Two Modes of Secretion in Pancreatic Acinar Cells: Involvement of Phosphatidylinositol 3-Kinase and Regulation by Capacitative Ca²⁺ Entry

Manuel Campos-Toimil,[1,3,4] Tanya Bagrij,[1,3]
J. Michael Edwardson,[1] and Paul Thomas[1,2]
[1]Department of Pharmacology
University of Cambridge
Tennis Court Road
Cambridge, CB2 1PD
United Kingdom

Summary

In pancreatic acinar cells, muscarinic agonists stimulate both the release of Ca^{2+} from intracellular stores and the influx of extracellular Ca^{2+}. The part played by Ca^{2+} released from intracellular stores in the regulation of secretion is well established; however, the role of Ca^{2+} influx in exocytosis is unclear. Recently, we observed that supramaximal concentrations of acetylcholine (≥ 10 μM) elicited an additional component of exocytosis despite reducing Ca^{2+} influx. In the present study, we found that supramaximal exocytosis was substantially inhibited ($\sim 70\%$) by wortmannin (100 nM), an inhibitor of phosphatidylinositol 3-kinase. In contrast, exocytosis evoked by a lower concentration of acetylcholine (1 μM) was potentiated ($\sim 45\%$) by wortmannin. Exocytosis stimulated by 1 μM acetylcholine in the absence of extracellular Ca^{2+} was, like supramaximal exocytosis, inhibited by wortmannin. The switch to a wortmannin-inhibitable form of exocytosis depended upon a reduction in Ca^{2+} entry through store-operated Ca^{2+} channels, as the switch in exocytotic mode could also be brought about by the selective blockade of these channels by Gd^{3+} (2 μM), but not by inhibition of noncapacitative Ca^{2+} entry by SB203580 (10 μM). We conclude that supramaximal doses of acetylcholine lead to a switch in the mode of zymogen granule exocytosis by inhibiting store-dependent Ca^{2+} influx.

Results and Discussion

Responses of Acinar Cells to Different Doses of Acetylcholine

Secretion of digestive enzymes into the lumen of the pancreatic duct occurs by regulated exocytosis of zymogen granules at the apical plasma membrane of acinar cells. We have recently developed the methodology to visualize the exocytosis of zymogen granules in intact acinar cells in response to brief applications of secre-

[2]Correspondence: pjt28@cam.ac.uk
[3]These authors contributed equally to this work.
[4]Present address: Departamento de Farmacoloxia, Facultade de Farmacia, Campus Universitario Sur, 15782 Santiago de Compostela, Spain.

tagogs (see Figure 1 and [1, 2]). We observed that the secretory response appeared to peak at 0.25 μM ACh, with 1 μM ACh giving no more exocytosis [1]. Curiously, however, application of 10 μM ACh led to a further enhancement of exocytosis. The effects of a supramaximal dose of ACh on $[Ca^{2+}]_i$ and exocytosis are shown in Figures 2A and 2B. Figure 2A shows the $[Ca^{2+}]_i$ responses evoked in pancreatic acinar cells by the application of 1 and 10 μM ACh; because of the importance of the spatial aspects of $[Ca^{2+}]_i$ signaling in the control of physiological function [3, 4] and because exocytotic activity takes place exclusively at the apical plasma membrane, we have restricted our analysis of $[Ca^{2+}]_i$ to the apical domain (see the Experimental Procedures). It can be seen that the initial transient increase in $[Ca^{2+}]_i$ was very similar with either dose of agonist; in contrast, the plateau phase of the response was clearly suppressed at the higher dose of ACh. The plateau phase is generally accepted to mainly reflect entry of Ca^{2+} from the extracellular space (also see Figure 3A). Despite the reduction in Ca^{2+} influx, the exocytotic response was enhanced by $\sim 70\%$ (Figure 2B; 22.6 ± 1.65 exocytotic events/cell with 10 μM ACh, n = 41 versus 13.3 ± 1.29 with 1 μM ACh, n = 36; p < 0.05). To account for this increased secretory response, we explored the potential role of signaling pathways other than $[Ca^{2+}]_i$ in the effect of supramaximal doses of ACh. Cholecystokinin (CCK; another acinar cell secretagog) has been shown to activate phosphatidylinositol 3-kinase (PI 3-K) in acinar cells [5]. We therefore decided to look at the effects of wortmannin, a selective inhibitor of this kinase, on the cellular responses to 10 μM ACh.

