\Delta t) \right]^2 \right\}
\]

where \(x(t)\) and \(y(t)\) are the coordinates of a granule at time \(t\), and \(N\) is the total number of analyzed images. Both \(n\) and \(j\) are positive integers between 1 and \(N-1\). The apparent diffusion coefficient in each condition was calculated as \(\Delta\text{MSD}/\Delta t\) for the first pair of points in such records (at baseline, 1 min after Ca\(^{2+}\) elevation, or 5 min after FSK addition).

All numerical values are given as mean ± SEM. \(N\) and \(n\) denote the number of measured cells and granules, respectively. Statistical significance was determined by Student’s t test; \(P < 0.05\) was considered significant.

**Modeling**

A kinetic model of steps leading to exocytosis was constructed and solved by first-order Euler integration in Igor Pro (WaveMetrics). The reaction model includes sequential steps between cytoplasm, a near-membrane vesicle pool, a docked vesicle pool, and exocytosis, and was modified from that of Fujita-Yoshigaki (2000), which modeled actions of cAMP on exocytosis from epithelial cells of the parotid gland.

**Online supplemental material**

Fig. S1 shows exocytosis induced by cAMP. To find out the optimal cAMP level in the cell, we tested the effect of a broad range of FSK on exocytosis. Fig. S2 presents the effect of [Ca\(^{2+}\)] on granule mobility in the cytoplasm. Cumulative distance traveled, speed, and MSD of a representative granule are analyzed. Fig. S3 shows a TIRF experiment demonstrating no effect of FSK on the granule mobility near the plasma membrane. Fig. S4 illustrates no effect of FSK on Ca\(^{2+}\)-induced translocation of granules toward the plasma membrane. Fig. S5 estimates rates of granule translocation toward the plasma membrane upon UTP stimulation and Ca\(^{2+}\) influx via ionomycin. Video clips for TIRF experiments demonstrate the membrane translocation of granules by [Ca\(^{2+}\)], increase (Movie 1) but not by FSK (Movie 2). Figs. S1–S5 and Movies 1 and 2 are available at http://www.jgp.org/cgi/content/full/jgp.200910355/DC1.

**RESULTS**

We previously demonstrated that exocytosis in PDECs can be promoted by intracellular Ca\(^{2+}\), PKA, and PKC (Koh et al., 2000; Jung et al., 2004; Kim et al., 2008). We now determine whether there is a cross talk between Ca\(^{2+}\) and cAMP/PKA effects on exocytosis. We increase cAMP concentrations by pharmacological agents or by activating endogenous receptors while concomitantly increasing intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)])

FSK increases cAMP

We first verified that cAMP is increased by our FSK treatments. PDECs were transfected with the Epac1-camps probe, which contains a cAMP-binding site flanked by tethered CFP and YFP. Binding of cAMP to the probe decreases the fluorescence of YFP relative to CFP, decreasing the FRET ratio \(F_{\text{YFP}}/F_{\text{CFP}}\) (Nikolaev et al., 2004). Fig. 1 A plots \(F_{\text{CFP}}, F_{\text{YFP}}\) and the FRET ratio (on an inverted axis) for a single cell. When 20 µM FSK is added to activate adenyl cyclase, there is a slow fall of \(F_{\text{YFP}},\) a slow rise of \(F_{\text{CFP}},\) and a slow decrease of the FRET ratio, indicating that cAMP rises considerably, but only gradually, over a 3–4 min period. Fig. 1 B plots averaged

![Figure 1](http://www.jgp.org/cgi/content/full/jgp.200910355/DC1)

**Figure 1.** FSK evokes cAMP increase. Optical measurements of cAMP production in PDECs transfected with Epac1-camps, a FRET probe. (A) Time courses of YFP (dotted olive line) and CFP (dotted cyan line) fluorescence from a single cell treated with 20 µM FSK to stimulate adenyl cyclase. When the FRET ratio \(F_{\text{YFP}}/F_{\text{CFP}}\) (red line, plotted on a reversed axis) decreases, cytoplasmic cAMP concentration increases. (B) Mean normalized (Norm.) FRET ratio with 20 µM FSK (red line, \(n = 6\)) or 100 µM UTP (black line, \(n = 9\)). The gray bar indicates the duration of treatment with FSK or UTP in normal control solution. (C) The effect of 1 µM FSK on cAMP production in cells exposed to a solution free of Ca\(^{2+}\) (0 Ca\(^{2+}\), including 100 µM EGTA) in the presence 1 µM ionomycin for at least 5 min, and then treated with solution containing 2 mM Ca\(^{2+}\) (2 Ca\(^{2+}\), black bar). In this measurement, we used 1 µM FSK as in the later experiments. \(n = 5.\)
Effect of cAMP on Ca\(^{2+}\)-dependent exocytosis

FSK potentiates Ca\(^{2+}\)-evoked exocytosis

We turn to exocytosis. Fig. 2 A displays in a single cell, the simultaneous monitoring of exocytosis, measured as amperometric current, and of [Ca\(^{2+}\)]\(_i\), calculated from fluorescence of the indo-1 Ca\(^{2+}\) indicator. The cell was preincubated with 1 µM ionomycin to allow [Ca\(^{2+}\)]\(_i\), to be regulated by extracellular Ca\(^{2+}\) concentrations. Raising extracellular Ca\(^{2+}\) from 0 to 2 mM led to a modest increase in [Ca\(^{2+}\)]\(_i\), peaking below 1 µM within ~1 min. Exocytosis, shown both as single fusion events (spikes) and mean rates per 30 s time bins (filled circles), was not strongly affected by such small [Ca\(^{2+}\)]\(_i\) increases. Fig. 2 B shows the mean time courses of [Ca\(^{2+}\)]\(_i\), increase (peak: 0.64 ± 0.1 µM) and of exocytosis from several cells. During the first 3 min after Ca\(^{2+}\) influx, mean relative exocytosis was about the same (0.9 ± 0.2) as in the 3 min control period (n = 6; Table I), and after several minutes, it drifted slightly above the control level.

FSK (1 µM) pretreatment had a clear effect on Ca\(^{2+}\)-evoked exocytosis (Fig. 2, C and D). FSK itself gradually evoked a small increase in exocytosis. A subsequent [Ca\(^{2+}\)]\(_i\) increase (peak: 0.78 ± 0.12 µM) achieved by raising extracellular Ca\(^{2+}\) to 2 mM evoked a dramatic increase in exocytosis. In these experiments, the [Ca\(^{2+}\)]\(_i\) rose to a peak and relaxed back to an elevated plateau with 2 mM Ca\(^{2+}\) still in the bath, and the rate of exocytosis paralleled this time course. Mean exocytosis during the first 3 min in 2 mM Ca\(^{2+}\) rose 6.5-fold compared with the initial low-Ca\(^{2+}\) control period, and 3.1-fold compared with FSK alone (Fig. 2 D and Tables I and II). Both values were significantly larger than the exocytosis with Ca\(^{2+}\) alone (Fig. 2 B; P < 0.05). In another set of experiments, we varied FSK concentration from 0.01 to 10 µM (Fig. S1). Exocytosis induced by Ca\(^{2+}\) influx via ionomycin was gradually increased with a half-maximal effect at 0.4 µM FSK. In summary, FSK sensitizes exocytosis in a graded manner to modest pharmacological Ca\(^{2+}\) elevations, and without FSK, these Ca\(^{2+}\) elevations barely stimulate any exocytosis.

