A Role for Phosphorylation of Inositol 1,4,5-Trisphosphate Receptors in Defining Calcium Signals Induced by Peptide Agonists in Pancreatic Acinar Cells

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Stimulation of pancreatic acinar cells with acetylcholine (ACh) and cholecystokinin (CCK) results in an elevation of cytosolic calcium ([Ca\(^{2+}\)]\(_c\)) through activation of inositol 1,4,5-trisphosphate receptors (InsP\(_3\)R). The global temporal pattern of the [Ca\(^{2+}\)]\(_c\) changes produced by ACh or CCK stimulation differs significantly. The hypothesis was tested that CCK stimulation results in a protein kinase A (PKA)-mediated phosphorylation of InsP\(_3\)R and this event contributes to the generation of agonist-specific [Ca\(^{2+}\)]\(_c\) signals. Physiological concentrations of CCK evoked phosphorylation of the type III InsP\(_3\)R, which was blocked by pharmacological inhibition of PKA. Imaging of fura-2-loaded acinar cells revealed that the rate of [Ca\(^{2+}\)]\(_c\) rise during CCK-evoked oscillations slows with each subsequent oscillation, consistent with a developing modulation of release, whereas the kinetics of ACh-evoked oscillations remain constant. Stimulation of cells with ACh following activation of PKA resulted in a slowing of the ACh-evoked [Ca\(^{2+}\)]\(_c\) rise, which now resembled a time-matched CCK response. PKA activation also resulted in a slowing of [Ca\(^{2+}\)]\(_c\), increases elicited by photolysis of caged InsP\(_3\). Targeted, PKA-mediated phosphorylation of type III InsP\(_3\)R is involved in a physiological CCK response, as disruption of the targeting of PKA with the peptide HT31 resulted in marked changes in the CCK-evoked [Ca\(^{2+}\)]\(_c\) signal but had no effect on ACh-evoked responses. Stimulation of cells with bombesin, which evokes [Ca\(^{2+}\)]\(_c\) oscillations indistinguishable from those produced by CCK, also results in PKA-mediated phosphorylation of type III InsP\(_3\)R. Thus, we conclude that PKA-mediated phosphorylation of type III InsP\(_3\)R is a general mechanism by which the patterns of [Ca\(^{2+}\)]\(_c\) oscillations are shaped in pancreatic acinar cells.

Agonist-induced increases in cytosolic calcium ([Ca\(^{2+}\)]\(_c\)) exert control over a multitude of physiological responses including exocytotic and fluid secretion as well as gene transcription (1, 2). In non-excitable cells, specificity is believed to be encoded by differences in the frequency, amplitude, and subcellular localization of the [Ca\(^{2+}\)]\(_c\) oscillations (3–5). A topical question in cell biology is how similar signal transduction machinery can be utilized to produce physiologically divergent [Ca\(^{2+}\)]\(_c\) signals and cellular responses (6–9). The acinar cells of the exocrine pancreas offer an ideal system in which to study the generation of agonist-specific [Ca\(^{2+}\)]\(_c\) signals. Stimulation of acinar cells with low doses of acetylcholine (ACh) or cholecystokinin (CCK) produces oscillations in [Ca\(^{2+}\)]\(_c\) that differ in terms of their frequency and temporal pattern as well as in the physiological processes controlled (9). The global temporal pattern of ACh-evoked oscillations consists of regular oscillations with a frequency of 4–6/min superimposed on an elevated [Ca\(^{2+}\)]\(_c\), plateau, whereas CCK-evoked oscillations exhibit slower kinetics, typically less than 1 oscillation each min, and the spikes are excursions from at or near the base line [Ca\(^{2+}\)]\(_c\). In terms of the spatial aspects of the agonist-induced signal, reports have suggested that responses stimulated by both ACh and CCK initiate in similar but not identical regions of the apical trigger zone and, at physiologically relevant concentrations, spread as a wave throughout the cell (10, 11). These agonist-specific [Ca\(^{2+}\)]\(_c\) signals may be important in encoding specific physiological endpoints. ACh stimulation tends to result predominately in increased exocytotic secretion, whereas CCK stimulation results in increased gene transcription and protein synthesis in addition to secretion (12, 13). Several mechanisms have been proposed as underpinning the differences in [Ca\(^{2+}\)]\(_c\) signaling patterns stimulated by various agonists. One hypothesis suggests that these differences arise from CCK receptor coupling to the production of a novel putative second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) (14). The primary evidence for this contention is that treatment of cells with NAADP results in Ca\(^{2+}\) release and that exogenously applied NAADP results in cross-desensitization of both CCK responses and subsequent NAADP-induced [Ca\(^{2+}\)]\(_c\) signals. Interestingly, stimulation of acinar cells with bombesin, which results in a pattern of [Ca\(^{2+}\)]\(_c\) signals very similar to those produced by CCK, is not antagonized by this maneuver (15, 16), indicating that a signaling pathway involving NAADP, although possibly contributing to CCK-induced signaling, cannot account for the general pattern of [Ca\(^{2+}\)]\(_c\) signals initiated by these agonists.