Effects of Wortmannin on Acinar Cell Responses to Acetylcholine

Wortmannin (100 nM) had a significant effect on Ca^{2+} signaling (Figure 2C) and exocytosis (Figure 2D) elicited by 10 μM ACh. The plateau phase of the $[Ca^{2+}]_i$ response was slightly enhanced by the PI 3-K inhibitor. Despite this enhancement of the Ca^{2+} signal, wortmannin reduced zymogen granule exocytosis by $\sim 70\%$ (Figure 2D; 7.0 ± 0.87 events, n = 43; p < 0.05), and 25 nM wortmannin also inhibited exocytosis, but to a lesser extent (15.3 ± 1.14 events/cell; n = 21). Surprisingly, when we looked at the effects of wortmannin on the responses to 1 μM ACh (Figures 2E and 2F), we observed no significant effect of the drug on Ca^{2+} signaling, but there was an $\sim 45\%$ enhancement in the amplitude of the secretory response (19.2 ± 1.38 events, n = 39; p < 0.05). It would appear, then, that ACh utilizes at least two distinct signaling pathways to regulate zymogen granule exocytosis. Furthermore, the relative usage of these two pathways varies according to the agonist concentration, with a wortmannin-inhibitable pathway making the major contribution at higher agonist concentrations.

The Effect of Reducing Ca²⁺ Influx on the Responses to 1 μM Acetylcholine

The release of Ca^{2+} from intracellular stores is critical for amylase secretion from acinar cells [6]; furthermore,
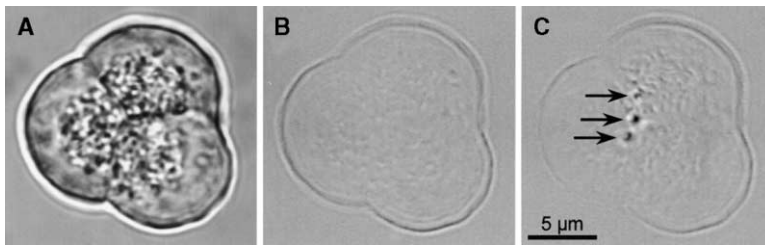
Figure 1. Zymogen Granule Exocytosis Revealed by Time-Differential Analysis

(A) Bright field image of a small acinus consisting of three acinar cells.
(B) Differential image of the same acinus during a 6-min application of 10 μM ACh. This image was formed by the subtraction of one frame from its predecessor.
(C) Image obtained as in (B), but showing three exocytotic events (arrows), two events in one cell (lower arrows) and one in another cell (upper arrow).

the influx of extracellular Ca^{2+} is indirectly involved in the regulation of secretion by helping to refill the empty stores. Nevertheless, whether this influx is directly involved in the exocytosis of zymogen granules is uncertain. It has been suggested that capacitative Ca^{2+} influx is critical for immunoglobulin-E-mediated secretion in mast cells [7], and it has also been demonstrated that a similar entry pathway in adrenal chromaffin cells can both modulate and trigger granule exocytosis [8]. The fact that increasing the concentration of ACh from 1 to 10 μM in our experiments increased exocytosis but suppressed the plateau phase of the Ca^{2+} response argues against a role for Ca^{2+} influx in the secretory response of acinar cells. To determine whether extracellular Ca^{2+} truly plays no role in the exocytotic response, we measured both $[Ca^{2+}]_i$ and zymogen granule exocytosis in response to 1 μM ACh in cells bathed in nominally Ca^{2+}-free medium.

As would be expected, the removal of extracellular Ca^{2+} led to a significant reduction in the plateau phase of the Ca^{2+} response (Figure 3A). Interestingly, however, the exocytotic response was also reduced in Ca^{2+}-free medium (Figure 3B; 9.8 ± 1.14 events/cell, n = 25; p < 0.05), suggesting that Ca^{2+} influx during the plateau phase of the $[Ca^{2+}]_i$ response has a significant potentiating effect on secretion at 1 μM ACh. It would appear, therefore, that the reduction of the plateau phase of the $[Ca^{2+}]_i$ response with 10 μM ACh is offset by a Ca^{2+} influx-independent enhancement of exocytosis activated by the higher agonist concentration. The results observed in Figure 2D would also suggest that such a

pathway is sensitive to wortmannin. Remarkably, when we examined the effect of wortmannin on exocytosis stimulated by 1 μM ACh in Ca^{2+}-free medium, we found that the PI 3-K inhibitor no longer enhanced, but inhibited exocytosis (Figure 3B). These data imply that, in the absence of Ca^{2+} influx, the phenotype of secretion in response to 1 μM ACh switches to the phenotype observed with 10 μM ACh.