FSK potentiates exocytosis evoked by purinergic and protease-activated receptor 2 (Par-2) receptors

PDECs express P2Y (purinergic) and PAR-2 (protease-activated) G\(_q\)-coupled GPCRs whose activation can elevate [Ca\(^{2+}\)]\(_i\), and stimulate exocytosis (Nguyen et al., 1999; Jung et al., 2004, 2006; Kim et al., 2008). Can exocytosis mediated by these endogenous receptors also be potentiated by FSK? We first used UTP as a P2Y2 agonist in PDECs. At 2 µM, UTP elicited oscillating elevations in [Ca\(^{2+}\)]\(_i\), whose peak values were higher than those
despite the continued presence of trypsin (Fig. 3 C and Table II). This short Ca\(^{2+}\) spike elicited only a small and brief exocytotic response (Fig. 3 D and Table I). On the other hand, after FSK pretreatment, the trypsin treatment evoked a very large exocytotic response (Fig. 3 D and Table I). In conclusion, FSK strongly potentiates exocytosis elicited by UTP and trypsin.

Potentiation by FSK occurred over the full range of UTP concentrations. Fig. 4 explores UTP concentrations from 0.5 to 100 µM. Progressively higher concentrations of UTP elicited progressively intensifying \([\text{Ca}^{2+}]_i\) oscillations, and at 100 µM, a \([\text{Ca}^{2+}]_i\) plateau (Fig. 4, A–D). UTP elicited progressively larger exocytosis, but without FSK, the normalized exocytosis achieved using ionomycin and 2 mM Ca\(^{2+}\) in the bath (Fig. 3 A). Adding 1 µM FSK did not significantly affect the peak amplitudes, duration, or number of Ca\(^{2+}\) peaks with UTP (Table II). In the absence of FSK, the oscillatory \([\text{Ca}^{2+}]_i\) elevations almost doubled the rate of exocytosis relative to rest (Fig. 3 B and Table I), but this exocytotic response was slow, taking a few minutes to develop. On the other hand, after FSK pretreatment, the exocytotic response to UTP was robust and developed more quickly (Fig. 3 B and Table I).

We obtained similar results when stimulating with trypsin as an agonist of the endogenous PAR-2 receptors. In response to 0.1 µM trypsin, \([\text{Ca}^{2+}]_i\), rose briefly to a high level and fell quickly back to baseline without a plateau, despite the continued presence of trypsin (Fig. 3 C and Table II). This short Ca\(^{2+}\) spike elicited only a small and brief exocytotic response (Fig. 3 D and Table I). On the other hand, after FSK pretreatment, the trypsin treatment evoked a very large exocytotic response (Fig. 3 D and Table I). In conclusion, FSK strongly potentiates exocytosis elicited by UTP and trypsin.

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### Table I

<table>
<thead>
<tr>
<th>Stimulatory treatment</th>
<th>Relative exocytosis</th>
<th>Stimulatory treatments and inhibitors</th>
<th>Relative exocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Ca(^{2+}) with ionomycin</td>
<td>0.9 ± 0.2 (n = 6)</td>
<td>0.5 µM UTP + FSK with ionomycin</td>
<td>6.5 ± 2.4* (n = 12)</td>
</tr>
<tr>
<td>0.5 µM UTP</td>
<td>1.2 ± 0.3 (n = 10)</td>
<td>2 µM UTP + FSK</td>
<td>5.6 ± 0.6** (n = 25)</td>
</tr>
<tr>
<td>2 µM UTP</td>
<td>1.9 ± 0.3 (n = 14)</td>
<td>10 µM UTP + FSK</td>
<td>8.1 ± 1.5** (n = 15)</td>
</tr>
<tr>
<td>100 µM UTP</td>
<td>3.5 ± 0.3 (n = 15)</td>
<td>100 µM UTP + FSK</td>
<td>12.7 ± 2.1** (n = 15)</td>
</tr>
<tr>
<td>0.1 µM Trypsin</td>
<td>1.6 ± 0.2 (n = 8)</td>
<td>0.1 µM trypsin + FSK + thapsigargin</td>
<td>9.7 ± 2.0** (n = 10)</td>
</tr>
<tr>
<td>0.1 µM VIP</td>
<td>2.1 ± 0.3 (n = 11)</td>
<td>2 µM UTP + VIP</td>
<td>1.8 ± 0.6*** (n = 10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 µM UTP + FSK + H-89</td>
<td>8.0 ± 1.5** (n = 11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 µM UTP + FSK + thapsigargin</td>
<td>1.6 ± 0.6** (n = 7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 µM UTP + FSK + H-89</td>
<td>1.7 ± 0.5** (n = 9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 µM UTP + FSK + Rp-8-Br-cAMPS</td>
<td>3.2 ± 0.8** (n = 6)</td>
</tr>
</tbody>
</table>

Relative exocytosis was calculated for the first 3 min after application of agonist treatment in the absence or presence of FSK (1 µM) or VIP (0.1 µM). Concentrations of reagents: ionomycin (1 µM), thapsigargin (1 µM), H-89 (30 µM), and Rp-8-Br-cAMPS (1 mM). *, P < 0.05, **, P < 0.005 significantly different compared to UTP or trypsin alone at the same concentrations. For ionomycin, we compared the values with and without FSK. *, P < 0.05, **, P < 0.005 significantly different compared to 2 µM UTP + FSK. ###, P < 0.005 significantly different compared to 0.1 µM trypsin + FSK. n is the number of cells.

### Table II

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Peak ([\text{Ca}^{2+}]_i) (µM)</th>
<th>Duration (s)</th>
<th>Number of peaks</th>
<th>Period (s)</th>
<th>Percentage of oscillatory cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 µM UTP</td>
<td>1.5 ± 0.3</td>
<td>288 ± 38</td>
<td>10 ± 1</td>
<td>27 ± 1.1</td>
<td>100% (5/5)</td>
</tr>
<tr>
<td>0.5 µM UTP + FSK</td>
<td>1.7 ± 0.3</td>
<td>230 ± 14</td>
<td>7 ± 0.7</td>
<td>32 ± 1.4</td>
<td>100% (3/3)</td>
</tr>
<tr>
<td>2 µM UTP</td>
<td>1.9 ± 0.2</td>
<td>240 ± 20</td>
<td>12 ± 1</td>
<td>20 ± 1.2</td>
<td>88% (14/16)</td>
</tr>
<tr>
<td>2 µM UTP + FSK</td>
<td>2.0 ± 0.4</td>
<td>239 ± 41</td>
<td>11 ± 2</td>
<td>23 ± 1.5</td>
<td>75% (6/8)</td>
</tr>
<tr>
<td>2 µM UTP + VIP</td>
<td>2.7 ± 0.1</td>
<td>218 ± 34</td>
<td>10 ± 2</td>
<td>21 ± 2.2</td>
<td>82% (9/11)</td>
</tr>
<tr>
<td>2 µM UTP + FSK + H-89</td>
<td>2.9 ± 0.4</td>
<td>193 ± 38</td>
<td>6 ± 1</td>
<td>31 ± 2.9</td>
<td>86% (6/7)</td>
</tr>
<tr>
<td>2 µM UTP + FSK + Rp-8-Br-cAMPS</td>
<td>1.8 ± 0.4</td>
<td>206 ± 60</td>
<td>10 ± 4</td>
<td>20 ± 1.5</td>
<td>75% (3/4)</td>
</tr>
<tr>
<td>10 µM UTP</td>
<td>2.6 ± 0.3</td>
<td>186 ± 38</td>
<td>11 ± 2</td>
<td>17 ± 0.8</td>
<td>89% (8/9)</td>
</tr>
<tr>
<td>10 µM UTP + FSK</td>
<td>2.4 ± 0.2</td>
<td>193 ± 35</td>
<td>11 ± 2</td>
<td>17 ± 0.6</td>
<td>45% (5/11)</td>
</tr>
<tr>
<td>100 µM UTP</td>
<td>3.3 ± 0.1</td>
<td>155 ± 36</td>
<td>8 ± 2</td>
<td>22 ± 1.6</td>
<td>57% (4/7)</td>
</tr>
<tr>
<td>100 µM UTP + FSK</td>
<td>3.5 ± 0.3</td>
<td>74 ± 7</td>
<td>4 ± 1</td>
<td>18 ± 3.3</td>
<td>38% (3/8)</td>
</tr>
<tr>
<td>0.1 µM trypsin</td>
<td>3.6 ± 0.7</td>
<td>3.2 ± 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Duration, number of peaks, and period were defined as total time lapsed from the first to the final peaks, the total number of peaks, and their quotient. For these three parameters, only cells showing oscillations were included for analysis. The concentrations of FSK, VIP, H-89, and Rp-8-Br-cAMPS were 1 µM, 0.1 µM, 30 µM, and 1 mM, respectively. The \([\text{Ca}^{2+}]_i\) did not oscillate with 0.1 µM trypsin in the absence or presence of FSK. Total number of cells for the experiments with trypsin with and without FSK was five and seven, respectively.
remained modest, peaking below 5 in 100 μM UTP (Fig. 4, E–H, and Table I). In the presence of FSK, the characteristics of the [Ca\(^{2+}\)]\(_e\), elevations elicited by UTP were unchanged (Table II), but normalized exocytosis was strongly potentiated at all UTP concentrations, reaching a peak of 16 at 100 μM (Fig. 4, E–H, and Table I).

