Spacial Compartmentalization of Ca\(^{2+}\) Signaling Complexes in Pancreatic Acini* 

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Imaging [Ca\(^{2+}\)], at high temporal resolution and measuring the properties of Ca\(^{2+}\) signaling in streptolysin O (SLO)-permeabilized cells were used to study the spacial organization of signaling complexes. Sequential stimulation of single cells within pancreatic acini with several Ca\(^{2+}\)-mobilizing agonists revealed an agonist-specific pattern and propagation rate of Ca\(^{2+}\) waves in the same cells, with CCK8 stimulating the fastest and bombesin the slowest waves. More importantly, each agonist initiated the wave in a different region of the same cell. On the other hand, repetitive stimulation with the same agonist induced Ca\(^{2+}\) waves of the same pattern that were initiated from the same region of the cell. The agonist-specific Ca\(^{2+}\) signaling does not appear to be the result of coupling to different G proteins as infusion of an anti-Gq antibody into the cells through a patch pipette equally inhibited Ca\(^{2+}\) signaling by all agonists. Further evidence for compartmentalization of signaling complexes was developed in permeabilized cells. The time-dependent loss of Ca\(^{2+}\) signaling due to SLO permeabilization occurred in an agonist-specific manner in the sequence cabachol > bombesin > cholecystokinin. Signaling by all agonists could be completely restored with as low as 2 \(\mu\)g guanosine 5’-3-O-(thio)triphosphate (GTP\(_{\gamma}\)S). At this low concentration GTP\(_{\gamma}\)S recoupled inositol 1,4,5-trisphosphate production and Ca\(^{2+}\) release, rather than enhancing phospholipase C activity. Priming of Ca\(^{2+}\) signaling by GTP\(_{\gamma}\)S was agonist-specific. Guanosine 5’-O-(thio)diphosphate (GDP\(_{\gamma}\)S) uncoupled the ability of signaling complexes to release Ca\(^{2+}\) much better than stimulating inositol 1,4,5-trisphosphate production. The uncoupling of Ca\(^{2+}\) signaling by GDP\(_{\gamma}\)S was also agonist-specific. The combined findings of agonist-specific initiation sites of the Ca\(^{2+}\) wave and differential access of guanine nucleotides to signaling complexes suggest spacial compartmentalization of Ca\(^{2+}\) signaling complexes. Each complex must include a receptor, G protein, and phospholipase C that are coupled to a specific portion of the Ca\(^{2+}\) pool. 

Transduction of the Ca\(^{2+}\) signal can occur through G protein-coupled or G protein-independent receptor complexes (1, 2). The G protein-independent complexes are proteins containing SH2 and SH3 domains which couple plasma membrane-localized receptors to the \(\gamma\) isofrom of phospholipase C (PLC). The G protein-dependent receptor complexes use heterotrimeric G proteins from the Gq/11 family that couples plasma membrane receptors to the \(\beta\) isofrom of PLC (1–4). Like other heterotrimeric G proteins, Gq/11 is composed of Ga and G \(\beta\)y subunits (2, 4). Both subunits can activate various PLC\(_{\beta}\) isoforms (4–10) to potentially transduce different types or patterns of Ca\(^{2+}\) signaling. Additional diversity of the system is provided by the ability of a single receptor to couple to several combinations of Ga and G\(\beta\)y (11–14) to transduce different information. 

A central question in recent years is how the diversity in signal transduction is manifested while maintaining specificity in cellular signaling. Many cells express a combination of signaling pathways and several receptors that activate the same signaling pathway (4). In the case of the exocrine pancreas, acinar cells respond to cholecystokinin (CCK), acetylcholine, and bombesin (BS) (15) by activation of PLC to generate IP\(_3\). 

Signaling due to SLO permeabilization from the aberrant behavior of antagonists acting on streptolysin O-permeabilized pancreatic acini stimulated with GTP\(_{\gamma}\)S (16). To obtain further evidence for compartmentalization of signaling complexes, we measured the patterns of the [Ca\(^{2+}\)]i changes evoked by several Ca\(^{2+}\)-mobilizing agonists and their sensitivity to Gq/11 antibodies in the same intact single cells. We also used SLO-permeable cells to determine the sensitivity of the signaling complexes to GTP\(_{\gamma}\)S and GDP\(_{\gamma}\)S. The clear differences observed with the various agonists show that agonist-specific [Ca\(^{2+}\)]i signaling can be attributed to spacial compartmentalization of signaling complexes. 

