Localization of Ca\(^{2+}\) Extrusion Sites in Pancreatic Acinar Cells*

(Pavel V. Belanšt, Oleg V. Gerasimenko, Alexei V. Tepikin, and Ole H. Petersen§)

From the Medical Research Council Secretory Control Research Group, The Physiological Laboratory, University of Liverpool, P. O. Box 147, Crown Street, Liverpool, L69 3BX, United Kingdom

We have investigated the localization of Ca\(^{2+}\) extrusion sites in mouse pancreatic acinar cells. Employing a new technique, in which high resolution localization of cellular Ca\(^{2+}\) exit is achieved by confocal microscopy and a Ca\(^{2+}\)-sensitive fluorescent probe coupled to heavy dextran to slow down diffusion of extracellular Ca\(^{2+}\), it is shown directly that the secretory pole (secretory granule area) is the major site for Ca\(^{2+}\) extrusion following agonist stimulation. This Ca\(^{2+}\) extrusion appears not to be a consequence of exocytosis, as assessment of secretion under our experimental conditions (low external Ca\(^{2+}\) concentration, room temperature) using the technique of monitoring quinacrine fluorescence shows little loss of secretory granules in spite of sustained Ca\(^{2+}\) exit. We conclude that Ca\(^{2+}\) is primarily extruded by Ca\(^{2+}\) pumps from the secretory pole and propose that this process is useful for maintaining a high Ca\(^{2+}\) concentration in the acinar lumen, which is necessary for promotion of endocytosis.

Hormone or neurotransmitter-evoked intracellular Ca\(^{2+}\) release is rapidly followed by activation of plasma membrane Ca\(^{2+}\) pumps extruding a considerable fraction of the Ca\(^{2+}\) liberated from the stores (1–4). Pancreatic acinar cells are highly polarized with an apical secretory pole that is particularly sensitive to Ca\(^{2+}\) mobilizing messengers (5–7). Recent indirect evidence suggests that continuous maximal agonist stimulation evoking a sustained global rise in the cytosolic Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_{i}\))\(^1\) results in activation of plasma membrane Ca\(^{2+}\) pumps in all regions of these cells but that the basolaterally located pumps are partly switched off after only a few seconds (8).

We have now devised a new method for direct visualization of the regional Ca\(^{2+}\) extrusion sites from single cells. To detect Ca\(^{2+}\) extruded from the stimulated cells as well as to slow down diffusion of Ca\(^{2+}\) in the external milieu, we use confocal microscopy and a Ca\(^{2+}\)-sensitive fluorescent probe linked to heavy dextran in the extracellular solution. We show directly that the secretory pole is the major Ca\(^{2+}\) extrusion site in pancreatic acinar cells following agonist stimulation. This arrangement would appear to have substantial physiological advantages. Ca\(^{2+}\) extrusion across the luminal membrane would help to confine physiological Ca\(^{2+}\) signals to the apical part of the cell. Furthermore, endocytosis, which must follow Ca\(^{2+}\)-activated exocytosis, requires extracellular Ca\(^{2+}\) (9, 10), and the Ca\(^{2+}\) pumping into the acinar lumen would secure this.

**MATERIALS AND METHODS**

Fragments of mouse pancreas were digested by pure collagenase to obtain single acinar cells or small clusters as described previously (11). In some experiments, cells were loaded with the fluorescent indicator fura-red acetoxyethyl ester for 30 min at room temperature as described previously for fura-2 loading (6, 11). A group of fura-red containing single cells/cell clusters or non-loaded cells/cell clusters were centrifuged twice in Ca\(^{2+}\)-free solution and then placed in a small experimental chamber (approximately 200 μl) containing nominally calcium-free solution and 30–100 μM Calcium Green 1 bound to dextran (M, 500,000) (Molecular Probes). The free calcium concentration in the extracellular solution under such conditions was around 0.2 μM. The substantial difference between the emission spectra of Calcium Green 1 dextran and fura-red allowed us to monitor simultaneously intracellular and extracellular calcium levels. The fluorescent signals from the intracellular and extracellular dyes were recorded using a Noran Odyssey confocal microscope (Noran Instruments) with the excitation wavelength of 488 nm and emission wavelengths of 530 and 650 nm for Calcium Green 1 dextran and fura-red, respectively. Several boxes were placed in different areas of the field, and the average fluorescence levels from these boxes were acquired during the course of experiments. Measurements of Ca\(^{2+}\) extrusion were performed on both loaded and unloaded cells. Images were analyzed and processed by two-dimensional Intervision analysis software (Noran Instruments). The experimental data are presented as fluorescence intensity rather than free calcium concentration.

