Mechanism of Ca\(^{2+}\) Wave Propagation in Pancreatic Acinar Cells*

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An increase in cytosolic Ca\(^{2+}\) often begins as a Ca\(^{2+}\) wave, and this wave is thought to result from sequential activation of Ca\(^{2+}\)-sensitive Ca\(^{2+}\) stores across the cell. We tested that hypothesis in pancreatic acinar cells, and since Ca\(^{2+}\) waves may regulate acinar Cl\(^{-}\) secretion, we examined whether such waves also are important for amylase secretion. Ca\(^{2+}\) wave speed and direction was determined in individual cells within rat pancreatic acini using confocal line scanning microscopy. Both acetylcholine (ACh) and cholecystokinin-8 induced rapid Ca\(^{2+}\) waves which usually travelled in an apical-to-basal direction. Both caffeine and ryanodine, at concentrations that inhibit Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), markedly slowed the speed of these waves. Amylase secretion was increased over 3-fold in response to ACh stimulation, and this increase was preserved in the presence of ryanodine. These results indicate that 1) stimulation of either muscarinic or cholecystokinin-8 receptors induces apical-to-basal Ca\(^{2+}\) waves in pancreatic acinar cells, 2) the speed of such waves is dependent upon mobilization of caffeine- and ryanodine-sensitive Ca\(^{2+}\) stores, and 3) ACh-induced amylase secretion is not inhibited by ryanodine. These observations provide direct evidence that Ca\(^{2+}\)-induced Ca\(^{2+}\) release is important for propagation of cytosolic Ca\(^{2+}\) waves in pancreatic acinar cells.

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**MATERIALS AND METHODS**

**Animals and Materials—**Male Sprague-Dawley rats (80–100 g; Camar Research Lab Animals, Wayne, NJ) were maintained on Purina rodent chow under a constant light cycle and used for all experiments. Acetylcholine (ACh), caffeine, and atropine were obtained from Sigma; fluo-3/AM and ryanodine were obtained from Molecular Probes (Pittsford, OR); and sincalide, a synthetic C-terminal octapeptide of cholecystokinin-8 (CCK), was a gift of Squibb Diagnostics (Princeton, NJ). All other chemicals were of the highest quality commercially available.

**Preparation of Pancreatic Acini—**Pancreatic acini were prepared from male Sprague-Dawley rats (80–100 g) as described previously (11). Briefly, the pancreas was removed following an overnight fast and placed in a Ca\(^{2+}\)- and Mg\(^{2+}\)-free buffer (pH 7.4) containing NaCl (97 mM), KCl (5 mM), glucose (20 mM), HEPES (20 mM), soy bean trypsin inhibitor (0.1 mg/ml), and bovine serum albumin (0.1%). The pancreas was finely diced, transferred to a siliconized flask in buffer containing CaCl\(_{2}\) (2 mM), MgCl\(_{2}\) (1.2 mM), and collagenase (400 units/ml), and gently agitated for 5 min at 37 °C. This pancreatic tissue was placed in a Corex tube and shaken by hand for 5–10 min then filtered through 200-μm nylon mesh and rinsed in collagenase-free buffer. This resulted in acini which contained over 50 cells each. In selected experiments, pancreatic acini were dispersed into clusters of only two to four cells each (6, 14), by incubating for an additional 10 min in Ca\(^{2+}\)-free medium containing EGTA (1 mM) and trypsin (1.5 mg/ml) followed by 5 min in medium containing collagenase (400

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1. The abbreviations used are: CICR, Ca\(^{2+}\)-induced Ca\(^{2+}\) release; IP\(_{3}\), inositol 1,4,5-trisphosphate; ACh, acetylcholine; CCK, cholecystokinin-8; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrito)]tetracetic acid.

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units/5 ml). These cells were passed through a second nylon filter and rinsed.

