Long Distance Communication between Muscarinic Receptors and Ca\textsuperscript{2+} Release Channels Revealed by Carbachol Uncaging in Cell-attached Patch Pipette*  

Michael C. Ashby§§, Cristina Camello-Almaraz¶, Oleg V. Gerasimenko, Ole H. Petersen, and Alexei V. Tepikin§  

From the Medical Research Council Secretory Control Research Group, The Physiological Laboratory, University of Liverpool, Liverpool L69 3BX, United Kingdom  

We have investigated the characteristics of cytosolic Ca\textsuperscript{2+} signals induced by muscarinic receptor activation of pancreatic acinar cells that reside within intact pancreatic tissue. We show that these cells exhibit global Ca\textsuperscript{2+} waves and local apical Ca\textsuperscript{2+} spikes. This is the first evidence for local Ca\textsuperscript{2+} signaling in undissociated pancreatic tissue. The mechanism of formation of localized Ca\textsuperscript{2+} signals was examined using a novel approach involving photolysis of caged carbachol inside a patch pipette attached to the basal surface of an acinar unit. This local activation of basolateral muscarinic receptors elicited local cytosolic Ca\textsuperscript{2+} spikes in the apical pole more than 15 \textmu m away from the site of stimulation. In some experiments, local basal receptor activation elicited a Ca\textsuperscript{2+} wave that started in the apical pole and then spread toward the base. Currently, there are two competing hypotheses for preferential apical Ca\textsuperscript{2+} signaling. One invokes the need for structural proximity of the cholinergic receptors and the Ca\textsuperscript{2+} release channels in the apical pole, whereas the other postulates long distance communication between basal receptors and the channels. Our intrapipette uncaging experiments provide definitive evidence for long distance communication between basolateral muscarinic receptors and apical Ca\textsuperscript{2+} release channels.  

In response to agonist stimulation, many cell types produce transient elevations in the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]), which remain localized to a specific subcellular domain (1). In isolated pancreatic acinar cells, a low concentration of acetylcholine (ACH)\textsuperscript{1} can evoke repetitive local cytosolic Ca\textsuperscript{2+} spikes, which are confined to the granule containing apical pole (2). These local Ca\textsuperscript{2+} spikes can activate exocytosis through the apical plasma membrane (3) and open Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channels present exclusively in the apical plasma membrane, thereby regulating acinar fluid secretion (4).  

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† Present address: Dept. of Anatomy, Medical Research Council Centre for Synaptic Plasticity, University of Bristol, Medical School, University Walk, Bristol BS8 1TD, United Kingdom.  

§ Supported by a Wellcome Trust Prize Studentship. To whom correspondence may be addressed. E-mail: M.C.Ashby@bristol.ac.uk (M. C. A.) or a.tepikin@liv.ac.uk (A. V. T.).  

¶ Present address: Dept. of Physiology, Faculty of Veterinary Science, P. O. Box 643, 10071 Caceres, Spain.  

1 The abbreviations used are: ACh, acetylcholine; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; CCh, carbachol; AM, acetoxymethyl ester.

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EXPERIMENTAL PROCEDURES

Cell Preparation, Solutions, Dye Loading, and Chemicals—All of the experiments were performed at room temperature (22–24 °C). The pancreas was removed from mice killed by cervical dislocation. The isolated pancreas was immediately placed into extracellular solution, which contained (in mM): 140 NaCl, 4.7 KCl, 1.13 MgCl₂, 10 HEPES, 10 glucose, 1 mM CaCl₂, pH 7.2 (adjusted by NaOH). Freshly isolated mouse pancreatic clusters of acinar cells were prepared as described previously (2) and used within 4 h. All of the experiments were performed during continuous superfusion of extracellular solution. Agonists and antagonists were added to the extracellular solution at the stated concentrations. Fluo-4 AM, Fura Red AM, caged fluorescein dextran-10K, H₂9251-carboxy-2-nitrobenzyl-caged carbachol, Mitotracker Green FM, and Lysotracker Red were from Molecular Probes. Hoechst 33342 was from Calbiochem. Loading/staining of cells with fluorescent compounds was achieved by incubation of cells/tissue with the particular dye in darkness at room temperature (except when specifically mentioned) under the following conditions: Fluo-4 AM (2.5 μM for 25 min); Fura Red AM (10 μM for 30 min); Mitotracker Green FM (500 nM for 25 min at 37 °C); Lysotracker Red (200 nM for 5 min); Hoechst 33342 (100 μg/ml for 2 min). Collagenase was obtained from Worthington, and all other chemicals were purchased from Sigma.