Quinacrine was loaded into the cells by incubation in Ca\(^{2+}\)-containing solution (1 mM) with 1 μM of the dye for 7–10 min at 37 °C. After loading, cells were centrifuged and resuspended twice in the nominally calcium-free solution (except for experiments of the type shown in Fig. 5C).

ACh applications were made either by a small droplet addition of a concentrated ACh stock solution to achieve a concentration of about 10 μM in the experimental chamber or by iontophoresis from a microelectrode filled with 20 mM ACh chloride (a second electrode was in this case placed in the experimental chamber). The iontophoretic currents used were from 10 to 100 nA and lasted from 1 to 20 s.

The extracellular solution contained (in mM): NaCl 140, MgCl\(_2\) 0.66–1.13, KCl 4.7, glucose 10, Hepes 10, pH 7.2, adjusted with NaOH. Experiments were carried out at room temperature with the exception of one series specifically mentioned in the text.

**RESULTS**

Fig. 1 shows simultaneous measurements of Ca\(^{2+}\)-sensitive fluorescence of fura-red loaded into a pancreatic acinar cell and Calcium Green 1 dextran present in the external solution. It can be seen that the rapid rise in [Ca\(^{2+}\)]\(_i\), evoked by ACh, causing a decrease in the intensity of fura red fluorescence, is followed by an increase in the fluorescence of Calcium Green 1 dextran signifying extrusion of Ca\(^{2+}\) from the cell to the surrounding medium. Stimulation of this type (with 10 μM ACh) always evokes a [Ca\(^{2+}\)]\(_i\) signal that is initiated in the secretory pole but then rapidly (within a few seconds) spreads to the whole of the acinar cell (11, 12). It is theoretically possible that there could be an uneven intracellular distribution of fura-red,
which might influence the time courses of Ca$^{2+}$ extrusion in different parts of the cell, and for that reason the majority of the experiments described in what follows were done on cells that had not been loaded with any fluorescent indicators. In these experiments only extracellular indicator was present monitoring Ca$^{2+}$ extrusion.

Fig. 2 shows the result from an experiment on a cluster of acinar cells bathed in a solution containing Calcium Green 1 dextran, where the confocal microscope was used to locate the sites of Ca$^{2+}$ exit from stimulated cells. Fig. 2A shows the transmitted light picture of the cluster demonstrating the central localization of the secretory granules. Fig. 2B shows a fluorescence image with the optical slice going through part of the cluster consisting of 4 cells surrounding a pseudolumen. The three boxes shown represent the sites from which the Ca$^{2+}$-sensitive fluorescence was recorded. A brief but vigorous period of ionophoretic ACh stimulation caused rises of the extracellular Ca$^{2+}$ concentration in all of the three areas in which fluorescence was monitored, but as seen in Fig. 2C by far the steepest rise occurs in box 1 placed close to the center of the cluster near the pseudolumen.

It could be argued that the results shown in Fig. 2 might be explained by restrictions to the diffusion of Ca$^{2+}$ away from the lumen. We therefore decided to do experiments in which the optical slice did not go through the cell cluster itself. In the following experiments the fluorescent light was collected from a layer just above the upper surface of the cluster.

Fig. 3 shows the result from such an experiment on a cluster of four acinar cells bathed in a solution containing Calcium Green 1 dextran. Fig. 3A shows the transmitted light picture of the cluster demonstrating the central localization of the secretory granules. All other pictures in this set show pseudocolor representations of the fluorescence intensity of Calcium Green 1 dextran in the extracellular solution. The outline of the cell cluster taken from the transmitted light picture has for convenience been placed on all the fluorescence images. Fig. 3, b–h, represents images taken at 10-s intervals immediately before b and following ACh stimulation (c–h), whereas Fig. 3i shows the situation more than 100 s after the maximal external Ca$^{2+}$ concentration had been reached. It is seen in Fig. 3 that the maximum fluorescence intensity is close to the secretory granule area. The rise in extracellular Ca$^{2+}$-sensitive fluorescence is transient, lasting only about 200 s, in spite of continuous ACh stimulation. This is expected as the external Ca$^{2+}$ concentration in our experiments is kept sufficiently low to prevent Ca$^{2+}$ entry into the cell so that the cell runs out of mobilizable Ca$^{2+}$ as shown previously (1, 2). Fourteen experiments of this type shown in Fig. 3 were carried out with similar results.