Confocal Microscopic Measurements of Cytosolic Calcium—Isolated rat pancreatic acini were prepared as described above, then loaded with the Ca2+-sensitive fluorescent dye fluo-3/AM (15) (6 μM) for 20 min at 37 °C, and then incubated in Leibovitz L-15 medium containing 10% fetal calf serum. The cells were transferred to a chamber on the stage of a Zeiss Axiovert microscope, perfused at 37 °C with a HEPES-buffered solution, and observed using a Bio-Rad MRC-600 confocal imaging system. An argon laser was used to excite the dye at 488 nm, and emission signals above 515 nm were collected. Optical sections between 0.5 and 1.0 μm in thickness were obtained of individual cells within acini; these cells have a diameter of 10–20 μm and are located within a cluster of cells with a typical aggregate diameter of ~100 μm. Neither autofluorescence nor other background signals were detectable at the machine settings (i.e. aperture, gain, and black level) that were used. There was also no change in size, shape, or location of cells during the experiments. Acini were stimulated under one of the conditions described below, and the resulting Ca2+ signals were detected by confocal line-scanning microscopy (7, 8, 13). In this type of confocal microscopy, fluorescence is determined at each point along a single line across the image, rather than at each point across the entire image (Fig. 1A), permitting images to be obtained as frequently as every 4 ms without loss of spatial resolution of fluo-3 fluorescence along the line that is scanned. Fluo-3 fluorescence in response to each stimulus was recorded in this way and the resulting line scan was convolved with the following 3 × 3 smoothing filter as follows.

|   | 1 | 2 | 1 |
|---|---|---|---|
| 2 | 8 | 2 |
| 1 | 2 | 0 |

Line scans were displayed as images consisting of 512 × 512 pixels, with a spatial resolution of 0.28 μm/pixel (in the x direction) and a temporal resolution of 4 ms/pixel (in the y direction). Because of this high degree of resolution relative to the typical Ca2+ wave speeds that were detected, the low pass filter served only to reduce noise. The change in fluorescence over time at each point along the scan line was determined from the recorded image using an Itex Series 151 image processor. Velocities of intracellular Ca2+ waves were determined from the rate at which initial increases in fluorescence progressed along the scan line. Specifically, an apical and basolateral point were identified within the cytosol, and the wave speed between the two points was calculated as (distance between the two points) / (interval between the initial increase in fluorescence at each of the points). For these measurements, intracellular points not clearly located within the cytosol were avoided. The line-scanning approach may overestimate the speed of Ca2+ waves, which do not travel along the scan line (by ~20% on average) (7), but scan lines were chosen to be oriented along the apical-to-basal axis, which is the direction that Ca2+ waves travel in this cell type. Acinar cells stimulated with either ACh or CCK were scanned at a frequency of 250 Hz (every 4 ms) for 512 consecutive scans. Cells stimulated with ACh in the presence of ryanodine or caffeine were instead scanned at a frequency of 10 Hz (every 100 ms), because a frequency of 250 Hz was too rapid to adequately define these slower transcellular Ca2+ waves, waves within 512 consecutive scans. For comparison, some Ca2+ waves induced by ACh in the absence of ryanodine or caffeine were also measured at a line-scanning frequency of 10 Hz, and their average speed was no different from that measured in ACh-stimulated cells at 250 Hz (Mann-Whitney test). Absolute Ca2+ concentrations were not estimated because fluo-3 cannot be ratio-imaged (15). Since the velocity of a Ca2+ wave is determined independent of Ca2+ concentration, measurements of wave speeds were not limited by use of fluo-3.

Ca2+ waves were measured in individual cells within acini in response to the following stimuli: 1) ACh (0.1, 1, and 10 μM), 2) ACh (10 μM) in the presence of atropine (1 μM), 3) ACh (1 μM) in the presence of ryanodine (10 and 50 μM), 4) ACh (1 μM) in the presence of caffeine (20 μM), 5) CCK (500 μM), and 6) CCK in the presence of caffeine (20 μM). Ca2+ wave speeds were also measured in individual acinar cells that had been further dispersed into pairs, triplets, and quadruplets, in response to stimulation with ACh (10 μM). Measurement of Amylase Secretion—Isolated rat pancreatic acini were prepared as described above, then 50-μl aliquots of the acini were preincubated with 50 μl of buffer (tryptorhodamine) for 5 min. An additional 100 μl of buffer ± agonist was added to the cells, and acini were incubated under these conditions for 25 min. All steps were carried out at 37 °C. Cells were then centrifuged for 2 min at 2,000 × g, and the Bernfeld assay (16) was used to measure amylase in each supernatant and lysed cell pellet as described previously (17). Amylase release was calculated as a percentage of the total amylase present. Amylase release was measured in acinar cells prepared from three rats. For each of these three preparations measurements were made in quadruplicate.