**Potentiation requires coincident elevation of Ca\(^{2+}\), cAMP, and PKA**

Thus far, in describing the pharmacological actions of FSK, we implicitly assumed that the action of FSK on exocytosis was caused by cAMP elevation and downstream PKA activation. This assumption was verified through experiments described in this study. PDECs express endogenous VIP receptors that couple to adenyl cyclase and elevate cAMP (Oda et al., 1996; Zhang et al., 2000). The addition of 100 nM VIP did not change the [Ca\(^{2+}\)]\(_e\), pattern evoked by 2 μM UTP, but it did change exocytosis. Addition of VIP stimulated exocytosis modestly by itself (2.1 ± 0.3, n = 11), as previously reported (Oda et al., 1996), and upon further addition of 2 μM UTP, exocytosis was strongly augmented (8.0 ± 1.5; n = 11; Table I). 100 nM VIP increased the rate of UTP-induced exocytosis by 4.2-fold. Similarly, 1 μM FSK increased the rate of UTP-induced exocytosis 2.9-fold. Thus, VIP and FSK both potentiate exocytosis induced by UTP (Fig. 2), which is consistent with the hypothesis that their common action, to raise cAMP, is responsible for their actions on exocytosis. These experiments also show that the potentiation we have described can be induced by an endogenous physiological receptor that raises cAMP.

We then considered the effects of two agents that block PKA action pharmacologically. As anticipated, pretreatment for 6 min with 30 μM of a PKA inhibitor, H-89, before exposure to 2 μM UTP, eliminated the potentiation of UTP-induced exocytosis by FSK (Fig. 5 B and Table I) without affecting the [Ca\(^{2+}\)]\(_e\), increase induced by UTP (Fig. 5 A and Table II). Similarly, the cAMP antagonist, Rp-8-Br-cAMPS (1 mM), partially eliminated potentiation of exocytosis by FSK without affecting [Ca\(^{2+}\)], (Tables I and II). These experiments show that the underlying mechanism for FSK potentiation of UTP-induced exocytosis requires cAMP and PKA.

If potentiation of exocytosis requires cAMP and PKA, does it also require [Ca\(^{2+}\), elevation? We tested whether FSK can potentiate actions of UTP or trypsin under conditions that abrogate the receptor-induced Ca\(^{2+}\), elevations. [Ca 2+]\(_i\) release from intracellular sources was eliminated by emptying the Ca\(^{2+}\), stores with thapsigargin, an inhibitor of sarco- and endoplasmic reticulum Ca\(^{2+}\), pumps, whereas Ca\(^{2+}\), influx from outside was abolished with a Ca\(^{2+}\)-free extracellular medium (Kim et al., 2008). Under these Ca\(^{2+}\)-free conditions, [Ca2+]i remained at the 0.1 μM resting level while cells were treated with UTP or trypsin (Fig. 6, A and C). Before purinergic or PAR-2 receptor stimulation, FSK slightly increased the resting exocytosis and further stimulation of the purinergic or PAR-2 receptor caused only minimal additional stimulation (Fig. 6, B and D). Thus, receptor activation alone is not sufficient to potentiate FSK; downstream [Ca\(^{2+}\)], signals are required.

cAMP does not change granule mobility in the cytoplasm

In other cell types, potentiation of exocytosis by different kinases has variously been attributed to an increase in the speed of granule movement (insulin-secreting β-cells: Hisatomi et al., 1996; Tsuboi et al., 2003), to an increase in the pool size of granules ready for release (β-cells and adrenal chromaffin cells: Gillis et al., 1996; Nagy et al., 2004; Wan et al., 2004), or to an increased sensitivity of these granules to Ca\(^{2+}\), (pituitary gonadotropes; Zhu et al., 2002). We tested some of these possibilities in PDECs.

To investigate the possibility that cAMP may potentiate Ca\(^{2+}\)-induced exocytosis by increasing the mobility...
of secretory granules, we studied the movements of granules labeled with a fluorescent dye. PDECs were incubated for 6–12 h in 4 µM FM 1–43, a membrane-impermeant lipophilic dye that labels intracellular membranes after endocytosis (Fig. S2; Jung et al., 2009). Most labeled granules were similar in size to mucin granules previously identified in PDECs by electron microscopy (Oda et al., 1996; Jung et al., 2009), and we have previously shown immunohistochemically that mucin is colocalized in granules labeled with the fixable analogue FM 1-43FX (Jung et al., 2009). Movement of FM 1–43–labeled granules in the cytoplasm was analyzed in a series of fluorescence images (Fig. S2 A, a). A representative single-granule trajectory is shown on an expanded scale in Fig. S2 A, b. The cumulative distance traveled, speed, and MSD are plotted in the remaining panels. The steeper the slope of cumulative distance traveled, the higher the mean speed. Fluctuations in granule speed are deemphasized in this cumulative plot. The speed of granule motion was defined as the distance traveled per unit time (µm/s or nm/s). Plots of MSD as a function of time would rise linearly for a random walk, sublinearly for caged motion, or supralinearly for directed motion (Jung et al., 2009). For the quasistationary granules, the plot was convex, suggesting that their movement was somehow restricted. Faster granules had linear MSD plots, indicating random motion. This apparently random motion was driven less by thermal agitation than by molecular motors attached to the granules (Jung et al., 2009). The initial slope of an MSD curve is proportional to the apparent diffusion constant of a granule. Sometimes, we observed long-range movement of granules that underwent stop-and-go motion on the cytoskeleton. In these cases, plots of the MSD included both convex and concave curvature as in Fig. S2 D.

