Functional Mapping of Ca\textsuperscript{2+} Signaling Complexes in Plasma Membrane Microdomains of Polarized Cells\textsuperscript{*S}

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Many cells cluster signaling complexes in plasma membrane microdomains. Polarized secretory cells cluster all Ca\textsuperscript{2+} signaling proteins, including GPCRs, at the apical pole. The functional significance of such an arrangement is not known because of a lack of techniques for functional mapping of signaling complexes at plasma membrane patches. In the present work, we developed such a technique based on the use of two patch pipettes, a recording and a stimulating pipette (SP). Including 20% glycerol in the SP solution increased the viscosity and the hydrophobicity to prevent leakage and formation of tight seals on the plasma membrane. This allowed moving the SP between sites to stimulate multiple patches of the same cell and with the same agonist concentrations. Functional mapping of Ca\textsuperscript{2+} signaling in pancreatic acinar cells revealed that the M3, cholecystokinin, and bombesin signaling complexes at the apical pole are much more sensitive to stimulation than those at the basal pole. Furthermore, at physiological agonist concentrations, Ca\textsuperscript{2+} signals could be evoked only by stimulation of membrane patches at the apical pole. \([\text{Ca}^{2+}]_i\) imaging revealed that Ca\textsuperscript{2+} waves were invariably initiated at the site of apical membrane patch stimulation, suggesting that long range diffusion of second messengers is not obligatory to initiate and propagate apical-to-basal Ca\textsuperscript{2+} waves. The present studies reveal a remarkable heterogeneity in responsiveness of Ca\textsuperscript{2+} signaling complexes at membrane microdomains, with the most responsive complexes confined to the apical pole, probably to restrict the Ca\textsuperscript{2+} signals to the site of exocytosis and allow the polarized functions of secretory cells.

Many cells, in particular polarized cells such as neurons and epithelial cells, assemble signaling complexes in cellular microdomains (1–4). Indeed, previous work has shown that all Ca\textsuperscript{2+} signaling proteins are expressed at the apical pole of secretory epithelial cells (5–10), including pancreatic acinar cells (3). These include the plasma membrane G protein-coupled receptors and Ca\textsuperscript{2+} ATPase pump, the cytoplasmic G proteins and phospholipase C, and the endoplasmic reticulum-resident inositol 1,4,5-trisphosphate receptors (IP\textsubscript{3R}1) and sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase pump (Ref. 3; and see supplemental Fig. 1 for GPCRs and IP\textsubscript{3}R). The functional significance of this pattern of expression is not known.

Ca\textsuperscript{2+} signaling is particularly suitable to address questions related to polarized expression of signaling complexes, because [Ca\textsuperscript{2+}]\textsubscript{i} can be measured with an excellent spatial and temporal resolution. Stimulation of GPCRs in polarized cells generates propagating Ca\textsuperscript{2+} waves (5, 6, 9, 11–13), which always initiate at the apical pole and propagate to the basal pole. Two models have been proposed to explain the polarized Ca\textsuperscript{2+} wave. The first model proposes that clustering of Ca\textsuperscript{2+} signaling proteins at the apical pole generates an apical-to-basal gradient of responsiveness, leading to an apical to basal Ca\textsuperscript{2+} wave (9, 13). This model does not require long range diffusion of second messengers but rather differential sensitivity of apical and basal signaling complexes to stimulation by agonists. The second model proposes that long range diffusion of second messengers transmit signals generated at the basal pole to the apical pole to initiate the Ca\textsuperscript{2+} waves (14, 15).