MATERIALS AND METHODS 

Preparation of Pancreatic Acini and Single Acinar Cells—Acini were prepared from the pancreas of 100–150-g rats by limited collagenase digestion as described previously (17). The acini were resuspended in a standard solution A containing (in mM) 140 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 Hepes (pH 7.4 with NaOH), 10 glucose, and 0.1% bovine serum albumin, and kept on ice until use for [Ca\(^{2+}\)]i measurement in intact cells or Ca\(^{2+}\) release in permeable cells. Single acinar cells were obtained by incubation of a minced pancreas in an 0.025% trypsin, 0.02% EDTA solution for 5 min at 37 °C. After washing the tissue with solution A supplemented with 0.02% soybean trypsin inhibitor, single acinar cells were liberated by a 5-min incubation at 37 °C in the same solution that also contained 160 units/ml pure collagenase. The cells were washed with solution A and kept on ice until use. 

Measurement of [Ca\(^{2+}\)]i—Acini in solution A were incubated with 5 \(\mu\)M Fura 2/AM for 30 min at 37 °C, washed once with solution A, and 

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1 The abbreviations used are: PLC, phospholipase C; IP\(_i\), inositol 1,4,5-trisphosphate; CCK, cholecystokinin; BS, bombesin; SLO, streptolysin O; GTP\(_{\gamma}\)S, guanosine 5’-3-O-(thio)triphosphate; GDP\(_{\gamma}\)S, guanosine 5’-O-(thio)diphosphate.
kept on ice until plating on glass coverslips that formed the bottom of a perfusion chamber. The plated cells were incubated for 2 min at room temperature to allow attachment to coverslips and then perfused at a rate of 10–12 ml/min for at least 10 min at 37 °C prior to the first stimulation. Acinar cell clusters consisting of three to five cells and forming a single layer in the bottom of the coverslip were selected for experimentation. Fura 2 fluorescence was measured by a PTI Int. image acquisition and analysis system as described elsewhere (18). To maximize the temporal resolution, fluorescence was measured at a single excitation wavelength of 380 nm and without image averaging. The light was directed by a dichroic mirror to an image intensifier and a video camera. The images were digitized and stored in CASM memory for further analysis. Under these conditions using a frame size of 256 × 240 pixels allowed recording the fluorescence of the entire cluster at a resolution of 2.0 Hz. A resolution of 4.7 Hz was obtained by recording the fluorescence from one cell within the cluster which could be fitted in a frame size of 128 × 128 pixels. During the perfusion with the control solution and just before the first stimulation, the image of the resting cells was acquired by averaging eight consecutive images. This was taken as the fluorescence at time 0 ($F_0$). All subsequent images were divided by this image, and the traces in Figs. 1–3 are the calculated $F/F_0$ where $F$ is the fluorescence at time $t$.

**Measurement of $Ca^{2+}$ Release in Permeable Cells—Agonist-mediated $Ca^{2+}$ release from SLO-permeabilized pancreatic acini was measured exactly as described previously (16, 19). The SLO permeabilization medium was composed of Chelax-treated 145 mM KCl and 10 mM Hepes solution and supplemented with 0.02% soybean trypsin inhibitor, 3 mM ATP, 5 mM MgCl₂, 10 mM creatine phosphate, 5 units/ml creatine phosphokinase, 10 μM antimycin A, 10 μM oligomycin, 1 μM fluo 3, and 3 mM/ml SLO (Difco). Fluor 3 fluorescence was recorded and calibrated as detailed previously (19).

**Measurement of $IP_3$—$IP_2$ levels were measured by a radioligand assay as described elsewhere (19). Acini washed with the Chelax-treated solution and incubated in the SLO permeabilization medium with or without GDPβS, were stimulated with the indicated agonist for 20 s at 37 °C. The reactions were stopped by the transfer of 100-μl samples to 100 μl of an ice-cold 15% perchloric acid, vigorous mixing, and incubation on ice for at least 10 min to allow precipitation of proteins. The supernatants were then collected by 2-min centrifugation at 10,000 × g and transferred to clean tubes. Standards of $IP_3$ were prepared in permeabilization medium and processed in the same manner. The perchloric acid was removed and $IP_3$ extracted by the addition of 0.2 ml of Freon and 0.2 ml of tri-n-octylamine. After mixing and phase separation by centrifugation, between 15 and 25 μl of the upper layer were used to measure $IP_3$ content with micromoles prepared from bovine brain cerebella.