In acinar cells, Ca^{2+} influx, in response to ACh, has been reported to occur via two separate pathways: one sensitive to lanthanides, and another that is insensitive to these trivalent cations [9]. The former pathway has the same properties as the classic capacitative entry pathway [10], but the lanthanide-insensitive pathway is more similar to the so-called noncapacitative pathway [11]. Camello and coworkers found that this noncapacitative pathway played the major role in Ca^{2+} entry during agonist stimulation [9]. We have now found that SB203580 (SB, 10 μM), which has previously been shown to inhibit the p38 mitogen-activated protein kinase [12], reduced Ca^{2+} influx during ACh application to a similar extent as the removal of extracellular Ca^{2+} (Figure 3A). Nevertheless, when we examined exocytosis in the presence of SB, we found that the responses, either with or without wortmannin, were not significantly different from those observed with 1 μM ACh in the presence of extracellular Ca^{2+} (Figure 3B). These data suggest that the effects of extracellular Ca^{2+} on exocytosis must be mediated by Ca^{2+} entering via a different pathway from that blocked by SB. Despite the fact that Camello and coworkers were unable to demon-
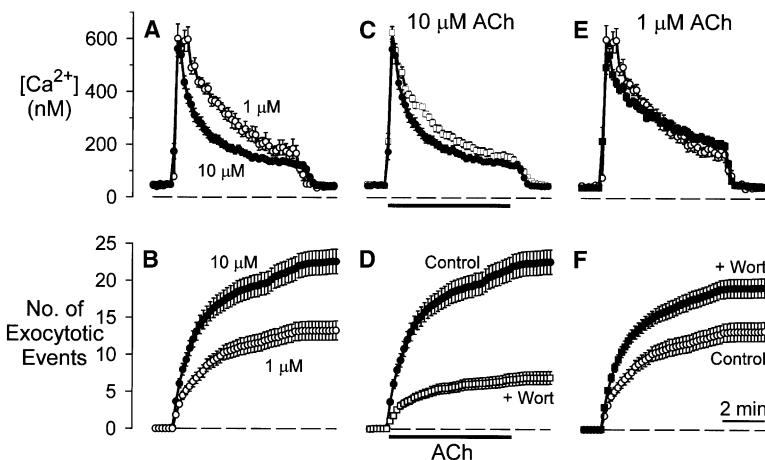

Figure 2. ACh-Evoked Changes in Apical Calcium Concentration, $[Ca^{2+}]_i$, and Zymogen Granule Exocytosis

(A) The $[Ca^{2+}]_i$ responses of acinar cells to 6-min applications of 1 (open circle) and 10 μM (solid circle) ACh; means ± SEM, n = 28 and 22, respectively.
(B) The exocytotic responses of acinar cells to 1 and 10 μM ACh expressed as numbers of exocytotic events per cell. The symbols are the same as in (A); n = 36 and 41, respectively.
(C) The effect of 100 nM wortmannin (open square; Wort) on the $[Ca^{2+}]_i$ response to 10 μM ACh, n = 35.
(D) The effect of wortmannin (open square) on the exocytotic response to 10 μM ACh, n = 43.
(E) The effect of wortmannin (solid square) on the $[Ca^{2+}]_i$ response to 1 μM ACh, n = 27.
(F) The effect of wortmannin (solid square) on the exocytotic response to 1 μM ACh, n = 39.
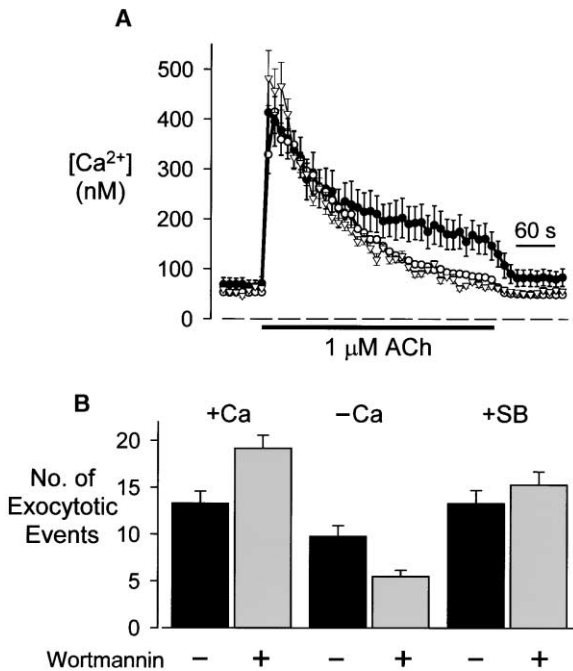
A