Functional mapping of Ca\textsuperscript{2+} signaling complexes in cellular microdomains is necessary to distinguish between the two models. Importantly, this has not been examined for any signaling pathway. Limited access and difficulties in reliably and repeatedly stimulating receptors in defined membrane patches have made it difficult to functionally map signaling complexes in all cell type, particularly in polarized cells. In the present work, we overcame these difficulties by developing a two-patch pipette procedure to alternately stimulate multiple plasma membrane patches of the same cell, with the same agonist concentration, to functionally map the activity of Ca\textsuperscript{2+} signaling complexes at the apical and basal poles of pancreatic acinar cells. We report that clustering of GPCR Ca\textsuperscript{2+} signaling complexes at the apical pole resulted in a higher responsiveness of the apical than the basal pole to agonist stimulation. Furthermore, stimulation of apical pole membrane patches evoked Ca\textsuperscript{2+} waves that were initiated at the site of stimulation, indicating that long distance diffusion of second messengers is not obligatory to evoke apical-to-basal Ca\textsuperscript{2+} waves. These findings provide direct evidence for functional heterogeneity of GPCR signaling complexes in plasma membrane microdomains and an explanation for the consistency of apical-to-basal Ca\textsuperscript{2+} waves in polarized cell.

EXPERIMENTAL PROCEDURES

Preparation of Pancreatic Acini—Acinar cell doublets were obtained by collagenase and trypsin digestion as described (9). After isolation, the cells were suspended in solution A (in mM, 140 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 10 HEPES (pH 7.4 with NaOH), 10 glucose, 0.1% bovine serum albumin, and 0.02% soybean trypsin inhibitor) and kept on ice until used.

Electrophysiology—The Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current was measured as described (23) using a pipette solution containing (in mM) 140 KC\textsubscript{1}, 0.1 EGTA, 5 ATP, 10 HEPES (pH 7.3 with KOH). Cell stimulation was done by touching the cells with a second patch pipette filled with solution A containing 20% glycerol and agonist. The stimulating

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\textsuperscript{S} The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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\textsuperscript{2} The abbreviations used are: IP\textsubscript{3}, inositol 1,4,5-trisphosphate receptor; GPCR, G protein-coupled receptor; SP, stimulating pipette; BS, bombesin; MI1, megohm; GI, gighohm; CCK, cholecystokinin.
pipette (SP) was advanced close to the patched cell until it gently touched the cell. Stimulation was started by a further slight advancement of the SP to firmly touch the plasma membrane. After about 4–7 min the SP was withdrawn and moved to a second site, and the cell was stimulated again in the same manner. The proximity of the SP to the membrane was evaluated from the increase in pipette resistance. Bright field images were acquired at the end of each of the stimulation events to avoid perturbation of the current recording.

Measurement of $[\text{Ca}^{2+}]_{i}$—$[\text{Ca}^{2+}]_{o}$, was measured by imaging Fura2 fluorescence (9). The cells were placed in a perfusion chamber, a doublet was selected, and the SP was advanced close to the stimulated cell without touching. Because visual inspection during final advancement of the SP was not possible while recording fluorescence, preliminary experiments trained the experimenter as to how much to advance the pipette (SP) was advanced close to the patched cell until it gently touched the cell. Stimulation was started by a further slight advancement of the SP to firmly touch the plasma membrane. After about 4–7 min the SP was withdrawn and moved to a second site, and the cell was stimulated again in the same manner. The proximity of the SP to the membrane was evaluated from the increase in pipette resistance. Bright field images were acquired at the end of each of the stimulation events to avoid perturbation of the current recording.

**RESULTS AND DISCUSSION**

Many signaling complexes are clustered in cellular microdomains (1, 2). Polarized secretory cells cluster $\text{Ca}^{2+}$-signaling complexes at the apical pole (3). This is illustrated in supplemental Fig. 1, showing expression of the M3 and IP$_3$ receptors at the apical pole of pancreatic acini. This is the site from which $\text{Ca}^{2+}$ waves are initiated when the cells are stimulated by any GPCR (9, 11–13). Because of technical limitations, the functional significance of such clustering of signaling complexes is not known. In the present work we addressed this question directly by functionally mapping the responsiveness of $\text{Ca}^{2+}$ signaling complexes in membrane microdomains.