As previously reported for PDECs (Jung et al., 2009), when [Ca^{2+}], was elevated by external 2 mM Ca^{2+} solution in the presence of ionomycin, the speed of granule motion decreased sharply (Fig. 7 A; see Fig. S2 C for a representative single granule). The mean speed slowed from 74 ± 18 nm/s in low Ca^{2+} basal conditions to 38 ± 4 nm/s in elevated Ca^{2+} with a time delay of ~10 s (Fig. 7 A). To our surprise, FSK affected neither the basal speed of granule motion in the cytoplasm (59 ± 11 nm/s with FSK vs. 58 ± 9 nm/s without FSK; Fig. 7 B) nor the Ca^{2+}-induced reduction of granule mobility (66 ± 13 nm/s before vs. 57 ± 5 nm/s after the increase in Ca^{2+}; Fig. 7 C). Then granule motion was assessed as apparent diffusion constant estimated from the MSD, FSK
cAMP also does not change granule movement close to the plasma membrane.

An alternative to potentiation of exocytosis was that cAMP might selectively affect movement of the granules close to the plasma membrane. We used TIRF microscopy to visualize FM 1–43 labeled granules that were within a few hundred nanometers of the membrane–glass interface (Öheim, 2001; Jung et al., 2009). Again, the cells were treated with 1 µM ionomycin. As shown in Fig. 8 A and Video 1, bright FM 1–43–loaded granular structures could be visualized near the plasma membrane. The trajectories of granules in Fig. 8 B were plotted in the x–y plane before and after intracellular Ca²⁺ rise. Granule “1” moved over a larger x–y territory than granule “2” (Fig. 8 B), it had steeper slope on the x–y distance traveled plot (Fig. 8 C), it showed higher bursts of speed (Fig. 8 D), and it slowed down more with elevated Ca²⁺.

The mobility of granules in the z axis could be gauged from intensity changes in TIRF microscopy because intensity rises as granules come closer to the plasma membrane. In this example, granule “2,” which was “less mobile” in the x–y plane showed greater movement on the z axis during Ca²⁺ rise than granule “1” (Fig. 8 E). As we reported before (Jung et al., 2009), when [Ca²⁺]ᵢ was increased by application of external 2 mM Ca²⁺ in the presence of 1 µM ionomycin, most granules visible in TIRF gradually brightened after their x–y movements stopped, and then their intensity increased saturated, presumably reflecting arrival near the plasma membrane or even docking (Fig. 8 F and Video 1). Evaluated in this manner, granules started to migrate toward the surface ~20 s after the [Ca²⁺]ᵢ increase and reached steady state with a time constant (τ) of 46 s (Fig. 9 A).

For further description see Fig. S5.
the patterns of x–y motion, cumulative distance traveled, and speed of granules in the presence of FSK as shown in Fig. S3 were similar to those in control conditions without FSK. Also, by itself, FSK did not induce migration of granules toward the plasma membrane (Fig. 9 B, Fig. S3, and Video 2). When combined with Ca\textsuperscript{2+} elevation, FSK did not alter the characteristics of Ca\textsuperscript{2+}-induced migration toward the plasma membrane, such as migration onset (\(\sim 20\) s delay after Ca\textsuperscript{2+} rise) and time constant (45 s) of translocation. In addition, the changes of trajectory, cumulative distance traveled, and speed of granules in the x–y plane during Ca\textsuperscript{2+} rise (Fig. S4, B–D) were similar to those in the absence of FSK. After the Ca\textsuperscript{2+} stimulus was removed, the granules drifted back to the cytoplasm; however, their return took much longer (\(\tau = 552\) s with and 720 s without FSK, respectively) than the decline of [Ca\textsuperscript{2+}], to baseline (\(\tau = 26\) s with and 23 s without FSK; Fig. 9 D).

Finally, the number of granules next to the plasma membrane was tallied, at baseline and following an increase in [Ca\textsuperscript{2+}], (with ionomycin) or cAMP (with FSK), alone or in combination. As shown in Fig. 9 E, granules accumulated near the plasma membrane following an increase in [Ca\textsuperscript{2+}], but no further accumulation was observed with FSK, either alone or together with increased [Ca\textsuperscript{2+}]. A correspondence between normalized granule counts (Fig. 9 E) and the overall normalized increase in brightness of the TIRF image (Fig. 9, A and C) allows us to consider the time course of brightness as a reasonable indicator of the time course of granule number near the cell surface.

In summary, although activation of the cAMP pathway potentiates Ca\textsuperscript{2+}-dependent exocytosis, we could detect no changes in the movements of secretory granules or in the number of granules near the plasma membrane during FSK treatment. Instead, potentiation must occur at some late steps after granules are near the plasma membrane, a change that would not be detected by our optical techniques.

A mathematical model for potentiation of Ca\textsuperscript{2+}-dependent exocytosis by cAMP

Where does cAMP act? To develop a working hypothesis for the effects of cAMP elevation, we considered minimal kinetic schemes for the steps leading to exocytosis. Fig. 10 shows the result. In the cartoon of Fig. 10 A, secretory granules in the cytoplasm can translocate to a near-membrane pool, become docked and primed at the membrane, and be released by exocytosis in response to appropriate stimuli. The more formal kinetic scheme in Fig. 10 B says that: Ca\textsuperscript{2+}, UTP, and trypsin
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First, consider the intracellular messengers in the model: actions of three different messengers need to be described. Their assumed time courses for a single example (the FSK and 100 µM UTP case) are shown as 3 traces from the simulation in Fig. 11. Application of FSK leads to a slow rise of cAMP as reported by the Epac1-camps probe in Fig. 1. In the model, it rises from an initial value of 0.05 to saturation at a value of 3 with a time constant of 200 s after FSK is applied. The Epac probe measurements are uncalibrated, but for illustrative purposes we will consider that the units for cAMP in the graph in Fig. 11 are approximately micromolar. Application of UTP leads to a Ca\(^{2+}\) rise. For its time course, we simply take the actual recorded Ca\(^{2+}\) trace for the appropriate condition and filter it with a time constant of 15 s because most of the relative exocytosis data are averages over 10 s segments. Again, the units are micromolar. Finally, we found improvements in the fit of exocytosis time courses if we also assumed the existence of a third messenger, UM, produced by stimulation of affect recruitment to the cell surface (pool A); cAMP and an unspecified messenger (UM) produced by receptor activation promote docking and priming (adding to pool B); and Ca\(^{2+}\), cAMP, and UM act in concert to promote exocytosis of vesicles from pool B. As is usual with kinetic models, pools A and B are formal kinetic states that we can suggest correspond to membrane and docked/primed vesicles, but any success of the model does not prove that that identification is correct. Mathematical details of the model are given below and in the Supplemental text. The implementation was guided by trying to use a single set of parameters to generate simulations for all conditions. Fig. 10 C compares actual exocytotic data (symbols) with predictions from the model (blue lines) for 10 conditions. For each case (ionomycin, three concentrations of UTP, and trypsin), both the exocytosis without FSK and the effects of cAMP elevation are reasonably described. We now outline the logic for each step that yields the equations listed in the Supplemental text.

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G_{q}-coupled receptors (P2Y or PAR-2) that increased with a time constant of 40 s during receptor stimulation. Its value started at 1.0 (dimensionless) at rest, which means no activation of UM and, depending on the intensity of the stimulus (i.e., concentration of receptor agonist), rose to a maximum of no more than 5.0 during receptor activation (1, 1.5, 2.5, and 5 for 2, 10, and 100 µM UTP and for 0.1 µM trypsin, respectively). In the experiments with Ca^{2+} plus ionomycin, there is no increase of UM (1.0).