B

+Ca   −Ca   +SB

Figure 3. Effect of Removing Extracellular Ca^{2+} on Changes in Apical Calcium Concentration, $[Ca^{2+}]_i$, and Inhibition of Exocytosis by Wortmannin

(A) The effect of 1 μM ACh on $[Ca^{2+}]_i$ in acinar cells in the presence (solid circle) or absence (open circle) of extracellular Ca^{2+}, or in the presence of both Ca^{2+} and 10 μM SB203580 (triangle); means $\pm$ SEM, n = 21, 27, and 24, respectively.
(B) The effect of 100 nM wortmannin on exocytosis evoked by 1 μM ACh in the presence (+Ca; data replotted from Figure 2F) or absence of extracellular Ca^{2+} (−Ca; −wortmannin, n = 25; +wortmannin, n = 41), or in the presence of both Ca^{2+} and SB203580 (+SB; −wortmannin, n = 15; +wortmannin, n = 28).

strate activity of the capacitative pathway during agonist application [9], we decided to determine whether this pathway might play a role in zymogen granule exocytosis.

Responses to 1 μM Acetylcholine in the Presence of Gd^{3+}

Studies in smooth muscle cells suggest that the capacitative pathway can be almost completely blocked by Gd^{3+} at concentrations as low as 1–5 μM, whereas the noncapacitative pathway is unaffected by such concentrations [11]. Figure 4 demonstrates the effects of 2 μM Gd^{3+} on both the $[Ca^{2+}]_i$ response ([A]) and the secretory response ([B]) to 1 μM ACh in the presence or absence of wortmannin. We observed no significant effects on the $[Ca^{2+}]_i$ signal, either with Gd^{3+} alone or with Gd^{3+} and wortmannin together (Figure 4A). These findings are in agreement with the results of Camello and colleagues, who demonstrated that the major influx pathway in the presence of agonist was the lanthanide-resistant pathway [9]. They suggested that the lanthanide-inhibitable pathway is only activated after prolonged stimulation. Nevertheless, Gd^{3+} has the same effect on exocytosis as removing extracellular Ca^{2+}; i.e., there is both a reduction in the amplitude of the secretory response and a
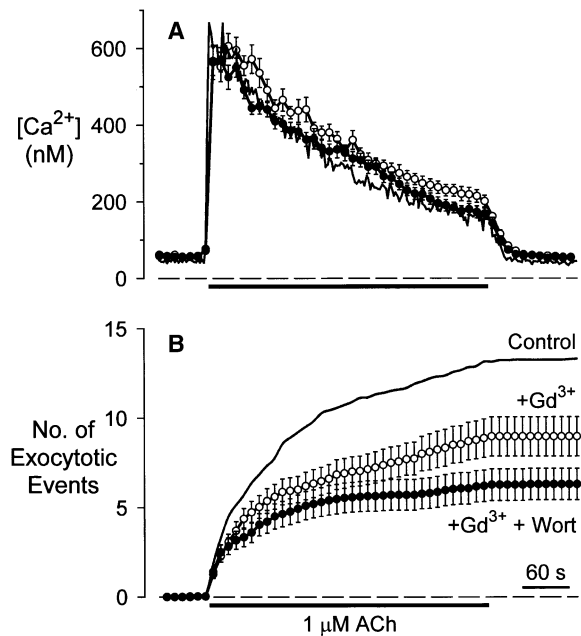
A

B

Figure 4. The Effects of Gd^{3+} on Ca^{2+} Signaling and Exocytosis

(A) Changes in apical calcium concentration ($[Ca^{2+}]_i$) evoked by 1 μM ACh in the presence of 2 μM Gd^{3+} (open circle) or 100 nM wortmannin + Gd^{3+} (solid circle); means $\pm$ SEM, n = 16 and 18, respectively.
(B) Exocytosis evoked by 1 μM ACh in the presence of 2 μM Gd^{3+} or 100 nM wortmannin (Wort) + Gd^{3+}. The symbols are the same as in (A); n = 30 for both groups. For comparison, the solid lines show the data from Figures 2E and 2F obtained in the absence of drugs.

switch to a wortmannin-inhibitable mode of exocytosis (Figure 4B). These results suggest that it is the Gd^{3+}-sensitive pathway that contributes more to secretory activity and dictates the mode of secretion. Indeed, the effects of Gd^{3+} on exocytosis would suggest that this pathway is active at earlier times than suggested previously [9], albeit contributing very little to the increase in $[Ca^{2+}]_i$. The detectable effect that 10 μM ACh has on the plateau phase of the Ca^{2+} signal suggests that high agonist concentrations inhibit the noncapacitative entry pathway; nevertheless, the effect of 10 μM ACh on the phenotype of the secretory response would suggest that supramaximal doses also lead to a reduction in entry through the Gd^{3+}-sensitive pathway.