**Whole-cell Current Recording—**The tight-seal whole-cell current recorded by the patch clamp technique was used (20) for measurement of $Ca^{2+}$—activated $Cl^{-}$ current, which directly correlates with changes in [Ca$^{2+}$]i (21). The experiments were performed with single acinar cells perfused with solution A. The standard pipette solution contained (in mM): KCl 140, MgCl₂ 1, EGTA 0.2, Na₂ATP 5, and Hepes 10 (pH 7.3 with KOH) as described in previous studies (21). In some experiments this solution also contained either 80 μg/ml immune serum or IgG anti-Gqα/11 antibodies, both of which were a generous gift from Dr. Paul Sternweis, University of Texas Southwestern Medical Center, Dallas (22). Seals of 6–10 gigohms were produced on the cell membrane, and the whole cell configuration was obtained by gentle suction or voltage pulses of 0.5 V for 0.3–1 ms. The patch clamp output (Axopatch-1B, Axon Instruments) was filtered at 20 Hz. Recording was performed with a Digidata 1200 A Digitizing Interface (Axon Instruments). The cells were voltage clamped at ~30 mV, and depolarizing voltage jumps of 100-mas duration at a frequency of 1 Hz to a membrane potential of +10 mV were applied throughout the experiments to measure the leak current. In all experiments the $Cl^{-}$ and cation equilibrium potentials were about 0 mV. All the traces shown were at a holding potential of ~50 mV and were corrected for leak currents.

**RESULTS**

**Agonist Specific Initiation Sites of $Ca^{2+}$ Release—**Pancreatic acini express several distinct receptors for $Ca^{2+}$-mobilizing agonists in the basolateral membrane, which stimulate the exocytotic secretion of digestive enzymes through the apical membrane (15). The most potent $Ca^{2+}$-mobilizing agonists are acetylcholine, bombesin, and CCK, all of which cause a large increase in $IP_3$ to mobilize all the agonist-sensitive intracellular $Ca^{2+}$ pool (15, 23–26). Mobilization of $Ca^{2+}$ from internal stores by one of these agonists, acetylcholine, was shown to be initiated in the apical and spread to the basolateral pole of the cell (27–29). To determine whether all agonists acting on acinar cells initiate the $Ca^{2+}$ signal at the same site, we compared the effects of the three agonists on [Ca$^{2+}$]i, of the same cells. All the experiments were performed on clusters of three to five cells to ensure the maintenance of intact tight junctions and cellular polarity, and then [Ca$^{2+}$], was analyzed in single cells within the cluster. Fig. 1 shows the experimental protocol used. The cells were stimulated with supermaximal concentration of agonists to mobilize all the $Ca^{2+}$ in the intracellular stores. Shortly after stimulation, the cells were inhibited with the relevant antagonist to force $Ca^{2+}$ back into the intracellular stores and cause rapid and maximal reloading (25, 30, 31). In the first set of experiments images were collected from the entire acinus at a resolution of 2.0 Hz, which was sufficient to determine the rate of the $Ca^{2+}$ wave. The upper panel of Fig. 1 shows the averaged [Ca$^{2+}$], changes in the entire cell. The bottom panels show the rising phase of [Ca$^{2+}$], for each agonist in an expanded time scale. In each case the solid line depicts the [Ca$^{2+}$], in the initiation region and the dashed line in the region in which [Ca$^{2+}$], increased last. In all experiments CCK8 caused the fastest and bombesin the slowest [Ca$^{2+}$], waves. In 34 cells from nine experiments the waves initiated by CCK8, carbachol, and bombesin propagated within 0.47 ± 0.06, 0.92 ± 0.13, and 2.3 ± 0.15 s, respectively.