Why was the UM messenger added to the model? As the assumptions for UM just given suggest, UM produces a graded increase of exocytosis at only higher concentrations of the agonists that stimulate G_{q}-coupled receptors. The experimental data do show much more exocytosis, for example, with 100 µM UTP than with 2 µM UTP. One might have imagined that this larger exocytosis could be predicted from a larger Ca^{2+} signal with 100 µM UTP. However, the growth of the Ca^{2+} signal between 2 and 100 µM UTP is small (Fig. 4), and we were unable to find a mathematical expression based on Ca^{2+} alone that gave the required growth of exocytosis and yet was consistent with the other observations.

Next consider A, the membrane pool of granules. We reasoned that pool A should include granules in the vicinity of the membrane at rest plus some of the additional granules we saw migrating from the cytoplasm during Ca^{2+} elevations, as in Figs. 8, 9, S4, and S5. Such additional migration occurs whenever Ca^{2+} rises as for ionomycin with Ca^{2+}, for UTP, and for trypsin. Thus, pool A was clamped to a fixed time course equal to its resting value (arbitrarily 500) plus a fraction of the extra granules seen translocating by TIRF microscopy. For all simulations, a reasonable choice for that fraction was 1/3, as if 2/3 of the observed translocated granules did not join pool A and 1/3 did. An example of the assumed time course of A (normalized) is shown in Fig. 11 again for a calculation representing FSK plus 100 µM UTP. The smooth time course was obtained by fitting an empirical function to the granule intensity time course to mimic the delay, steepness, amplitude, and late decay of TIRF measurements for 100 µM UTP (Fig. S5). Implicit here is the approximation that the number of granules near the surface is proportional to their brightness measured in TIRF microscopy.

Figure 9. cAMP does not affect granule migration. Before each experiment, cells were preincubated with 1 µM ionomycin for at least 5 min in Ca^{2+}-free solution (0 Ca^{2+}), and all test solutions contained 1 µM ionomycin in a Ca^{2+}-free solution. Treatments with different concentrations of extracellular Ca^{2+} and with FSK are indicated by bars at the top of each graph. The open, black, and gray bars indicate 0 mM Ca^{2+} (0 Ca^{2+}), 2 mM Ca^{2+} (2 Ca^{2+}), and 1 µM FSK, respectively. (A and C) Mean time course of intensity of granules (A, N = 24, and C, N = 10, black line) approaching to the plasma membrane during an increase in [Ca^{2+}] (A, n = 13, and C, n = 11, green line) in the absence (A) or presence (C) of 1 µM FSK. (B) Mean time course of intensity change of granules (black line) and [Ca^{2+}] (green line) after the FSK treatment (n = 11). (D) Mean time course of return of granule intensity after Ca^{2+} removal. The black and red lines indicate kinetics of granule moving away from the plasma membrane in the absence or presence of FSK, respectively. [Ca^{2+}] level is shown as green dotted line without FSK and green solid line with FSK. (E) Number of granules near the plasma membrane under the conditions 0 Ca^{2+}, 2 Ca^{2+} (n = 23), FSK + 0 Ca^{2+} (n = 9), and FSK + 2 Ca^{2+} (n = 9), normalized to that in 0 Ca^{2+} in each experiment.
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by a saturation function as cAMP rises, and the acceleration is augmented linearly by any rise of UM. UM acts only on the potentiation caused by cAMP. For example, in the calculation for Fig. 11, $k_{AB}$ is 0.0028 min\textsuperscript{-1} at rest, rises slowly to 0.0088 during FSK, and rises again to 0.0196 with further addition of 100 µM UTP. Thus, the combination of FSK and receptor activation strongly accelerates the docking/priming step. The forward flux is opposed by a small reverse flux (undocking) with $k_{BA} = 0.034$ min\textsuperscript{-1}.

Figure 11. Details of a sample calculation with the model showing the time course of the internal variables. All traces are simulations for experiments with perfusion of 1 µM FSK (Fig. 10 B), 1 µM FSK plus 100 µM UTP, and back to 1 µM FSK alone. (A) The three messengers, Ca\textsuperscript{2+}, cAMP, and unidentified messenger (UM). The Ca\textsuperscript{2+} record is the mean recorded Ca\textsuperscript{2+} but filtered (smoothed). (B) The normalized near-membrane granule pool A (initially 500 granules, blue line) and the normalized docked/primed pool B (initially 10 granules, black line). The initial percent of docked/primed granules was 2%. The time course of pool A is dictated by the measurements of granule migration. The time course of pool B reflects stimulation of docking/priming by FSK and depletion of docked/primed vesicles as they are exocytosed. (C) Predicted exocytosis reflecting strong stimulation during UTP because of the simultaneous presence of all three messengers: Ca\textsuperscript{2+}, cAMP, and UM.
The last step is exocytosis from the docked/primed pool B. The resting pool size is (arbitrarily) 10 and the rate constant for exocytosis ($k_{BC}$) in units of min$^{-1}$ is:

$$k_{BC} = 0.1 + 0.3 \times UM \left[ \frac{[Ca^{2+}]^4}{[Ca^{2+}]^4 + K_{Ca}^{4}(cAMP)} \right]$$

Again, this rate constant is accelerated directly by two messengers, UM and Ca$^{2+}$, where Ca$^{2+}$ enters as a saturating Hill function with an exponent of 4. The fourth power gives exocytosis in these epithelial cells the kind of cooperative dependence on Ca$^{2+}$ seen in neurons and endocrine cells (Dodge and Rahamimoff, 1967; Heinemann et al., 1994; Tse et al., 1997). An additional feature here adds cAMP sensitivity. At rest $K_{Ca}$, the concentration for half-maximal effect is 0.9 µM Ca$^{2+}$, and with cAMP elevation it falls to 0.5 µM. In this way, cAMP sensitizes exocytosis to Ca$^{2+}$ elevations, lowering the concentration of agonists (UTP) needed to attain certain rates of exocytosis. In the simulation of Fig. 11, the rate constant $k_{BC}$ has a value of 0.1 (min$^{-1}$) at rest, rises only slightly to 0.107 during FSK, and increases to a peak of 0.82 during the peak of Ca$^{2+}$ evoked by 100 µM UTP.

The effect of these messenger-dependent rate constants can be seen in the simulated traces for the docked/primed granule pool (B) and for exocytosis in Fig. 11 (B and C). Upon addition of FSK, the docked/primed pool B starts to grow slowly because $k_{AB}$ is increased, and exocytosis rises a little as B grows without any change of $k_{BC}$. Further addition of UTP produces a strong Ca$^{2+}$ signal that quickly increases $k_{BC}$ and promotes exocytosis. The rapid exocytosis depletes some of pool B, but in the meantime pool A is growing as more vesicles migrate to the membrane so the supply of vesicles to B gets faster. Receptor activation also produces messenger UM, which augments both exocytosis and the rate of docking/priming. As the Ca$^{2+}$ falls from its peak toward a plateau, it drops to near the half-activation point and exocytosis declines a bit. Upon removal of UTP, Ca$^{2+}$ and UM drop, and the burst of exocytosis comes to an end.

**DISCUSSION**

Using carbon fiber microamperometry, we demonstrated that Ca$^{2+}$-dependent exocytosis in PDECs is synergistically amplified by coactivation of cAMP signaling. This effect was observed when [Ca$^{2+}$]$_i$ was increased pharmacologically using ionomycin as a Ca$^{2+}$ ionophore or more physiologically by activating endogenous P2Y$_2$ or PAR-2 receptors. Stimulation of P2Y$_2$ receptors under conditions that prevented a [Ca$^{2+}$]$_i$ increase did not speed exocytosis. The potentiation of exocytosis occurred whether adenyl cyclase was activated pharmacologically with FSK addition or more physiologically through endogenous VIP receptors. The effect of cAMP elevation was blocked by H-89 and partially blocked by Rp-8-Br-cAMPS, inhibitors of PKA. Hence, the potentiation requires protein phosphorylation as well as Ca$^{2+}$. Several hypotheses could be ruled out. Thus, we found that increased cAMP concentrations did not modify the intensity or pattern of Ca$^{2+}$ signals induced by ionomycin or purinergic receptors. Further, with direct imaging of the secretory granules, we detected no effect of cAMP on the trafficking of these granules to the plasma membrane. We conclude that cAMP has key influence(s) on steps that take place at or very close to the plasma membrane.