A Procedure for Stimulation of Multiple Membrane Patches—A technique suitable for the functional mapping of signaling complexes must allow stimulation of multiple membrane patches of the same cell and with the same agonist concentration. A patch pipette is ideal for such a task. However, the pipette tip should have a surface sufficient to generate a measurable stimulus while having little to no leakage. Another critical feature is that the pipette should allow firm attachment to the cell surface without generation of a GH$_1$ seal so that the pipette can be moved between membrane patches. We reasoned that increasing the viscosity and hydrophobicity of the pipette solution would enable us to meet these requirements. Trials showed that good results could be obtained with a pipette tip of 1.5–2.0 $\mu$m and when the SP is filled with a solution containing 20% glycerol. Under these conditions the SP resistance was 3.31 ± 0.15 MΩ, and a GH$_1$ seal was not formed in any of the experiments ($n > 150$).

All of the experiments in the present work were performed with two-cell clusters, which maintain excellent cell polarity and intact tight junctions (18). In addition, it was necessary to
minimize propagation of the stimulus between cells to avoid complications in interpreting the results. In pancreatic acini the stimulus propagates through gap junctions (16, 17), and the configuration of the doublets minimizes gap junction communication (see supplemental Fig. 1D).

Controls—Several controls were performed to test the applicability of the technique. First, a visible dye and a brightly fluorescent dye were included in the SP, and while not perfusing the bath, leakage was inspected. No leakage was detected in 10 such experiments with each dye. Second, using SP without agonists, in six of seven experiments firmly touching the cells at the apical pole had no effect, and in one experiment small current oscillations were observed, indicating that touching the SP is not sufficient to evoke a stimulus. Third, in nine experiments similar to that in supplemental Fig. 1D, SP containing 100–250 μM carbachol were used to stimulate by touching apical patches of the adjacent cell and then the cell from which current was recorded. A much stronger (4 of 9 cells, as in supplemental Fig. 1D) or exclusive (5 of 9 cells) stimulation was observed by touching the cell from which current was recorded. These findings further indicate a minimal, if any, gap junction communication between the couplets. Fourth, in all experiments it was necessary to firmly touch the cell. The extent to which the SP touched the cell was evaluated from the change in SP resistance. Advancing the SP to lightly touch the apical pole increased the resistance from 3.32 ± 0.15 to 7.3 ± 0.3 MΩ. Lightweight touching the basal pole of the same cells with the same pipettes during the second stimulation event resulted in SP resistance of 7.1 ± 0.3 MΩ (n = 44). Touching the cell in this manner did not evoke a stimulus. To initiate a stimulus the SP was advanced further, which increased the SP resistance to 11.0 ± 0.4 and 10.8 ± 0.4 MΩ when touching the apical and basal poles, respectively. In 22 of the experiments the apical pole was touched first, and in 22 of the experiments the basal pole was touched first. The identical increase in membrane resistance indicates that the same extent of stimulus is applied to the two membrane patches. Firmly touching the cells was also observed as a small membrane indentation and was always observed before a stimulus could be detected. This is illustrated in supplemental Fig. 1E by demarcation of the SP and the cell borders with a green and an orange line, respectively. Conversely, when the SP was withdrawn from the cell, the membrane patch was frequently stretched out before snap-

Differential Responsiveness of Apical and Basal Membrane Patches—Fig. 1 shows how the technique was used to map the M3 receptor signaling complexes in the polarized pancreatic acinar cells. Relatively high concentrations of agonists had to be included in the SP, probably because small membrane patches were stimulated, and there was restricted access of the agonist to the patches because of the increased hydrophobicity of the SP solution. To ensure that the sequence of patch stimulation was not the reason for any observed differences, receptors at the apical pole were stimulated first and at the basal pole second in one-half of the experiments (Fig. 1, A, D, and F); in the second half, the reverse sequence was used (Fig. 1, B, E, and G). It is clear from Fig. 1 that the apical pole membrane patches were much more sensitive to stimulation of the M3 receptors than the basal pole patches. Hence, in eight experiments with 100 μM carbachol in the SP, stimulation of apical pole patches evoked repetitive oscillations in the Ca2+-activated Cl- current, whereas in all experiments stimulation of basal pole patches of the same cells failed to generate a Ca2+ signal (Fig. 1, A and B).