To further analyze the differences between the agonists, the same protocol was used but now [Ca$^{2+}$], was imaged in one cell and only during the rising phase at a resolution of 4.7 Hz (maximal possible with our recording system). Fig. 2 shows the results of such an experiment. The upper bright-field image is a 600 × magnification of the three-cell cluster used for the experiment. The bottom left panel shows a 1000 × magnification of the cell enclosed by the broken line in the 600 × image. The remaining images are the Fura 2 fluorescence ratios re-
Agonist-specific initiation and patterns of \( \text{Ca}^{2+} \) waves in single pancreatic acinar cells. The experimental protocol was similar to that in Fig. 1 except that a three-cell cluster (middle left, magnification, \( \times 600 \)) was used and Fura 2 fluorescence was recorded only during the rising phase of \( \text{Ca}^{2+} \), from one of these cells, as marked by the dashed line in the middle left panel and shown in \( \times 1000 \) magnification in the bottom left panel. Fluorescence imaging was performed at the \( \times 1000 \) magnification used to record the bright-field image in the bottom left. Fluorescence image ratios are displayed in pseudocolor. The top left panel shows the \( \text{Ca}^{2+} \) of the cell under resting conditions. The remaining images in the upper row show three images recorded during stimulation of the cell with 100 \( \mu \text{M} \) carbachol. The fluorescence images in the middle row are from the period of stimulation with 25 \( \mu \text{M} \) bombesin and the fluorescence images in the bottom row are from the period of stimulation with 10 \( \text{nm} \) CCK8. The bottom panels show the change in \( F_0/F_3 \) fluorescence ratios during the first 1.4 or 2.2 s of the \( \text{Ca}^{2+} \), rise phase for each agonist. Traces 1–5 show the \( \text{Ca}^{2+} \), changes in the areas marked 1–5 in the bottom left, bright-field image. The apical (A) and basal (B) poles of the cells are also marked.

A significant difference between the agonists is the pattern of the \( \text{Ca}^{2+} \) wave as illustrated in the traces in the bottom panels of Fig. 2. The traces show the pattern of [\( \text{Ca}^{2+} \)], changes during the first 1–2 s of cell stimulation for each agonist. The carbachol-induced \( \text{Ca}^{2+} \), wave propagated from areas 1 to 2, 3, 4, and 5. The bombesin-induced wave appears to progress simultaneously in two directions increasing first at area 2 and then at areas 1 and 3 at about the same time and then at areas 4 and 5. The CCK8-induced wave was almost in the opposite direction from that of carbachol, starting at area 4 and rapidly progressing through areas 3, 2, 1, and then 5. Similar agonist-specific patterns were recorded in the experiments mentioned above that were designed to identify the initial site of [\( \text{Ca}^{2+} \)], increase for each agonist. As reported before for acetylcholine (27–29), carbachol and bombesin always initiated the [\( \text{Ca}^{2+} \)], increase in the apical region (marked with A in bright-field images) and the wave tended to spread through the cell periphery to the basal region (marked with B). In the case of CCK8 the [\( \text{Ca}^{2+} \)], increase was initiated either in the apical (Fig. 1) or basolateral (Fig. 2) regions, although in most experiments (~76%) it started in the apical region of the cell.

An important control experiment in assigning a specific portion of the pool to a \( \text{Ca}^{2+} \)-mobilizing agonist is to demonstrate that repetitive stimulation with the same agonist initiates \( \text{Ca}^{2+} \) waves of the same pattern and from the same region of the cytoplasm. To demonstrate this behavior, we selected carbachol, since stimulation by this agonist can be readily reversed with a very low concentration of atropine, and atropine can then be easily washed to allow repetitive stimulations. Fig.
Ca$^{2+}$-Signaling Complexes in Pancreatic Acini

Fig. 3. Constant initiation site and pattern of [Ca$^{2+}$], waves induced by repetitive stimulation with the same agonist. The upper and lower bright-field images are the $\times$ 600 and $\times$ 1000 magnification of the cluster and the cell from which fluorescence was recorded, respectively. The dotted line in the upper image marks the cell shown in the lower image. The apical and basal pole of the cluster and the cells are indicated. Regions 1–5 in the lower image indicate the areas analyzed. The corresponding traces are shown in the right panel. For this experiment the cell was stimulated first with 10 $\mu$M carbachol (CAP), and at peak [Ca$^{2+}$], it was inhibited with 2 $\mu$M atropine. After an additional 5.5 min of continuous perfusion at 37°C with solution A, the cell was stimulated with 100 $\mu$M carbachol and at peak [Ca$^{2+}$], inhibited with 2 $\mu$M atropine. Finally, after an additional 5.5 min-perfusion with solution A, the cell was restimulated with 100 $\mu$M carbachol. The traces show the changes in [Ca$^{2+}$], during 1.8 s of the [Ca$^{2+}$] rising phase in regions 1–5.