**Epithelial exocytosis probably differs from excitable-cell exocytosis in details**

Regulated exocytosis mediates rapid vesicular secretion in neurons and endocrine cells. In the classical description, secretory granules bud off from late Golgi structures and traffic to the proximity of the plasma membrane where they form a reserve pool. Subsequently the granules dock at the plasma membrane, become primed, and with appropriate signals, quickly fuse with the membrane to release their contents. Each stage includes physical and chemical reactions (Südhof, 2004). Exocytic signals, including Ca$^{2+}$ and protein kinases modulate steps before fusion, such as granule trafficking (Hisatomi et al., 1996; Niwa et al., 1998; Tsuboi et al., 2003), granule mobility (e.g., through dynein, kinesin, and myosin; Rodionov et al., 2003; Kashina et al., 2004), and maintenance of the reserve pool (e.g., synapsin I; Bonanomi et al., 2005; Menegon et al., 2006). The final vesicular fusion requires interactions between the vesicular v-SNARE, VAMP/synaptobrevin, and plasma-membrane t-SNAREs syntaxin and SNAP-25 (Südhof, 2004). This step is powerfully regulated by Ca$^{2+}$ with further modulation by protein kinases (Trudeau et al., 1998; Ilardi et al., 1999; Chheda et al., 2001; Seino and Shibasaki, 2005). The synaptotagmin-1 calcium sensor for fast exocytosis shows steep cooperativity but low affinity, with half saturation ranging from 10 to 100 µM Ca$^{2+}$. It triggers release of granules in a burst from an immediately releasable pool within milliseconds and regulates exocytosis over an enormous dynamic range (Südhof, 2004). The synaptotagmin-7 calcium sensor shows higher affinity for Ca$^{2+}$, binds Ca$^{2+}$ more slowly, and in chromaffin cells mediates slow exocytosis (Schonn et al., 2008).

Exocytosis from exocrine secretory cells is not as well characterized as for excitable cells. A different subset of underlying proteins is found in epithelial cells: rab3D (Valentin et al. 1996), synaptotagmin-2 and MUNC13-2 (Davis and Dickey, 2008), syntaxin 2 (Hansen et al., 1999), and SNAP-23 (Gaisano et al., 1997). A pool of docked or primed secretory granules is less evident (Oda et al., 1996; Chen et al., 2005), although the concept of an immediately releasable pool of granules has...
not been tested rigorously. The apparent steps of regulation are impressively slower both in onset and in termination, taking tens of seconds rather than a few milliseconds, and the physiological dynamic range of secretory rates is much smaller, typically only one order of magnitude. Thus, mechanisms for different steps of exocytosis in PDECs are likely to have different quantitative properties and different synergy between cAMP and Ca\(^{2+}\). Exocytosis from PDECs can be triggered by [Ca\(^{2+}\)]\(_i\) rise (Koh et al., 2000; Jung et al., 2004, 2006). The secretion we see is more like slow, asynchronous release. According to our current modeling, the calcium sensor for exocytosis has a much higher affinity for Ca\(^{2+}\) (\(\sim 1\) µM without FSK versus submicromolar with FSK). However, the latter conclusion should be tempered by the absence so far of experiments with caged Ca\(^{2+}\) in PDECs that could explore high Ca\(^{2+}\) concentrations.

Unexpectedly, our recent studies of the intracellular trafficking of secretory granules in these cells revealed that [Ca\(^{2+}\)]\(_i\), increase hinders granule mobility through Ca\(^{2+}\)-dependent F-actin formation and then initiates a concerted, slow granule translocation toward the plasma membrane by F-actin and myosin motors (Jung et al., 2009). The slow onset kinetics of vesicular migration, paralleling that of exocytosis, led us to wonder whether the rate-limiting step for Ca\(^{2+}\)-induced exocytosis is vesicular migration rather than plasma membrane fusion, but we did not find evidence that the migration is rate limiting. More broadly, for some epithelia, the principal secretagogues (VIP, secretin) may be ones that act on GPCRs that elevate cAMP rather than Ca\(^{2+}\), so cAMP is considered the primary regulator of exocytosis in those tissues (Hille et al., 1999). Further, other secretagogues acting via G\(_i\) and phospholipase C (PLC) activate PKC and raise Ca\(^{2+}\), and PKC alone can evoke exocytosis. Indeed cAMP or activators of PKC alone suffice to evoke some secretion from epithelial cells (Koh et al., 2000; Nakahari et al., 2002; Yoshimura et al., 2002; Jung et al., 2004, Kim et al., 2008).

**Dissection of targets for Ca\(^{2+}\) and cAMP synergy**

In this study, we have measured the time course of two messengers, Ca\(^{2+}\) and cAMP, the translocation of granules to a juxta-membrane position, and exocytosis. Such measurements do not clearly identify the biochemical steps that are synergistically regulated by cAMP. However, based on generally accepted ideas, we have divided trafficking and exocytosis into three steps (Fig. 10, A and B) with a membrane pool A and a docked/primed pool B, and tried to model all our results in one self-consistent scheme. Fujita-Yoshigaki (2000) introduced a similar model to describe the dual regulation of salivary amylase secretion by Ca\(^{2+}\)- and cAMP-elevating agonists and concluded that docked/primed pool B was very small, that cAMP acted primarily to speed the docking/priming step, and that Ca\(^{2+}\) acted primarily to speed the exocytosis step. The validity of these models could be tested more strongly if one had an independent reporter for pool B such as a FRET reporter that indicated formation of SNARE core complexes.

Each of our model calculations started with a Ca\(^{2+}\)- dependent time course of pool A (Fig. 10 C) given in part by the TIRF observations of translocation for most studied conditions. As the resting pool A was supplemented by only 33% of the observed translocated granules, the time variation of A was small and contributed only modestly to regulation of exocytosis.