Statistical Analysis—Outliers were identified a priori using an F test based on the Mahalanobis distance (18). Comparisons between groups were then made using a one-tailed two-sample t test for Ca2+ wave speeds, and using a paired t test for amylase secretion. Ca2+ wave speeds and measures of amylase secretion are expressed as mean ± S.E.

RESULTS AND DISCUSSION

Ca2+ Waves Induced by ACh and CCK—Ca2+ waves were induced in pancreatic acinar cells by stimulation with either ACh or CCK; ACh-induced Ca2+ waves were observed in a representative acinus are shown in Fig. 1. Waves induced by 1 μM (n = 20) and 10 μM ACh (n = 22) were equally rapid (95 ± 11 μm/s and 97 ± 13 μm/s, respectively; mean ± S.E.), although 0.1 μM ACh (n = 9) induced waves that were 40% slower (57 ± 10 μm/s, p < 0.05). No Ca2+ increase was detected in acinar cells stimulated with ACh (10 μM) in the presence of caffeine (1 μM) (n = 4). CCK (50 μM, n = 14) also induced rapid (60 ± 11 μm/s) Ca2+ waves, and most waves induced by either ACh (73%) or CCK (77%) traveled in an apical-to-basal direction. These observations demonstrate that stimulation of either muscarinic or CCK receptors induces rapid apical-to-basal Ca2+ waves, waves in pancreatic acinar cells. The wave speed appears to be dose-dependent, since submaximal stimulation with ACh (0.1 μM) induced slower waves. It has previously been reported that ACh (10 μM)-induced Ca2+ waves travel across pancreatic exocrine cells at ~15 μm/s (6), but these measurements were made in cells from acini that had been further digested and dispersed into groups of two to four cells. For comparison, we also examined Ca2+ waves in pancreatic acinar cells prepared and stimulated in this way (Fig. 2). We observed that stimulation with 10 μM ACh induced apical-to-basal Ca2+ waves, waves in 100% of these cells (n = 15), but at a speed of only 37 ± 4 μm/s (p < 0.0005 relative to those cells within intact acini stimulated with 10 μM ACh). Our observations thus indicate that the manner in which cells are dispersed may influence Ca2+ wave speed, since waves were markedly slower in cells from acini that were dispersed to a greater extent, even though these cells were maximally stimulated as well. Possible explanations for this behavior of such cells most likely relate to the way in which the cells are prepared, rather than to the fact that the cells were dispersed per se. First, prolonged incubation of cells in Ca2+-free medium may partially deplete their Ca2+-induced Ca2+ release (CICR) stores. Alternatively, fewer muscarinic receptors may be present on the basal membrane of such cells (so that the cells respond as if stimulated with a lower dose of ACh), possibly because of digestion of receptors by the collagenase and trypsin preparation, or because receptors may have relocalized across loosened tight junctions to the apical membrane (19). Ca2+ wave speeds measured in dispersed acinar cells in our study were nonetheless faster than previously reported (6), but this may relate to the temperature at which our study was performed (2) (37 °C, as opposed to room temperature in a previous study (6)). Together, our findings indicate that stimulation of pancreatic acinar cells results in a rapid apical-to-basal Ca2+ wave, regardless of the type of receptor that is stimulated, and the wave travels at a speed that is dependent upon the magnitude of the stimulus and the
Fig. 1. Subcellular changes in cytosolic Ca\(^{2+}\) in pancreatic acinar cells stimulated with ACh (1 \(\mu\)M). A, confocal microscopic image of acinar cells loaded with the Ca\(^{2+}\)-sensitive dye fluo-3 (depth of focus is 1 \(\mu\)m). Nine cells (outlined in the inset at lower left) can be seen in this optical section through the acinus, although the entire acinus contains \(>50\) cells. The confocal line scan in B was performed along the white horizontal line across this image. Fluorescence intensity along the white horizontal line across this image. Scale bar (lower right) is 25 \(\mu\)m. B, confocal line scan collected along the line indicated in A. Fluorescence intensity along the x axis reflects distance (along the scan line) and along the y axis reflects time (between serial scans). Horizontal lines comprising this scan were obtained every 4 ms for a total of 2 s (from top to bottom). Note that, in both cells scanned, the increase in fluorescence begins apically and spreads at a constant rate to the opposite (basal) pole. C, graphical representation of fluorescence intensity over time at an apical and a basal point (represented by open and closed circles, respectively) in one of the cells along the scan line. The increases in Ca\(^{2+}\) (arrows) occur 200 ms apart and the two points are separated by a distance of 16 \(\mu\)m; this corresponds to a wave speed of \(-80\) \(\mu\)m/s.