Confocal Imaging—Fluorescence images were obtained using either a Zeiss LSM 510 confocal microscope or a Leica SP2 confocal microscope with 40 oil-immersion or 63 water-immersion objective. During the experiments, intact pancreatic tissue was immobilized on a glass coverslip using either a needle through the tissue or a slice hold-down (Warner Instruments). Intact tissue or cells adhered to poly-L-lysine-coated slides were imaged on the stage of an inverted microscope (Axiovert 100M, Zeiss, or DMIRBE from Leica). Fluorescence images from tissue (acinar unit is outlined by dashed line) is loaded with Mitotracker Green FM, Hoechst 33342, and Lysotracker Red showing mitochondria, nuclei, and secretory granules, respectively.

**FIG. 1.** Local and global [Ca²⁺], signals and organelle distribution in acinar cells within intact pancreatic tissue. A, confocal Fluor-4 fluorescence recording from regions of a single acinar cell within intact pancreatic tissue (shown in transmitted light image). Upper section shows the time course and spatial distribution of the ACh-elicited [Ca²⁺], changes. A low ACh concentration (100 nM) caused small repetitive Ca²⁺ transients exclusively localized to the apical part of the cell. Stronger stimulation (1 μM ACh) generated larger Ca²⁺ signals, which spread throughout the cell. The spatial properties of the signals are shown in the lower section, which contains high magnification pseudocolor images of Fluor-4 fluorescence during single local and global signals in the cell shown in the accompanying transmitted light image. A change in color from blue to green and to yellow represents a [Ca²⁺] elevation. Images 1–3 show the time course of a single local Ca²⁺ transient, which is confined exclusively to the apical part of the cell. Images 4–7 show the evolution of a single global Ca²⁺ transient in which the [Ca²⁺] rise starts in the apical region and travels throughout the cell as an apical-to-basal wave. B, comparative intracellular positioning of mitochondria, secretory granules, and nuclei in acinar cells within intact pancreatic tissue. Confocal fluorescence images from tissue (acinar unit is outlined by dashed line) is loaded with Mitotracker Green FM, Hoechst 33342, and Lysotracker Red showing mitochondria, nuclei, and secretory granules, respectively.
Uncaging was achieved during the scanning procedure by exposure of basal plasma membrane of individual acinar cells in small clusters. Short lasting uncaging (points which was loaded into the cytoplasm of the cells, from the regions exclusively inside the pipette. Graph shows the time courses of fluorescence without triggering any cellular Ca$^{2+}$ release. Finally, ACh triggers a marked reduction in the Fura Red fluorescence intensity, which corresponds to a [Ca$^{2+}$] rise. The lower panel shows images of fluorescein (green) and Fura Red (red) fluorescence before and after uncaging. Intrapiette Uncaging—Standard patch clamp technique was adapted using the EPC-8 amplifier and Pulse software (HEKA). We used pipettes having resistances of 2–4 megohms. Pipettes were back-filled with the extracellular solution containing, in addition, either 5 mg/ml caged fluorescein dextran-10K or 50 μM-5 mM caged CCh. Seals of 5–10 giga-ohm resistance were obtained between the pipette and the basal plasma membrane of individual acinar cells in small clusters. Uncaging was achieved during the scanning procedure by exposure of pre-defined regions to UV laser light at 364 and 351 nm. The regions were selected to surround the pipette but not expose the cell.