The time courses of Ca\(^{2+}\) and cAMP were simple to implement. Calcium rose after receptor stimulation and during perfusion of Ca\(^{2+}\) with ionomycin. The ionomycin experiment showed that Ca\(^{2+}\) alone can evoke some exocytosis. In the model, the exocytosis rate constant k\(_{BC}\) was Ca\(^{2+}\) sensitive. For the model calculations, we always used the experimental Ca\(^{2+}\) time courses (filtered) measured simultaneously (ionomycin experiments) or in parallel (UTP or trypsin) with exocytosis in each condition. FSK and VIP raised cAMP slowly, gradually developing a strong potentiating effect on resting and stimulated exocytosis. We modeled FSK and VIP identically. The augmentation of resting exocytosis by cAMP was due primarily to acceleration of the docking/priming rate constant k\(_{AB}\), which increased the size of the docked/primed pool. The larger augmentation of Ca\(^{2+}\)-stimulated exocytosis was caused by increased Ca\(^{2+}\) sensitivity in the exocytosis step k\(_{BC}\), and by the continuing acceleration of k\(_{AB}\). Thus, we have adopted two mechanisms for cross talk by cAMP: increase of the docked/primed pool size and increase in the Ca\(^{2+}\) sensitivity. Both mechanisms have been invoked to explain protein kinase actions in other studies (Zhu et al., 2002; Nagy et al., 2004; Yang et al., 2005). In parotid acinar cells, cAMP with PKA activation is proposed both to stimulate the priming step and to enhance the sensitivity of secretory granules to Ca\(^{2+}\) (Fujita-Yoshigaki et al., 1999; Nakahari et al., 2002; Yoshimura et al., 2002). In some examples, a specific phosphorylation site on the t-SNARE (SNAP-25) has been shown to augment the pool size of a special highly Ca\(^{2+}\)-sensitive granule pool (Nagy et al., 2004; Yang et al., 2005). We previously established that high concentrations (> 100 µM) of nucleotide triphosphates (ATP or UTP) can evoke both mucin exocytosis and bicarbonate (HCO\(_3\)\(^-\)) secretion but low concentrations (< 10 µM) of UTP stimulated only HCO\(_3\)\(^-\) secretion and not exocytosis (Jung et al., 2000, 2009). We speculated that the difference might be because, with high UTP, the [Ca\(^{2+}\)]\(_i\), increase is sustained, whereas, with low concentrations, the [Ca\(^{2+}\)]\(_i\), increase was oscillatory. Further, already with low UTP, the x-y motions of secretory granules were frozen by Ca\(^{2+}\)-dependent formation of F actin, and a stronger stimulus was needed to overcome that effect. In our new model, this stronger stimulus comes not from the oscillatory versus steady nature of evoked
[Ca\textsuperscript{2+}], per se, but from the slightly higher average Ca\textsuperscript{2+} levels and from the unidentified messenger UM supposed to be generated only at higher agonist concentrations. This assumption in the model was introduced ad hoc to explain larger exocytosis with stronger receptor stimulation, but it fits well in offering a self-consistent description of exocytosis in many conditions.

What is UM? What is required is a receptor-dependent potentiating signal that comes on after some seconds of adding the high receptor agonist and decays after some seconds of washing away the agonist. Here, we gave this signal a rise and fall time constant of 40 s and an ability to potentiate docking/priming and Ca\textsuperscript{2+}-dependent exocytosis up to fivefold, depending on the agonist and agonist concentration. The hypothetical messenger arises through strong stimulation of G\textsubscript{q}-coupled receptors and could include many candidate signal acting separately or together: diacylglycerol-activated PKC (Koh et al., 2000; Kim et al., 2008; Satoh et al., 2009), diacylglycerol-potentiated Munc13 (Lou et al., 2008), further effects of IP\textsubscript{3}-released Ca\textsuperscript{2+}, depletion of phosphatidylinositol 4,5-bisphosphate, and generation of any of a very large number of lipid messengers that are derived from diacylglycerol. Production of all of these effects would be reduced in the experiments involving thapsigargin with Ca\textsuperscript{2+}-free medium because PLC, the enzyme activated by G\textsubscript{q}-coupled receptors, is a Ca\textsuperscript{2+}-requiring enzyme. This might explain why thapsigargin can cripple exocytosis so strongly. In fact, PMA, an activator of PKC, stimulates exocytosis and potentiates Ca\textsuperscript{2+}-dependent exocytosis in PDECs (Koh et al., 2000; Kim et al., 2008). We recently showed that PAR-2 activated PKC in addition to the Ca\textsuperscript{2+} signal, promoting exocytosis. Therefore, UTP activation of purinergic receptors may augment PKC activity because both P2Y2 and PAR-2 receptors are linked to PLC pathway (Jung et al., 2006; Kim et al., 2008). To address whether Ca\textsuperscript{2+}-independent exocytosis is involved in UTP-induced exocytosis, we blocked [Ca\textsuperscript{2+}], rise by loading cells with BAPTA-AM (20 μM, 1 h preincubation at 37°C). Exocytosis evoked by 100 μM UTP was not completely blocked, suggesting that Ca\textsuperscript{2+}-independent pathway exists (unpublished data). This Ca\textsuperscript{2+}-independent exocytosis was not blocked by 1 μM of calphostin C, a PKC blocker that inhibited PMA-induced exocytosis, suggesting that PKC is not the unknown messenger for the Ca\textsuperscript{2+}-independent exocytosis evoked by UTP. In addition to these signals, other possible candidates are activation of MAP kinases, src kinase, and G-protein coupled receptor kinases that do not depend on the G\textsubscript{q} subunit but do depend on receptor activation.

Two items should be noted for the simulation of trypsin action on PAR-2 receptors. First, for trypsin we had no TIRF measurements, so we used those from 100 μM UTP instead to obtain the time course for pool A. Second, the elevation of exocytosis and Ca\textsuperscript{2+} were obviously much briefer and more intense for trypsin than they were for UTP at any concentration, although, like UTP, trypsin was applied for 6 min. Initially, the model simulation predicted excess exocytosis lasting the full 6 min because of the protracted generation of messenger UM—even though the Ca\textsuperscript{2+} signal itself was brief. We recognized that because of their covalent cleavage by trypsin and very rapid internalization, PAR-2 receptors are irreversibly activated and quickly shut down (Böhm et al., 1996). Therefore, the simulation shown in Fig. 10 C assumes that PAR-2 receptors are active for only 60 s rather than the full 6 min of trypsin application and that their activity is more intense than that with 100 μM UTP. As in the simulations for UTP, the second messenger UM is rising and falling with a 40-s time constant during and after PAR-2 activation.

Physiological role of potentiation on exocytosis in pancreatic ductal system

Considering the low concentrations of ATP/UTP of ∼10 μM secreted from pancreatic acinar cells (Sørensen and Novak, 2001), we might expect mainly bicarbonate secretion with minimal mucin secretion during digestion. Our study suggests, however, that with the concomitant activation of the cAMP–PKA signaling pathway, even the weaker oscillatory [Ca\textsuperscript{2+}], increases can stimulate significant exocytosis. Because secretin, a classical activator of the cAMP–PKA pathway, is a major physiological agonist for pancreatic ductal secretion of mucin, such potentiation can play a major role in pancreatic physiology. Further, alkaline bicarbonate enhances the hydrodynamic properties of mucin (Smith et al., 1989), so activation of the cAMP pathway by secretion may have a dual role in optimizing mucin function in PDECs, by potentiating its release through exocytosis and optimizing its function by stimulating bicarbonate secretion. In addition, vesicular fusion may also be a mechanism whereby membrane proteins such as ion channels and transporters are delivered and inserted into the plasma membrane of these cells (Ameen et al., 1999; Peters et al., 2001; Butterworth et al., 2005).

In summary, we have shown far reaching synergy between cAMP and Ca\textsuperscript{2+} in mediating exocytosis from PDECs.

The authors thank Drs. Joseph G. Duman and Jill B. Jensen for comments on the manuscript, Dr. Manuel F. Navedo for assistance with TIRF microscopy, Thomas Wong for help with cell culture, and Lea M. Miller for technical assistance.

This work was supported by the International Research Internship Program of the Korea Science and Engineering Foundation (to S.-R. Jung, M7-2004-000-10106-0), grants from R&D Program of Advanced Technologies (to D.-S. Koh), the National Institutes of Health (GM089913 to B. Hille), and the Department of Veterans Affairs (Merit Review to T.D. Nguyen).

Angus C. Nairn served as editor.