Effects of Caffeine and Ryanodine on Ca\(^{2+}\) Wave Speed—To assess the importance of CICR for Ca\(^{2+}\) wave propagation, pancreatic cells within acini were stimulated with 1 \(\mu\)M ACh (n = 16, 15, and 35, respectively). We chose this concentration of caffeine because others had shown it to block CICR in this cell type (20). Caffeine affects ryanodine-sensitive rather than inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive stores (21–24), although there is conflicting evidence as to whether this action of caffeine is due to discharge of CICR stores (21–22) or caused by the release of Ca\(^{2+}\) from CICR stores (20, 23). We found that 20 mM caffeine markedly slowed the speed of both ACh-induced Ca\(^{2+}\) waves (11 ± 2 \(\mu\)m/s; p < 0.0005 relative to 1 \(\mu\)M ACh in the absence of caffeine) and CCK-induced Ca\(^{2+}\) waves (20 ± 3 \(\mu\)m/s, n = 13; p < 0.005 relative to CCK in the absence of caffeine). Ryanodine at a concentration of 50 \(\mu\)M, which blocks mobilization of CICR stores (23, 24), similarly slowed ACh-induced Ca\(^{2+}\) waves (16 ± 2 \(\mu\)m/s; p < 0.0005). This effect was not seen with 10 \(\mu\)M ryanodine (87 ± 21 \(\mu\)m/s), which may be because this concentration is near or below the \(K_d\) of the ryanodine receptor (24). These findings demonstrate that mobilization of caffeine- and ryanodine-sensitive Ca\(^{2+}\) stores are necessary for rapid propagation of Ca\(^{2+}\) waves in pancreatic acinar cells. Estimates of the diffusion constant for Ca\(^{2+}\) in cytosol have ranged from ~10 to 400 \(\mu\)m\(^2\)/s (10, 25, 26), suggesting that agonist-induced Ca\(^{2+}\) waves travel much faster than can be explained by diffusion. These observations are consistent with the hypoth-
esis that Ca²⁺ wave propagation in the acinar cell is mediated by sequential release of spatially distributed CICR stores, serving as a positive feedback mechanism to accelerate wave speed (1, 2).

Amylase Secretion—Since apical-to-basal Ca²⁺ waves are thought to regulate luminal Cl⁻ secretion in pancreatic acinar cells (6), we wished to determine whether such waves also affect amylase secretion, an indicator of apical exocytosis. Stimulation of amylase with 1 μM ACh induced a greater than 3-fold rise in amylase secretion relative to unstimulated controls (p < 0.025, paired t-test; Fig. 3). Amylase release in the presence of 50 μM ryanodine alone was no different than in unstimulated controls (Fig. 3). Secretion of amylase was increased almost 3-fold in acini incubated with both ryanodine and ACh (p < 0.005), identical to the effect observed with ACh alone (Fig. 3). These findings suggest that, unlike Cl⁻ secretion, secretion of amylase by pancreatic acinar cells is not dependent on Ca²⁺ wave speed. However, these findings merely relate the initial ~200 ms of ACh-induced Ca²⁺ signals to the amylase secretion that follows over the next ~20 min. There is considerable evidence that other features of ACh-induced Ca²⁺ signals are causally related to exocytosis (27).