Fig. 4. Effect of anti-Gq antibody on Ca$^{2+}$ signaling by all agonists. The Ca$^{2+}$-activated Cl$^{-}$ current was recorded as described under “Materials and Methods.” In experiment a, the pipette solution contained 80 $\mu$g/ml IgG anti-Gq/11 antibody. After either 7 (a and b) or 14 (c) min of cytosolic perfusion with the pipette solutions, the cells were sequentially exposed to 100 $\mu$M carbachol (Car), 10 $\mu$M atropine (Atrop), 25 nM BS, 100 nM bombesin antagonist (BS&Ant), and 10 nM CCK8. To show that the antibody had no effect on the channel itself, the cells were exposed to 2 $\mu$M ionomycin (Ion) (b and c) to elevate [Ca$^{2+}$], and activate the current. The number of experiments and controls performed are listed in the text.

Preliminary experiments showed that when the pipette solution contained between 80–240 $\mu$g/ml IgG anti-Gq/11 (the highest concentration tested) maximal inhibition of Ca$^{2+}$ signaling could be achieved. Concentrations of the antibody between 80 and 240 $\mu$g/ml affected only the time of dialysis required to achieve maximal inhibition. In Fig. 3, 80 $\mu$g/ml IgG was used. Trace b shows an experiment in which less than maximal inhibition was achieved. The trace is shown to demonstrate that the Gq/11 antibody inhibited the signal induced by the three agonists to a similar extent. The effect of carbachol was inhibited by 82%, that of bombesin by 86%, and that of CCK8 by 87%. In trace c the anti-Gq/11 antibody inhibited the effect of all the agonists by more than 95%. The antibody inhibited Ca$^{2+}$ signaling by all agonists and to a similar extent in all 17 experiments tested. Thus, it appears that all three receptors are coupled to Gq.

Compartmentalized Signaling Complexes in Permeable Cells—Spacial compartmentalization of signaling complexes was supported by testing the time-dependent effect of cell permeabilization with SLO toxin on Ca$^{2+}$ signaling by the three agonists. As we reported before (16, 19), after permeabilization with SLO, pancreatic acinar cells retain intact signaling systems, including the stimulated generation of IP$_3$ and Ca$^{2+}$ release from intracellular stores. However, continuous exposure to SLO resulted in a time-dependent loss of signaling, which was agonist-specific. Fig. 5 shows individual experiments with carbachol and CCK8, whereas Fig. 6 (closed symbols) summarizes the results of three to six experiments with all agonists. The ability of carbachol to signal Ca$^{2+}$ release was lost within 10 min of exposure to SLO. The lose of signaling by bombesin was slower with 60% inhibition after 10-min incubation with SLO, while CCK8 signaling was quite resistant to permeabilization by SLO with only about a 20% loss of signaling after 10 min of permeabilization.

The loss of Ca$^{2+}$ signaling could be completely restored by the addition of very low concentration of GTPyS. Fig. 7 shows individual experiments in which the cells were stimulated with carbachol or CCK8 and 2 $\mu$M GTPyS after 2 or 10 min of permeabilization with SLO. Fig. 6 (open symbols) summarizes the results obtained with all agonists after 10 min permeabilization. It is clear that the low concentration of GTPyS was able to restore maximal signaling by all agonists. G proteins can affect Ca$^{2+}$ signaling either by regulating PLC and IP$_3$ production (4), or by directly regulating the IP$_3$-activated Ca$^{2+}$ chan-
Signaling Complexes in Pancreatic Acini

**CA2\(^{+}\) Signaling Complexes in Pancreatic Acini**

**Fig. 5.** Effect of permeabilization with SLO on carbachol or CCK8-induced CA2\(^{+}\) signaling. Acini were incubated in SLO permeabilization medium for about 1.5 (a and b), 5 (b and c), or 10 min (c and d) at 37 °C prior to stimulation with either 2 mM carbachol (a–c) or 0.1 μM CCK8 (d–f). The continuous traces are from experiments c and f and show the [CA2\(^{+}\)] of the incubation medium during the 10-min incubation at 37 °C. All the experiments were performed are summarized in Fig. 6.