In order to improve the resolution of Ca$^{2+}$ exit sites we carried out a series of experiments on isolated single cells. In this case the single acinar cells were stimulated with 10–15-s pulses of ionophoretically applied ACh. In experiments with fura-red loaded cells it was established that this protocol always gave rise to a global elevation of [Ca$^{2+}$]$_i$ (12). One of the experiments designed to visualize the major Ca$^{2+}$ extrusion site from a single cell after this type of ACh stimulation is shown in Fig. 4. The polarization of the single cell is clearly seen in Fig. 4A, a. The confocal system collected the fluorescent light from a layer that went through the cell, which is shown as a black spot in each of the fluorescence images (Fig. 4A, b–i) that were obtained at 3-s intervals following the start of ACh stimulation. The intensity of the fluorescence of the extracellular Calcium Green 1 dextran increased much more near the secretory pole than near the basal pole, and this is also illustrated graphically in Fig. 4B. In every one of the six experiments of this type, the fluorescence intensity grew more markedly near the secretory pole than the basal pole, and in no case was the peak intensity at the basal pole more than 50% of that seen near the secretory pole, irrespective of the exact position of the stimulating ACh pipette. As seen in Fig. 4B there was a steep increase in the Ca$^{2+}$-sensitive fluorescence also at the basal pole, and we tried to assess whether this might be due to diffusion of Ca$^{2+}$ primarily extruded from the apical (secretory)
pole or whether it was more likely to arise from extrusion through the basal membrane. We therefore compared the rise in Ca$$^{2+}$$-sensitive fluorescence from three boxes placed equidistantly on a straight line with the box in the middle near the secretory pole and the two other boxes at the basal pole or in the exact opposite direction from the secretory pole, respectively. In such cases the rise near the basal pole occurred earlier and was always steeper and larger (at least 2 times) than in the box on the opposite side of the secretory pole, indicating that the rise in Ca$$^{2+}$$ concentration at the basal pole is mostly due to Ca$$^{2+}$$ extrusion through the basal pole rather than diffusion of Ca$$^{2+}$$ extruded at the secretory pole.

Can the Ca$$^{2+}$$ extrusion observed (Figs. 2-4) be explained simply as a result of exocytotic Ca$$^{2+}$$ release? From experiments with the droplet technique, in which the total amount of Ca$$^{2+}$$ extruded from maximally stimulated pancreatic acinar cells was measured, it is known that the whole of the mobilizable Ca$$^{2+}$$ pool, corresponding to about 0.7 mM of total cellular calcium concentration (1, 13), is exported in about 200 s (1). Within that time frame the pancreatic acinar cell is very far from being depleted of secretory granules. We could not observe any significant loss of quinacrine fluorescence from secretory granules (quinacrine is accumulated in the acidic granules, and loss of quinacrine fluorescence is used as a measure of secretion (14)) after our standard 10-15 s ACh pulse under the conditions of our experiments (room temperature, Ca$$^{2+}$$-free solution) (n = 6) (Fig. 5, A and B). In the presence of 1 mM external Ca$$^{2+}$$, sustained ACh stimulation (10 μM) at 37°C did evoke a measurable decrease in quinacrine fluorescence, indicating secretion (Fig. 5C).

**DISCUSSION**

The novel technique described here, in which high resolution localization of cellular Ca$$^{2+}$$ exit is achieved by confocal microscopy and a Ca$$^{2+}$$-sensitive fluorescent probe coupled to heavy dextran to slow down diffusion of extracellular Ca$$^{2+}$$, has enabled us to show directly that the secretory pole in pancreatic acinar cells is the major site of Ca$$^{2+}$$ extrusion following agonist stimulation.