It has been hypothesized that CICR provides a unifying mechanism for spatial and temporal organization of Ca²⁺ signals within the cytosol (1, 2, 10, 28). Documented effects of CICR on Ca²⁺ signals include increasing the magnitude of Ca²⁺ elevations (29) and establishing the threshold for (20) and maintaining (28, 30) Ca²⁺ oscillations. By demonstrating that this positive-feedback mechanism also greatly enhances Ca²⁺ wave speed independent of the receptor type stimulated, we provide experimental evidence that CICR is the common subcellular mechanism by which intracellular Ca²⁺ signals are coordinated.

These findings are in agreement with the previous observation that ACh-induced Ca²⁺ waves begin apically and spread to the basal pole in pancreatic acinar cells (6). In addition, we report that this subcellular pattern of Ca²⁺ release is elicited by CCK, which suggests that apical-to-basal Ca²⁺ waves occur independent of the type of receptor that is stimulated. Since both ACh- and CCK-induced increases in Ca²⁺ are initiated by release of Ca²⁺ from IP₃-sensitive stores (31), these findings would suggest that such stores are located in the apical region (6). Since ryanodine-sensitive (i.e. CICR) Ca²⁺ stores are thought to be distributed across the cell (1), our findings would also suggest that apical release of Ca²⁺ (by IP₃) leads to sequential release of additional Ca²⁺ from apically to basally distributed ryanodine-sensitive stores. This pattern of Ca²⁺ release would result in apical-to-basal Ca²⁺ waves, as observed. Selective inhibition of CICR would again result in apical release of Ca²⁺ from IP₃-sensitive stores, but followed by a slower apical-to-basal Ca²⁺ wave, as we also observed. The subcellular distribution of receptors for IP₃ and ryanodine in exocrine pancreas has not been reported, but IP₃ receptors in the hepatocyte are located apically while hepatocyte ryanodine receptors are located elsewhere (23, 32).

Ca²⁺ wave speeds of ~100 μm/s, as reported here in pancreatic acinar cells, have also been observed in excitable cells (e.g. myocytes (5)) and nonexcitable cells (hepatocytes (7, 8)). The significance of such waves for acinar cell function is not fully understood. The importance of Ca²⁺ waves for luminal Cl⁻ secretion has been suggested (6), but the present work suggests that amylase secretion is not controlled in a similar fashion. In hepatocyte couplets and triplets, agonist-induced Ca²⁺ signals travel across gap junctions in a rapid, synchronized, and wave-like fashion (7, 8), and cells within pancreatic acini also communicate across gap junctions (12, 33). Disruption of conductance across gap junctions influences exocrine pancreatic secretion (12) and agonist-induced Ca²⁺ oscillations are synchronized among cells within a single acinus (34). These considerations raise the question of whether CICR-induced Ca²⁺ waves in this cell type play a role in establishing intercellular communication and coordination. Further work will be needed to determine whether CICR indeed leads to synchronized and integrative behavior among cells comprising the pancreatic secretory epithelium.