**Fig. 6.** Time course of agonist-dependent CA2\(^{+}\) signaling loss by SLO permeabilization. Protocols similar to those in Figs. 5 and 7 were used to evaluate the loss of CA2\(^{+}\) signaling by each agonist. After the indicated permeabilization periods, the cells were stimulated with 2 mM carbachol (●), 2 mM carbachol and 2 μM GTPγS (○), 0.1 μM bombesin (■), 0.1 μM bombesin and 2 μM GTPγS (□), 0.1 μM CCK8 (▲), or 0.1 μM CCK8 and 2 μM GTPγS (△). The figure shows the mean ± S.E. of three to six experiments.

To distinguish between the two possibilities we measured the effect of SLO permeabilization on IP3 production. Table 1 shows that after 2-min permeabilization with SLO all agonists increase IP3 levels to the same extent and 2 μM GTPγS had no effect on IP3 production. A more significant finding is that after 10-min permeabilization IP3 production by all agonist was equally reduced by about 25–30% and GTPγS did not restore IP3 production to control levels. Thus, the loss or uncoupling of a GTP-dependent mechanism downstream from PLC stimulation was specific for the signaling complex of each agonist.

**Fig. 7.** Restoration of agonist-mediated CA2\(^{+}\) signaling by GTPγS. Acini were incubated in SLO permeabilization medium for about 2 (a and c) or 10 min (b and d) at 37 °C before stimulation with either 2 mM carbachol and 2 μM GTPγS (a and b) or 0.1 μM CCK8 and 2 μM GTPγS (c and d). The changes in medium [CA2\(^{+}\)] during the 10-min incubation at 37 °C is shown only for trace b. All the experiments were summarized in Figure 6.

**TABLE I**

| Conditions      | 2 min | 10 min |
|-----------------|-------|--------|
| IP3 in pmol/mg protein |       |        |
| Control         | 4.3 ± 0.2 \(^a\) | 4.7 ± 0.4 |
| 2 μM GTPγS      | 4.7 ± 0.3             | 4.7 ± 0.2 |
| Carbachol 2 mM | 64 ± 6                    | 45 ± 3     |
| + GTPγS 2 μM   | 69 ± 8                   | 52 ± 2     |
| Bombesin 0.1 μM| 62 ± 4                   | 43 ± 2     |
| + GTPγS 2 μM   | 65 ± 5                   | 48 ± 3     |
| CCK 8 0.1 μM   | 71 ± 3                   | 51 ± 4     |
| + GTPγS 2 μM   | 72 ± 2                   | 54 ± 2     |

\(^a\) Values are the mean ± standard error of the mean of triplicate determinations. Similar results were obtained in three additional experiments.

...about 1.48 mM. Hence, GDPβS has different access to the CA2\(^{+}\) signaling complexes coupled to the carbachol, bombesin, and CCK receptors. To determine if the inhibition of CA2\(^{+}\) signaling by GDPβS was due to inhibition of PLC we measured the effect of GDPβS on IP3 production. Fig. 9 shows that inhibition by GDPβS was agonist-specific. However, much higher GDPβS concentrations were needed to inhibit IP3 production (Fig. 9B) than CA2\(^{+}\) release (Fig. 9A). Even at 2.5 mM, GDPβS had no effect on IP3 production stimulated by CCK8. GDPβS only partially inhibited the effect of bombesin with about 32% inhibition at 2.5 mM. In the case of carbachol, GDPβS inhibited IP3 production by approximately 65% with an IC50 of 0.53 mM, which is about 13-fold higher than that required for 100% inhibition of CA2\(^{+}\) release (Fig. 9A).