How is it that Ca^{2+} entry can occur without eliciting a measurable Ca^{2+} signal? It should be borne in mind that only 1% of the Ca^{2+} entering the cytosol through Ca^{2+} channels may be detectable as an increase in free Ca^{2+} concentration [13, 14]. Thus, a small influx through the Gd^{3+}-sensitive pathway may not be detectable as a change in fura-2 fluorescence. But how could a pathway that allows entry of such a small amount of Ca^{2+} have such a large effect on the secretory response? One possibility is that the Gd^{3+}-sensitive channels preferentially colocalize with the sites of granule release. Alternatively, it has been proposed that Ca^{2+} entering acinar cells at the basolateral membrane may enter the basally located endoplasmic reticulum (ER) and may be channeled through this organelle to the apical pole via long

ER projections [15, 16]. In this way, Ca^{2+} entering through basolateral, Gd^{3+}-sensitive channels may be released into the cytoplasm via IP_3 receptors in close proximity to the site of exocytosis. Correspondingly, the local increase in $[Ca^{2+}]$ may be sufficient to activate the Ca^{2+}-sensitive proteins involved in exocytosis (and also those inhibiting the wortmannin-sensitive pathway, see below).

Our interpretation of the complex effects of wortmannin on exocytosis is that ACh activates two parallel signaling pathways: one involving phospholipase C type β (PLCβ), and the other involving PI 3-K. The pathway mediated by PLCβ controls exocytosis mainly through its effects on $[Ca^{2+}]_i$, while PI 3-K acts via an as-yet-uncharacterized mechanism. We would suggest that, through capacitative Ca^{2+} influx, the PLCβ-dependent pathway acts to limit exocytosis activated by PI 3-K. This inhibitory cross-talk provided by capacitative Ca^{2+} influx dominates at low ACh concentrations (≤ 1 μM) such that secretion is mainly dependent on the PLCβ pathway. However, at supramaximal concentrations (≥ 10 μM), ACh activates another signaling pathway that inhibits Ca^{2+} influx; this both removes the block on PI 3-K-dependent exocytosis and reduces Ca^{2+}-dependent exocytosis, leading to a situation in which PI 3-K-dependent secretion is the dominant mode of zymogen granule exocytosis. Thus, Gd^{3+}, by inhibiting capacitative Ca^{2+} entry, has the same effect as supramaximal doses of ACh; i.e., it switches exocytosis from a PLCβ-dependent mode to a PI 3-K-dependent mode.

The potentiating effect of wortmannin on exocytosis elicited by 1 μM ACh implies that there is inhibition of the PLCβ pathway from the PI 3-K pathway; i.e., there is mutual inhibitory cross-talk between the two pathways. This observation also implies that PI 3-K itself is not the target of Ca^{2+} influx and that there must be one, or more, intervening steps between PI 3-K and the site of action of Ca^{2+} influx in the PI 3-K-dependent pathway. In our experiments, 100 nM wortmannin inhibits exocytosis evoked by 10 μM ACh by ~70%, and 25 nM reduces the amplitude of the secretory response by ~30%. These results would suggest an IC_{50} for zymogen granule exocytosis of 30–60 nM. This value is similar to the values determined for the inhibition of PI 3-K-dependent processes in smooth muscle cells (10–55 nM) [17–19] and is lower than determined for the inhibition of either myosin light chain kinase (300–500 nM) [20, 21] or phosphatidylinositol 4-kinase (PI 4-K)-dependent processes in intact cells (≥ 300 nM) [22, 23]. Likewise, we have observed no reduction in Ca^{2+} signaling with wortmannin, as would be expected from an inhibition of PI 4-K [22, 23]. It would seem most likely, therefore, that the effects of wortmannin on zymogen granule exocytosis are due to inhibition of PI 3-K rather than these other kinases.