**Fig. 2. Intrapiette uncaging technique.** Upper transmitted light image shows the experimental setup with a single acinar cell within a cluster having a micropipette attached to the basal plasma membrane. The pipette forms a giga-ohm resistance seal with the membrane in cell-attached configuration. The pipette solution is identical to that used pipettes having resistances of 2–4 megohms. Pipettes were back-adapted using the EPC-8 amplifier and Pulse software (HEKA). We used pipettes having resistances of 2–4 megohms. Pipettes were back-filled with the extracellular solution containing, in addition, either 5 mg/ml caged fluorescein dextran-10K or 50 μM-5 mM caged CCh. Seals of 5–10 giga-ohm resistance were obtained between the pipette and the basal plasma membrane of individual acinar cells in small clusters. Uncaging was achieved during the scanning procedure by exposure of pre-defined regions to UV laser light at 364 and 351 nm. The regions were selected to surround the pipette but not expose the cell.

**RESULTS AND DISCUSSION**

**Subcellular Localized Calcium Signaling in Intact Epithelial Tissue**—Confocal fluorescence microscopy was used to image the Ca$^{2+}$-sensitive dye, Fluo-4, in the cytoplasm of cells that were stimulated by bath perfusion of physiological agonists. We used an undissociated pancreatic preparation to image large areas of intact pancreatic tissue (see Fig. 1A) and showed that application of ACh triggered repetitive [Ca$^{2+}$] elevations in nearly all of the cells (n = 25 experiments). An analysis of the fluorescence changes in individual cells revealed the spatiotemporal profile of these Ca$^{2+}$ oscillations (Fig. 1A). In some cells, lower levels of stimulation caused [Ca$^{2+}$] oscillations that were restricted entirely to the secretory granule region at the apical end of the cell (Fig. 1A, images 1–3 (n = 9)). Increasing the ACh concentration changed the profile of each transient from local to global. At higher doses, the [Ca$^{2+}$] transient became a wave, which was initiated at the apical pole and spread toward the basal plasma membrane (Fig. 1A, images 4–7 (n = 22)). As the intensity of stimulation increased, the oscillations became superimposed on an elevated baseline (Fig. 1A). These observations demonstrate that pancreatic acinar cells in their native environment can produce apically localized [Ca$^{2+}$] elevations and global Ca$^{2+}$ waves initiated in the apical region.

Specific patterns of Ca$^{2+}$ signaling are translated into the responses of cellular organelles such as exocytosis of secretory granules (19), changes of mitochondrial metabolism (20, 21), and nuclear gene expression (22). Also, the positioning of intracellular organelles can have profound implications for patterns of [Ca$^{2+}$] signaling (23, 24). We assessed the intracellular position of the major intracellular organelles in live cells within intact pancreatic tissue. The secretory (zymogen) granules were labeled using LysoTracker Red (based upon the affinity of the dye for acidic compartments), nuclei were stained using Hoechst 33342, and mitochondria were stained using Mitotracker Green FM. The majority of acidic compartments (mostly representing zymogen granules) were found near the apical (luminal) membrane (Fig. 1B). The acinar lumen was often visible as an unstained region between the cells. The nucleus or nuclei were always found outside the zymogen granule region toward the base (Fig. 1B). In isolated pancreatic acinar cells, several studies have shown that mitochondria have distinct and specific intracellular locations. The majority of the mitochondria are positioned in a perigranular belt, which limits the spread of apically localized Ca$^{2+}$ signals (23). This positioning was also apparent in the intact tissue, because the strongest mitochondrial staining was in the perigranular region (Fig. 1B). In addition, Mitotracker Green staining also revealed the perinuclear and subplasmalemmal mitochondria that have been identified in isolated cells (Fig. 1B) (24). The intracellular positioning of mitochondria is thought to have important functional interactions with nearby Ca$^{2+}$ signaling pathways (24–26). Our observations in the intact pancreas highlight the relevance of the polarity of organelles and Ca$^{2+}$ signaling in cells within their native environment.

**Long Distance Signaling Demonstrated by Intrapiette Uncaging of Carbachol**—Acinar cells within the intact pancreas can produce local apical [Ca$^{2+}$], spikes when ACh is applied to the basal side of the epithelium. This highlights the need for understanding the mechanism by which the local apical Ca$^{2+}$ signals are generated. Here we have studied the patterns of Ca$^{2+}$ signaling induced by exclusive stimulation of basally located receptors.