Submitted: 27 October 2009
Accepted: 31 March 2010
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Published April 26, 2010

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Model equations
This section gives an abbreviated core of the model calculations as computer code simplified from the C-like IGOR code. In the model, time units are in minutes and steps are as diagramed in Fig. 10 B. Euler integration was done in time steps of $dt = 0.02$ min. The equations below are all within the integration loop to be executed repeatedly. Values of several constants are given in the main text near the end of the Results.

// Start by updating the three messengers
// cAMP changes with time constant τ = 3.333 min = 200 s toward its target. Target is 0.05 μM at rest or 3 μM during forskolin or VIP.
cAMP = filter(cAMP, cAMPtarget, 3.333) // See function “filter” below.
// UM changes with τ = 0.6667 min = 40 s toward its target. Target is 1 at rest or 1.5, 2.5, 5 (see text).
UM = filter(UM, UMtarget, 0.6667) // See function “filter” below.
// Ca changes with τ = 0.25 min = 15 s toward its target. Target is measured Ca at that time.
Ca = filter(Ca, CaData(Time), 0.25) // See function “filter” below.
ScaledCa = (Ca)^CaExpon // Ca was filtered.
ScaledKCa = (KCa)^CaExpon // KCa is 0.9 or 0.5 μM for without or with forskolin, respectively. CaExpon was 4.
// Docking and priming step: (kAB = 0.002) A slow undocking step (kBA = 0.034) is included. KcAMP was 0.4 μM.
fluxAB = dt * (kAB* (1 + 4*UM*(cAMP)/(cAMP + KcAMP)))*A - kBA*B
// Exocytosis step: (kBC = 0.1; KBCCa = 0.3)
fluxBC = dt*(KBCCa*B/(ScaledCa + ScaledKCa))*B
// Integrate B dt
B = B - fluxBC + fluxAB
// Exocytosis per minute
exocytosis = fluxBC/dt

// Function Filter calculates exponential relaxation toward a target value for a single time step dt
Function filter(now, target, tau)
return now + (target-now)*dt/tau
end function
Figure S1. FSK increases Ca\textsuperscript{2+}-evoked exocytosis in a dose-dependent manner. (A) Exocytosis was evoked by Ca\textsuperscript{2+} influx via ionomycin upon the application of 2 mM Ca\textsuperscript{2+} (‘2 Ca\textsuperscript{2+}’) at various FSK concentrations. Average normalized rate of exocytosis in the presence of at 0.01 (green, N = 3), 0.1 (blue, N = 5), 1 (black, N = 16), or 10 μM (red, n = 5) FSK. Cells were preincubated for at least 5 min in 1 μM ionomycin before the measurements and all test solutions contained 1 μM ionomycin. (B) Relative exocytosis evoked by different concentrations of FSK (open circles, first 6 min) and by 2 mM Ca\textsuperscript{2+} in the presence of FSK (filled circles, next 6 min). The data were fitted with the Hill equation, with half-maximal concentrations of 0.5 and 0.4 μM FSK for exocytosis evoked by FSK alone and FSK plus Ca\textsuperscript{2+}, respectively.

Figure S2. Representative measurement of granule mobility during [Ca\textsuperscript{2+}]\textsubscript{i} rise induced by ionomycin. External Ca\textsuperscript{2+} was exchanged from 0 mM Ca\textsuperscript{2+} to 2 mM Ca\textsuperscript{2+} as indicated in B and C. All test solutions contained 1 μM ionomycin. (A-a) The first image of this experiment showing part of a single cell. Bar, 5 μm. (A-b) Enlargement of the image in panel a. Bar, 0.5 μm. A granule marked with an arrow showed trajectories as indicated with black line before and after [Ca\textsuperscript{2+}]\textsubscript{i} rise, and with red line during 2 mM Ca\textsuperscript{2+}. The granule marked with an arrow in A-b exhibited cumulative distance traveled (B), speed (C), and MSD (D) before (black circles) and during (red circles) [Ca\textsuperscript{2+}]\textsubscript{i} rise.
Figure S3. FSK does not affect the near-plasma membrane granule movement. All test solutions contained 1 μM ionomycin in a Ca²⁺-free solution. (A) TIRF images obtained before (A-a, at 40 s) and during (A-b, at 120 s) 1 μM FSK treatment. Bars, 10 μm. Background fluorescence was not subtracted. Granules indicated by arrowheads in A are analyzed. (B) Trajectory in x-y plane before (black) and during (red) Ca²⁺ rise. Cumulative distance traveled (C), speed of granules (D), and change of fluorescence intensity (E) of granules 1 and 2 (z axis movement) shown as arbitrary units (A.U.). Background fluorescence from a granule-free region was subtracted. Data are plotted at 2-s intervals. (F) Intensity of 17 randomly chosen granules from A.
**Figure S4.** FSK does not affect Ca\textsuperscript{2+}-induced granule migration. All test solutions contained 1 μM ionomycin in Ca\textsuperscript{2+}-free solution. (A) TIRF images obtained before (A-a, FSK, at 40 s) and during (A-b, ‘FSK+2 Ca\textsuperscript{2+}’, at 140 s) 2 mM external Ca\textsuperscript{2+} stimulation in the presence of 1 μM FSK. Bars, 10 μm. Background fluorescence was not subtracted. Granules indicated by arrowheads in A are analyzed. (B) Trajectory in x–y plane before (black) and during (red) Ca\textsuperscript{2+} rise. Cumulative distance traveled (C), speed of granules (D), and change of fluorescence intensity (E) of granules 1 and 2 (z axis movement) shown as arbitrary unit (A.U.). Background from a granule-free region was subtracted. Data are plotted at 2-s intervals. (F) Intensity of 28 randomly chosen granules from A.

**Figure S5.** Examples of empirical curves describing the translocation of granules during treatment of various stimuli for intracellular Ca\textsuperscript{2+} rise. The plot shows mean time course of normalized granule brightness plotted every 2 s (circles) and the empirical curves describing them used in calculations of the model. Data are for addition of 2 mM external Ca\textsuperscript{2+} (green) to ionomycin-treated cells and for addition of 2 or 100 μM UTP (blue or red, respectively) at t = 120 s. After t = 120 s, the curves have the following form in units of seconds: amplitude * ((1–exp(–(t–120)/τ\textsubscript{On}))\textsuperscript{expon} * exp(– (t–120)/τ\textsubscript{Decay})) For Ca\textsuperscript{2+} plus ionomycin, 2, and 100 μM UTP, respectively, the parameter values were: amplitude, 2.4, 1.25, 2; τ\textsubscript{On}, 84, 95, 50 s; exponent, 3.7, 1.3, 1.7; and τ\textsubscript{Decay}, 300, 390, 350 s. Averages of > 5 experiments for each condition.
Video 1. Concerted increase of granule fluorescence induced by \([Ca^{2+}]_i\), increase. Granules were labeled with FM 1–43 fluorescent dye (8 μM) and inspected under a TIRF microscope. Cells were incubated with 1 μM ionomycin in Ca\(^{2+}\)-free bath solution. Bath solution was exchanged from Ca\(^{2+}\)-free to 2 mM Ca\(^{2+}\) as indicated on the right side of the images. The brightness of granules gradually increased up to a steady-state level, indicating that most granules concertedly moved to the plasma membrane. The original interval between frames is 4 s and the movie displays at 5 frames/s.

Video 2. The granule mobility was not strongly affected by FSK-induced cAMP increase. Granules were labeled with FM 1–43 fluorescent dye and inspected under a TIRF microscope. Cells were incubated with 1 μM ionomycin in Ca\(^{2+}\)-free solution. FSK was applied as indicated on the right side of the images. The original interval between frames is 4 s and the movie displays at 5 frames/s.