What is the physiological relevance of the switch in the mode of exocytosis? Both CCK and cholinergic hyperstimulation have been used in models of acute pancreatitis, and it is generally considered that such supramaximal stimulation is a contributing factor to the disease [24–26]. The cellular consequences of supramaximal stimulation that are thought to contribute to pancreatic autodigestion are sustained elevation of $[Ca^{2+}]_i$, with subsequent intracellular activation of zymogens [26, 27], and inappropriate exocytosis of granules

at the basolateral plasma membrane [24]. The reduction in Ca^{2+} influx that we have observed at high agonist concentrations may be a protective mechanism to reduce the consequences of such sustained increases in $[Ca^{2+}]_i$ and may explain the observed disruption of normal Ca^{2+} signaling that occurs following prolonged hyperstimulation [28, 29]. Whether the switch in exocytotic mode from PLCβ- to PI 3-K-dependent exocytosis underlies the inappropriate exocytosis observed in less-dispersed pancreatic preparations [24] remains to be determined; nevertheless, the targeting of the PI 3-K-dependent pathway may prove of therapeutic value in the treatment of acute pancreatitis.

The presence of divergent signaling pathways from a single receptor, as well as cross-talk among pathways activated by different receptors, are emerging concepts in cell biology [30]. Clearly, the coexistence of the PLCβ pathway, capacitative Ca^{2+} entry, and PI 3-K is not restricted to the pancreatic acinar cell. Thus, our observations on the role of capacitative Ca^{2+} influx as a mediator of cross-talk between PLCβ signaling and PI 3-K signaling may provide a ubiquitous mechanism by which cells coordinate the activities of these parallel pathways.

Conclusions

We propose that the influx of Ca^{2+} through capacitative entry channels plays two critical functions in pancreatic acinar cells: one, to potentiate secretory activity; and two, to provide a switching device that determines which of the two distinct signaling pathways controls zymogen granule exocytosis. Furthermore, the switch to a wortmannin-sensitive mode of exocytosis at supramaximal agonist concentrations may be a contributing factor to the progression of acute pancreatitis, and this signaling pathway may prove to be a useful pharmacological target for the treatment of this disease.

Experimental Procedures

Measurement of Zymogen Granule Exocytosis
Pancreatic acinar cells were obtained from 5- to 7-week-old male Wistar rats (150–225 g) and were allowed to attach to modified, glass-bottomed Petri dishes as described previously [1]. For experiments with wortmannin and/or SB, cells were pretreated at 37°C for 15 min prior to stimulation. Following pretreatment, wortmannin was removed by washing; SB remained present throughout the experiments. Both wortmannin and SB were dissolved in DMSO and then added to the incubation medium [1] such that the final [DMSO] was <0.4%. Exocytosis was measured at 37 ± 2°C using time-differential analysis of bright field recordings as previously described [1]. This technique is based upon the loss of the dense granule contents during exocytosis and the resultant change in optical density; exocytotic events are then revealed by the successive subtraction of one image from another over time [2]. ACh was applied to the cells from glass micropipettes (2- to 5-μm diameter) using a PicoSpritzer II (General Valve). Images were acquired and analyzed using AQM and Lucida software, respectively (Kinetic Imaging).

Measurement of Intracellular Calcium
For measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), cells were loaded with fura-2/acetoxymethyl ester, and pairs of fluorescence images at 340 nm and 380 nm were obtained every 2.5 s as described previously [1]. For analysis, only the $[Ca^{2+}]$ in the apical region was considered. The apical domain was defined as that region of the cell visibly occupied by zymogen granules. Calibration

of the fura-2 signal was carried out using solutions of known $[Ca^{2+}]$ in 20-μm thick microslides (VitroCom) as described previously [1].

ACh chloride, BME amino acids, BSA fraction V, collagenase type IA, DMSO, Pluronic F-127, polyethyleneimine, SBTI, and wortmannin were obtained from Sigma. Fura-2/acetoxymethyl ester and potassium salt were obtained from Molecular Probes. SB203580 was obtained from Calbiochem. All general chemicals were obtained from Sigma; where possible, only cell culture-tested chemicals were used.

Except where otherwise stated, all values shown are means $\pm$ SEM. The significance of differences between means ($p < 0.05$) was determined by Student's t test for unpaired data, except when sample variances were unequal, in which case we used Welch's approximate t test. In the case of multiple treatments, data were analyzed by one-way analysis of variance, followed by Dunnett's t test for comparison of multiple treatments with a single control. However, when the variances of the samples were unequal, we used the nonparametric Kruskal-Wallis test and determined significance using the procedure of Dunn.

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