REFERENCES

1. Berridge, M. J., and Irvine, R. F. (1989) Nature 341, 197-205
2. Jeffe, L. F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9830-9837
3. Gilkey, J. C., Jaffe, L. F., Ridgway, E. B., and Reynolds, G. T. (1978) J. Cell Biol. 76, 448-456
4. Spekander, J. E., Sardet, C., and Cafa, L. F. (1990) Dev. Biol. 142, 246-250
5. Wier, W. G., Cannell, M. B., Berlin, J. R., Marban, E., and Lederer, W. J. (1987) Science 235, 325-328
6. Kasis, H. and Augustine, G. J. (1990) Nature 349, 735-738
7. Nathanson, M. H., and Burstahler, A. D. (1992) Mol. Cell. Biol. 3, 113-121
8. Nathanson, M. H., and Burstahler, A. D. (1992) Cell Calcium 13, 89-98
9. Saer, J. C., Connor, J. A., Spray, D. C., and Bennett, M. L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2708-2712
10. Berridge, M. J., and Irvine, R. F. (1984) J. Gen. Physiol. 93, 963-977
11. Bruzzone, R., Halban, P. A., Giunovci, A., and Trimble, E. R. (1990) Biochem. J. 226, 621-624
12. Meda, F., Bruzzone, R., Chanson, M., Bosco, D., and Orlic, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4901-4904
13. Herbrand, Cruz, A., Salinas, R. A., and Adams, P. R. (1990) Science 247, 858-862
14. Nathanson, Y. (1988) J. Physiol. 406, 298-315
15. Fao, J. F., Y., Harootanian, A. T., and Tsien, R. Y. (1989) J. Biol. Chem. 264, 5179-5184
16. Ferrell, P. (1985) Methods Enzymol. 149, 188-188
17. Padfield, P. J., Ding, T.-G., and Jamieson, J. D. (1991) Biochem. Biophys. Res. Commun. 174, 536-541
18. Aff, A. A., and Azem, S. P. (1979) Statistical Analysis: A Computer Oriented Approach, Academic Press, New York
19. Amsterdam, A., and Jamieson, J. D. (1974) J. Cell Biol. 63, 1037-1056
20. Wakui, M., Oshchepkov, Y. V., and Petersen, O. H. (1990) Cell 63, 903-923
21. Schmid, A., Dehghani-Kremer, M., Schuller, L., and Gogeblin, H. (1990) Nature 346, 374-378
22. Burgoyne, P. C., Cheek, T. R., Morgan, A., O'Sullivan, A. J., and Moreton, R. B. Berridge, M. J., Mata, A. M., Colyer, J., Lee, A. G., and East, J. M. (1990) Nature 342, 72-74
23. Shoshan-Barmatz, V., Zhang, G. H., Garretson, L., and Kraus-Friedmann, N. (1990) Biochem. J. 268, 699-705
24. Shoshan-Barmatz, V. (1990) FEBS Lett. 263, 317-320
25. Hodgkin, A. L., and Keynes, R. D. (1957) J. Physiol. 136, 253-281
26. Nakamichi, M. J., and Podolsky, R. J. (1969) Science 166, 1287-1289
27. Bruzzone, R. (1990) Gastroenterology 99, 1175-1178
28. Berridge, M. J. (1990) J. Biol. Chem. 265, 9583-9586
29. Reibmann, C., Bianchetti, G., and Trautmann, A. (1991) Cell Regul. 2, 513-522
30. Malgiroti, A., Feres, R., and Melo de Boi, L. (1990) J. Biol. Chem. 265, 3005-3008
31. Saluja, A. K., Dawa, R. K., Lerch, M. L., and Steer, M. L. (1992) J. Biol. Chem. 267, 11292-11297
32. Shears, S. B., Evans, W. H., Kirk, C. J., and Michell, R. H. (1988) Biochem. J. 256, 393-399
33. Chanson, M., Orlic, L., and Meda, P. (1991) Am. J. Physiol. 261, G28-G36
34. Pralong, W. F., Wolheim, C. B., and Bruzzone, R. (1988) FEBS Lett. 242, 79-84

FIG. 3. Amylase released (expressed as percentage of total cellular amylase) by rat pancreatic acini stimulated with ACh (1 μM) in the absence (left) or presence (right) of ryanodine (50 μM). ACh induces a 3-fold increase in amylase release relative to unstimulated controls, regardless of the presence of ryanodine. Amylase release is expressed as mean ± S.E. (*). Increase relative to unstimulated controls is significant (p < 0.025) by paired t test.