The relatively high rate of Ca$$^{2+}$$ extrusion at the secretory pole, uncovered in our experiments (Fig. 4), could be due to a relatively high density of Ca$$^{2+}$$ pumps in this region and/or be explained by the Ca$$^{2+}$$ pumps in this part of the cell having different characteristics from those in the basal membrane. A number of isoforms of the plasma membrane Ca$$^{2+}$$-ATPase have been described (15), and it is known that the Ca$$^{2+}$$ affinity of the pump can be controlled by alternative splicing via changes in the affinity for calmodulin (16). It is also possible that agonist stimulation could differentially regulate Ca$$^{2+}$$ pumps in the luminal and basolateral membranes.

The results shown in Fig. 5, A and B, should not necessarily be taken as an indication that there was no secretion in our experiments, although secretion would be expected to be reduced because of the low external calcium concentration (17). The quinacrine technique employed may not be sufficiently sensitive, but the crucial point is that the majority of secretory granules was still in the cell after the period of stimulation resulting in the Ca$$^{2+}$$ extrusion shown in Figs. 2-4. The total calcium concentration in isolated zymogen granules appears to be about 15 mM (18, 19). Taking into account the known magnitude of the acutely hormone-mobilizable Ca$$^{2+}$$ pool (1, 13) and the ratio of zymogen granule volume to cell volume (20), it can be estimated that the zymogen granules apparently contain about the same amount of calcium as the acutely hormone-mobilizable pool. Since the whole of the acutely hormone-mobilizable Ca$$^{2+}$$ pool is extruded within 200 s (1) it means that if this extrusion were to be accounted for solely in terms of exocytotic release, all the zymogen granules should have been lost within this period. This is obviously very far from being the...
case (Fig. 5) (1). The maximal rate of enzyme secretion, under optimal conditions, is such that only about 7% of the cellular enzyme content has been released after a half hour of continuous maximal stimulation (21).

Let us, for the sake of argument, suppose that most of the Ca\(^{2+}\) is extruded homogeneously from the entire cell membrane but that calcium secretion occurs only in the secretory region. Such an arrangement would lead to inhomogeneity of calcium release. But under the above mentioned assumptions, this inhomogeneity would not appear to explain the whole difference observed with regard to calcium release between basal parts of the cells and their secretory regions. In all our experiments the difference in values of fluorescent signals between these parts of the extracellular milieu was not less than a factor of 2. It means that there is at least a 2-fold difference in calcium flux, leaving a unit area of cell membrane in these two regions. The part of the cell perimeter associated with the secretory granule area where calcium efflux was at least 2 times higher than in the basolateral part was, in our experiments, about one-fifth of the whole cell perimeter. In this case, calcium secretion should be 10% of the total calcium efflux in order to account for the difference between the basal parts of the cells and their secretory regions, i.e. on the basis of previous estimations of the total calcium releasable pool and total calcium granule content, 10% of all granules should be released during ACh application at room temperature and low external Ca\(^{2+}\) concentration in less than 4 min. This is unlikely since only 7% of the granules could be released during a half hour of ACh stimulation at 37 °C with a high external Ca\(^{2+}\) concentration. We therefore conclude that the Ca\(^{2+}\) extrusion observed in our experiments is unlikely to be accounted for by exocytotic release of Ca\(^{2+}\), although there may well be a small contribution from this process, but is most likely due to plasma membrane Ca\(^{2+}\)-activated ATPases, since Na\(^{+}\)-Ca\(^{2+}\) exchange is virtually absent in pancreatic acinar cells (1, 13).