The findings in Fig. 1, A and B, are particularly significant because numerous studies in pancreatic acinar cells show that global stimulation with low, physiological agonist concentrations evoke only repetitive Ca2+ spikes (3, 19), similar to those triggered by stimulation of the apical pole membrane patches. This is also illustrated in Fig. 1C. Global stimulation by perfusing the cells with a solution containing 0.25 μM carbachol triggered Ca2+ spikes, whereas stimulation with 100 μM carbachol resulted in a sustained response. Hence, it appears that for the most part, if not exclusively, the Ca2+ signaling complexes at the apical pole generate Ca2+ signals at physiological agonist concentrations.

Fig. 1, D and E, shows that increasing the carbachol concentration to 250 μM increased the intensity of the response when apical membrane patches were stimulated; yet either no response or only low frequency/low amplitude [Ca2+], oscillations were evoked by stimulation of basal membrane patches of the same cells (n = 8). A response could be evoked routinely by stimulation of basal membrane patches when the SP contained 1 mM carbachol (Fig. 1, E and F). However, these responses were oscillatory and smaller than the responses evoked by stimulation of apical patches of the same cells (n = 16).

In a previous work we show that the CCK receptors are also expressed at high levels at the apical pole region (9). The plasma membrane distribution of the bombesin (BS) receptors is unknown. Because stimulation of both the CCK and BS receptors triggers an apical-to-basal Ca2+ wave (11, 13, 20, 21), it was of interest to map these receptors functionally. Representative experiments are depicted in Fig. 2. As found for the M3 receptors, stimulation of the CCK (Fig. 2, A and B) and BS (Fig. 2C) receptors at apical membrane patches generated a much more intense Ca2+ signal that stimulation of the receptors at basal patches. It is therefore clear that the uneven distribution of Ca2+ signaling complexes of both the CCK and BS receptors at the plasma membrane generates sites from which Ca2+ signals can be evoked at low physiological agonist concentrations.

Basal and Apical Stimulation Trigger Ca2+ Waves—Repetitive stimulation of pancreatic acinar cells with the same agonist at the same (13) or increasing agonist concentrations (9)
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evoked the exact same Ca\(^{2+}\) wave with the same initiation site and propagation pattern. Therefore, we asked whether stimulation of apical and basal membrane patches of the same cell would evoke the same Ca\(^{2+}\) wave. Fig. 3 shows the results of a typical experiment with an SP containing 1 mm carbacol. First, [Ca\(^{2+}\)]\(i\) imaging confirmed the results obtained by measuring the Ca\(^{2+}\)-activated Cl\(^{-}\) current, showing that stimulation of apical patches generated a much larger [Ca\(^{2+}\)]\(i\), increase than stimulation of basal patches of the same cells. Second, invariably, stimulation of apical membrane patches resulted in a Ca\(^{2+}\) wave that always initiated at the site of patch stimulation (n = 54/54). This was the case whether the apical or the basal pole patches were stimulated first. Stimulation of basal membrane patches of the same cells also generated an apical-to-basal Ca\(^{2+}\) wave. However, in most experiments (52 of 54), either stimulation of the basal pole did not evoke a Ca\(^{2+}\) signal (5 of 52) or the Ca\(^{2+}\) waves evoked by apical and basal pole stimulation of the same cell had different initiation sites (47 of 52; see arrowheads in Fig. 3).

As concluded previously (14), initiation of a Ca\(^{2+}\) waves at the apical pole upon stimulation of basal patches indicates that second messengers like IP\(_3\) must diffuse from the basal to the apical pole upon stimulation of basal patches indicates that the apical pole to all agonists that act on pancreatic acini. Furthermore, that the apical pole is much more responsive than the basal pole ensures that no events will take place at the basal pole before exocytosis take place at the apical pole.

Cells other than epithelial cells express Ca\(^{2+}\) and other type of signaling complexes unevenly at the plasma membrane, as in caveolae and membrane rafts (1). It will be of interest to use a technique similar to the one described here to functionally map signaling complexes in such cells and study their physiological significance.

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