To specifically excite basal receptors, we have combined electrophysiological and photoalytic (uncaging) techniques. A patch pipette containing caged agonist in standard extracellular solution was used to isolate a small area of the basal membrane.
of a single acinar cell within a cluster of cells (3–10 cells). We used isolated cell clusters because the patching of cells within the intact pancreas was impossible because of the capsule surrounding the tissue. During continuous superfusion of the cell clusters, a high resistance (5–10 giga-ohms) seal was formed between the basal cell membrane and the glass pipette. Such a configuration could then be used to photolyse caged carbachol by UV laser light exposure of pre-defined regions (shown as dashed orange lines throughout) surrounding the pipette but avoiding the cells.

To test this experimental configuration, we used caged fluorescein dextran-10K in the pipette and loaded the cytoplasm of the cells with the Ca\(^{2+}\)-sensitive dye, Fura Red. This allowed us to characterize the intrapipette uncaging and test whether UV exposure itself would cause any \([\text{Ca}^{2+}]\), elevation (Fig. 2). As expected, each UV exposure caused an increase in the fluorescence of fluorescein inside the pipette as the compound was uncaged (Fig. 2, \(n = 4\)). The increase in fluorescence peaked at the first frame obtained after uncaging and then declined, initially rapidly and then more slowly. This decline presumably reflects dilution of the product of uncaging in the solution in the rest of the pipette, which is much larger than the volume exposed to UV light. The uncaging itself did not cause any change in \([\text{Ca}^{2+}]\), whereas subsequent application of ACh to the bath triggered a large rapidly evolving \([\text{Ca}^{2+}]\), transient (Fig. 2). These experiments showed that we can selectively expose a small region of basal plasma membrane to a photo-lytically activated compound. This technique is equally applicable to studies using site-specific stimulation of many other cell types including neurons, which produce polarized responses to stimulation. The rapid time course and range of UV exposure makes intrapipette uncaging faster, more sensitive, and better regulated than other local stimulation techniques (2).

We tested the ability of CCh to trigger changes in \([\text{Ca}^{2+}]\), measured by Fluo-4 in the cytoplasm of the cells when uncaged inside a cell-attached pipette. We found that intrapipette uncaging of carbachol at the basal pole was often able to trigger short lasting \([\text{Ca}^{2+}]\), elevations, which were restricted to the extreme apex of the cell (Fig. 3A, \(n = 11\) cells). The distance between the basal membrane under the pipette and the point of Ca\(^{2+}\) release was always over 15 \(\mu\)m and up to a distance of 25 \(\mu\)m. Approximately 60% of the cells responded to uncaging by producing \([\text{Ca}^{2+}]\), signals, and each uncaging event usually only triggered a single \([\text{Ca}^{2+}]\), spike. Intriguingly, in the other experiments, it was not possible to trigger \([\text{Ca}^{2+}]\), release even with very strong and/or long lasting UV exposure. This inability of even strong uncagings to trigger \([\text{Ca}^{2+}]\), signals in 40% of the cells could be explained either by insufficient production of second messengers or by absence of receptors under the patch pipette because of receptor clustering in other regions of the basal membrane. The clustering of transmembrane receptors has been reported previously (27) and is proposed to underlie high sensitivity areas of membrane that are able to amplify low level extracellular stimuli.

We compared the size of the \([\text{Ca}^{2+}]\), rises triggered by intrapipette CCh uncaging to those caused by bath application of ACh (10 \(\mu\)m) (Fig. 3A). Short lasting uncaging events (UV exposure of 100–500 ms) consistently generated smaller responses than ACh in the same cell (\(n = 4\)). For these durations of uncaging, the \([\text{Ca}^{2+}]\), signals were localized to the apical part of the cells.