**DISCUSSION**

Multiple receptor types that use the same transduction system and second messengers are present in virtually every cell type (1, 2, 4). Cross-talk between the same and different signaling pathways has been known for many years (1, 4). Most recently such cross-talk was demonstrated between heterotrimeric G proteins and tyrosine kinase-dependent pathways (33, 34). The extensive and coupled network of signaling pathways requires mechanisms to achieve specificity and precision in signal transduction. Compartmentalization of signaling by localization of several (or all) components of signaling complexes is emerging as a control mechanism in achieving signaling specificity. For example, many components of the mitogen-activated protein kinase and other signaling pathways are clustered in caveolae (35, 36). In the case of CA2\(^{+}\) signaling, differential localization of protein kinase C isoforms is used to regulate different cellular activities (37). Clustering of specific CA2\(^{+}\) channels by binding to components of the exocytotic path-
analyze the levels of IP₃ by radioimmunoassay as described under "Materials and Methods." The figure shows the mean ± S.E. of three experiments performed in duplicate determinations.

FIG. 8. Effect of GDP/S on Ca²⁺ signaling in SLO permeabilized acini. Acini were incubated in SLO permeabilization medium containing 0 (a and d), 0.05 (b), 0.25 (c), 1 (e and f), or 2.5 mM GDP/S (g) for 2 min at 37°C. Where indicated, the cells were stimulated with 2 mM carbachol (a–c) or 0.1 μM bombesin (d–g). All the experiments performed are summarized in Fig. 9.

FIG. 9. Effect of GDP/S on agonist stimulated Ca²⁺ signaling and IP₃ production. Panel A, the protocols of Fig. 8 were used to determine the effect of GDP/S on Ca²⁺ release by 2 mM carbachol (●), 0.1 μM bombesin (■), or 0.1 μM CCK8 (▲). The figure shows the mean ± S.E. of four separate experiments. Panel B, acini were incubated in the SLO permeabilization medium containing between 0 and 2.5 mM GDP/S for 2 min at 37°C before a 20-s stimulation with 2 mM carbachol (●), 0.1 μM bombesin (■), or 0.1 μM CCK8 (▲). The reactions were stopped by transferring cells to a solution containing perchloric acid to analyze the levels of IP₃ by radioimmunoassay as described under "Materials and Methods." The figure shows the mean ± S.E. of three experiments performed in duplicate determinations.

The problem of Ca²⁺ signaling specificity is somewhat more acute in polarized secretory cells such as pancreatic and other acinar cells. For the most part receptors in the basolateral membrane transduce their signal to the apical membrane, where exocytosis of digestive enzymes and fluid secretion occurs (15). A partial solution to signaling specificity appears to be compartmentalization of the Ca²⁺ pool which results in quantal behavior of Ca²⁺ release (40) and localized changes in [Ca²⁺], next to the apical membrane (27, 28). In the present studies we extended the previous findings (27–29) to show that all major Ca²⁺-mobilizing agonists release Ca²⁺ first in the apical region which then propagates to the basolateral region. A difference between our studies and those reported before (27, 28) is that we used clusters of three to five cells to ensure intact tight junctions and the maintenance of cell polarity, whereas previous studies (27, 28) used single isolated cells. The use of cell clusters showed that the initial increase in Ca²⁺ tended to occur in the boundary between the basolateral and the apical membrane. In addition we find that, at least in the case of CCK receptors, the [Ca²⁺], wave can start in several regions of the cell, such as the apical, apical-lateral or lateral-basal regions (Fig. 2). These features suggest that the receptors for all agonists can be clustered in regions of the basolateral membrane from which they initiate localized Ca²⁺ signal close to the apical membrane. Thus, the Ca²⁺-liberating messenger IP₃ does not need to diffuse across the cell to initiate the Ca²⁺ signal in the apical region. Rather, it can act at the site of its production by the clustered receptors. Clustering of the receptors can be viewed as the first order of signal compartmentalization.