The marked Ca\(^{2+}\) extrusion through the secretory pole would appear to be advantageous for the overall function of the acinar cells. Ca\(^{2+}\) signals evoked by physiological agonist concentrations are essentially confined to the secretory granule area (5-7), and selective Ca\(^{2+}\) pumping across the luminal
membrane would help to prevent spreading of the signal into the basolateral regions. This may be important to avoid Ca\(^{2+}\)-induced Ca\(^{2+}\) release in the nucleus via the inositol triphosphate and ryanodine receptors localized in the inner nuclear membrane (22, 23). While Ca\(^{2+}\) extruded across the basolateral membrane would appear to have no special function, Ca\(^{2+}\) pumping into the acinar lumen would maintain a high Ca\(^{2+}\) concentration in this extracellular compartment. This is important as it is known that endocytosis in the pancreas cannot proceed in the absence of external Ca\(^{2+}\) (9, 10). We can now envisage an interesting Ca\(^{2+}\) cycle in the pancreas where agonist stimulation primarily releases Ca\(^{2+}\) from stores in the secretory granule area (24), most likely from the granules themselves (19, 25). The Ca\(^{2+}\) released into the cytosol is then mainly transported across the luminal membrane into the acinar lumen where it promotes endocytosis (9, 10), following the stimulant-evoked exocytosis, and may also in part be taken back into the cell via the endocytotic process or through a Ca\(^{2+}\) entry pathway, in this way being recycled into new secretory granules.

Acknowledgment—We thank Dave Berry for technical assistance.

REFERENCES
1. Tepikin, A. V., Voronina, S. G., Gallacher, D. V., and Petersen, O. H. (1992a) J. Biol. Chem. 267, 3569–3572
2. Tepikin, A. V., Voronina, S. G., Gallacher, D. V., and Petersen, O. H. (1992b) J. Biol. Chem. 267, 14073–14076
3. Zhang, B.-X., Zhao, H., Loeßberg, P., and Muallem, S. (1992) J. Biol. Chem. 267, 15419–15425
4. Berridge, M. J. (1993) Nature 361, 315–325
5. Kasai, H., Li, Y., and Miyashita, Y. (1993) Cell 74, 669–677
6. Thorn, P., Lawrie, A. M., Smith, P., Gallacher, D. V., and Petersen, O. H. (1993) Cell 74, 661–668
7. Thorn, P., Gerasimenko, O., and Petersen, O. H. (1994) EMBO J. 13, 2038–2043
8. Toescu, E. C., and Petersen, O. H. (1995) J. Biol. Chem. 270, 8528–8535
9. Maruyama, Y. (1989) Pfluegers Arch. Eur. J. Physiol. 413, 438–440
10. Maruyama, Y., Inooka, G., Li, Y., Miyashita, Y., and Kasai, H. (1993) EMBO J. 12, 3017–3022
11. Toescu, E. C., Lawrie, A. M., Petersen, O. H., and Gallacher, D. V. (1992) EMBO J. 11, 1623–1629
12. Kasai, H., and Augustine, G. J. (1990) Nature 348, 735–738
13. Muallem, S. (1989) Annu. Rev. Physiol. 51, 83–105
14. Bokvist, K., Eliasson, L., Ammala, C., Renstrom, E., and Rorsman, P. (1995) EMBO J. 14, 50–57
15. Carafoli, E. (1992) J. Biol. Chem. 267, 2115–2118
16. Enyedi, A., Verma, A. K., Heinm, R., Adamo, H. P., Filoteo, A. G., Strehler, E. E., and Penniston, J. T. (1994) J. Biol. Chem. 269, 41–43
17. Ueda, N., and Petersen, O. H. (1977) Pfluegers Arch. Eur. J. Physiol. 370, 179–183
18. Nicaise, G., Maggio, K., Thirion, S., Horoyan, M., and Keicher, E. (1992) Biol. Cell 75, 89–99
19. Gerasimenko, O. V., Gerasimenko, J. V., Belan, P. V., and Petersen, O. H. (1996) Cell 84, 473–480
20. Bölander, R. P. (1974) J. Cell Biol. 63, 269–287
21. Chang, A., and Jamieson, J. D. (1989) in Handbook of Physiology, Section 6: The Gastrointestinal System (Forst, J. G., ed) Vol. 3, pp. 531–547, American Physiological Society, Bethesda, MD
22. Gerasimenko, O. V., Gerasimenko, J. V., Tepikin, A. V., and Petersen, O. H. (1995) Cell 80, 439–444
23. Humbert, J.-P., Matter, N., Arnault, J.-C., Köppler, P., and Malviya, A. N. (1996) J. Biol. Chem. 271, 478–485
24. Petersen, O. H., Petersen, C. C. H., and Kasai, H. (1994) Annu. Rev. Physiol. 56, 297–319
25. Yoo, S.-H., and Albanesi, J. P. (1990) J. Biol. Chem. 265, 13446–13448