To be certain that only muscarinic receptors under the patch pipette would be responsible for generating the intracellular \([\text{Ca}^{2+}]\), release, we carried out experiments in which we made sure that even in the unlikely event that some of the uncaged CCh could escape the pipette, no activation of receptors outside the area of the plasma membrane covered by the patch pipette would occur. We blocked all muscarinic receptors outside the pipette by adding atropine (100 \(\mu\)m) after the gigaseal formation. This atropine concentration always blocked \([\text{Ca}^{2+}]\), elevations triggered by ACh (1 \(\mu\)m) applied to the bath (\(n = 12\)). Under these conditions, short intrapipette uncaging was still able to trigger a localized \([\text{Ca}^{2+}]\), elevation at the extreme apical pole of the cell (Fig. 3B, \(n = 6\) cells). This confirms that the pipette does indeed form an impenetrable seal with the
Ca\textsuperscript{2+} is able to produce significant activation of intracellular
strating that activation of a small area of basal plasma mem-
points marked by
stronger activation of receptors in a small area of basal plasma
spreading of the [Ca\textsuperscript{2+}] produced by intrapipette uncaging is sufficient to support the
activated basal receptor to the Ca\textsuperscript{2+} channels in the apical part of
the endoplasmic reticulum store ([31]).

In the majority of our experiments, it was possible to trigger
large [Ca\textsuperscript{2+}], transients despite the fact that only a small area
of plasma membrane underwent stimulation. We estimate that
the area of plasma membrane covered by the patch pipette is at
most 2% of the total surface area (based upon the O-shaped
membrane inside the pipette tip and the total cell surface
estimated from our confocal images). If the distribution of the
muscarinic receptors were reasonably even, this would suggest
that occupancy of a relatively small proportion of the receptors
is sufficient to trigger not only local but also global and even
intercellular [Ca\textsuperscript{2+}], signals. The ACh-detecting system in the
basal membrane must therefore be highly sensitive.

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Global Ca\textsuperscript{2+} Waves Triggered by Basal Intrapipette CCH Uncaging—The time course and size of the [Ca\textsuperscript{2+}] elevations described above correlate with a low level of ACh activation in studies on isolated cells (2). Therefore, we tested whether stronger activation of receptors in a small area of basal plasma membrane could generate larger global [Ca\textsuperscript{2+}] rises. Intrapipette uncaging of longer duration (800 ms-1.5 s) or repetitive short uncagings were able to trigger global Ca\textsuperscript{2+} waves (Fig. 4, n = 4). Such waves were always initiated in the apical region and propagated toward the basal plasma membrane, demonstrating that activation of a small area of basal plasma membrane is able to produce significant activation of intracellular Ca\textsuperscript{2+} signaling mechanisms. In some of our intrapipette uncaging experiments (n = 4), we could trigger a Ca\textsuperscript{2+} wave that propagated from the stimulated cell to other cells in the same cluster. This suggests that the level of second messenger produced by intrapipette uncaging is sufficient to support the spreading of the [Ca\textsuperscript{2+}] rise via gap junctions.

The long distance signaling demonstrated directly in this

FIG. 4. Global elevations of the cytosolic Ca\textsuperscript{2+} concentration triggered by basal intrapipette uncaging of caged carbaryl is initiated in the apical region. Fluorescence recording from regions of Fluoro-4-loaded cell, which is patched on the basal plasma membrane (shown in transmitted light image). Intrapipette uncaging of 1 mM caged CCH triggers a large global Ca\textsuperscript{2+} transient. The lower panel shows sequential pseudocolor images of Fluoro-4 fluorescence (from time points marked by black arrows in the graph shown in the upper panel) during the [Ca\textsuperscript{2+}] transient. The transient started in the apical region and spread toward the basal membrane. The muscarinic antagonist, atropine (100 μM), was present in the bath after establishment of the high resistance pipette seal.

study must rely on the production of some diffusible intracel-
lar messenger following receptor activation. IP\textsubscript{3}, cyclic ADP-
ribose, and nicotinic acid adenine dinucleotide phosphate are all able to release Ca\textsuperscript{2+} from intracellular stores in pancreatic acinar cells and are involved in agonist-evoked responses (11, 28–30). Such small water soluble messengers are highly dif-
usible in the cytoplasm (13), and it seems likely that one or
more of them transmits the Ca\textsuperscript{2+} release signal from the
activated basal receptor to the Ca\textsuperscript{2+} channels in the apical part of
the endoplasmic reticulum store (31).
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