Another aspect of signal specificity was revealed when the initiation, pattern and propagation of the Ca²⁺ signal evoked by each agonist was studied in the same cell. Previous studies have already shown that carbachol and CCK can initiate different patterns of Ca²⁺ spiking in acinar cells (41). At low agonist concentration this Ca²⁺ spiking can be confined to one pole of the cells (27, 28). Here we used supermaximal concentrations of agonists to access all the Ca²⁺ pool by each agonist to determine whether a) agonist specificity is maintained when IP₃ production is not limited and b) to be able to follow the initiation and propagation of the wave in the entire cell. The protocol of Fig. 1 revealed that each agonist initiates the Ca²⁺ signal in different region of the cell, even when high agonist concentrations are used. In addition, the pattern and propagation rate for each Ca²⁺ wave differs among the agonists used. The simplest interpretation of these findings is compartmentalization of the Ca²⁺ pool and coupling of individual compartments to specific signaling complexes. This coupling must be very tight as repetitive stimulation of the same cell with maximal or different concentrations of the same agonist always initiated the [Ca²⁺], wave at the same cellular site. In previous studies we provided evidence for such an arrangement in SLO-permeabilized cells (16, 19). Compartmentalized pools with variable affinities for IP₃ could be accessed by all antagonists in permeabilized but not intact cells (16). Furthermore, in salivary and isoproterenol, carbachol and ATP access non overlapping and limited portions of the internal Ca²⁺ pool, whereas isoproterenol and forskolin access the same overlapping portion of the pool (42). Compartmentalization of the pool and its coupling to specific signaling complexes can be viewed as the second order of signal compartmentalization.

A more direct evidence for spacial compartmentalization of signaling was obtained by studying the properties of Ca²⁺ signaling in permeable cells. Cell permeabilization with SLO caused time-dependent loss of Ca²⁺ signaling with the sequence (first to last) carbachol, bombesin, CCK. Signaling by all agonists could be completely restored at any time after permeabilization with low concentration of GTPγS. GTP was much less efficient than GTPγS in restoring signaling (not shown) despite the presence of an ATP/GTP regeneration system in the reaction medium. A tentative interpretation of these experiments is that the signaling complexes lose GTP needed for activation of G proteins at different rates. This is, however, not likely since ATP and IP₃ have complete access to the Ca²⁺ pump and the Ca²⁺ channel, respectively, a short time after cell permeabilization (16, 19). Heparin with a molecular mass of 6 kDa completely inhibits Ca²⁺ release induced by IP₃ and all agonists (ref 16 for carbachol and CCK and not shown for bombesin). The 5-kDa β thymosins and a 15-kDa S1 fragment of gelsolin can access the actin in the basal and luminal membranes (43). In addition, it is difficult to comprehend how GDP/S can access the signaling complexes (albeit at high concentrations) while GTP, which is likely to be in the µM range, will be retained by the CCK signaling complex. Indeed, the loss of Ca²⁺ signaling by carbachol and bombesin was not accompanied by a loss in the ability to activate a Goq-coupled PLC and generate IP₃, nor the ability of GTPγS to restore signaling was reflected in an increased agonist stimulated IP₃ production (Table 1).

A more likely explanation for the loss of signaling and its
Ca\(^{2+}\) Signaling Complexes in Pancreatic Acini

restoration by GTPyS may lie in our recent findings, showing a dual role for G proteins in Ca\(^{2+}\) signaling; activation of PLC and regulation of the coupling between IP\(_3\) production and Ca\(^{2+}\) release (32). Thus, it is possible that incubation in SLO leads to a time-dependent uncoupling between agonist-stimulated IP\(_3\) production and Ca\(^{2+}\) release, which can be restored by low [GTPyS] and inhibited by GDP\(\beta\)S. The rate of uncoupling in SLO is different for the various agonists. The agonist-specific uncoupling points to strict spacial compartmentalization of signaling complexes which must include, for each agonist, a receptor, a G protein, an effector (PLC), IP\(_3\), and its access to specific portion of the internal pool.

In summary, we used the measurement of [Ca\(^{2+}\)]\(_i\) at relatively high time resolution in the same single cells stimulated with several Ca\(^{2+}\) mobilizing agonists and characterized several properties of Ca\(^{2+}\) signaling by the various agonists in SLO-permeabilized cells to conclude the spacial compartmentalization of Ca\(^{2+}\) signaling complexes. Such compartmentalization is likely to be important in determining signaling specificity. Thus, the site of action of second messengers can be determined by compartmentalization so that the same second messenger can modulate different cellular activities or the same activities at different sites. We also provide evidence for multiple roles for G proteins in Ca\(^{2+}\) signaling, which regulate IP\(_3\) production and the ability of IP\(_3\) to release Ca\(^{2+}\) from internal stores.

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