An additional proposal is that InsP\(_3\) production is differentially regulated upon stimulation with different agonists. This could occur through the interaction of an individual G protein-coupled receptor with a specific RGS protein, thus exerting control over the activity of G\(_q\), and, subsequently, phospholipase C. In support of this idea, CCK and muscarinic receptor stimulation clearly show differential sensitivity to exogenously applied RGS proteins (17, 18). Although these proposals are not
mutually exclusive, an important starting point in evaluating any potential mechanisms is that the \([\text{Ca}^{2+}]_i\), response to all agonists appears to be mediated through coupling to the Goq family of heterotrimeric G proteins, as antagonism of Goq family members abolishes \([\text{Ca}^{2+}]_i\) signaling in response to CCK, muscarinic, and bombesin stimulation (19, 20). In addition, the importance of this pathway is emphasized by the observation that responses to all agonists can be inhibited by antagonism of InsP3R (14, 21), suggesting a critical role for \([\text{Ca}^{2+}]_i\) release via InsP3R in the production of these responses. Although other calcium release channels, such as ryanodine receptors, have been shown to play a role in agonist-induced signaling in acinar cells (22–24), it is clear that signals cannot be produced in the absence of \([\text{Ca}^{2+}]_i\) release through InsP3R.

Given that \([\text{Ca}^{2+}]_i\) release through InsP3R is a critical component of the cell’s response to all agonists, it is tempting to speculate that differential modulation of the release event provides a means of shaping the \([\text{Ca}^{2+}]_i\) signal. In pursuit of this hypothesis, we have recently demonstrated that CCK stimulation results in phosphorylation of type III InsP3R (25). Furthermore, PKA-mediated phosphorylation of this InsP3R can modulate the sensitivity of the receptor to InsP3, ultimately resulting in a diminished ability of the channel to release \([\text{Ca}^{2+}]_i\) (26). Although CCK is known to couple to Goq in pancreatic acinar cells (27), it is not known whether this can account for the phosphorylation of InsP3R produced upon CCK stimulation or, in turn, for the specific kinetics of \([\text{Ca}^{2+}]_i\) oscillations observed upon CCK stimulation. The goal of the present study was to determine whether agonist-induced phosphorylation of InsP3R contributes to the generation of agonist-specific patterns of \([\text{Ca}^{2+}]_i\) oscillations in pancreatic acinar cells.

**EXPERIMENTAL PROCEDURES**

**Preparation of Pancreatic Acini—**Cells were prepared from male NIH (Swiss) mice (21–24 g) by collagenase digestion essentially as described previously (28).

**Measurement of \([\text{Ca}^{2+}]_i\) and Photolysis of Caged InsP3—**Isolated acini were incubated with 2 \(\mu\)M fura-2 AM (Teflabs) at 25 °C for 30 min followed by washing and resuspension in physiological salt solution containing (mM): 127 NaCl, 0.56 MgCl2, 1.28 CaCl2, 10 HEPES-NaOH, and 11 D-glucose, pH 7.4. For measurement of \([\text{Ca}^{2+}]_i\), fura-2-loaded cells were placed in a recording chamber and mounted on the stage of a Nikon TE200 microscope equipped with a Nikon Super Fluor 40 ×, 1.3 NA oil immersion objective. Acini were locally superfused at a rate of 1 ml/min with physiological salt solution. Solution changes were accomplished by use of a multi-port solenoid manifold and electronic controller. The outflow of the superfusion was placed within 100 \(\mu\)m of the cells being imaged to ensure rapid solution presentation. \([\text{Ca}^{2+}]_i\) imaging was performed using a monochrometer-based illumination system and high speed CCD camera (T.I.L.L. Photonics). For measurement of \([\text{Ca}^{2+}]_i\), in cells loaded with fura-2, the dye was alternately excited at 340/380 nm ± 15 nm, and fluorescence emission was collected through a 510 ± 25 nm bandpass filter (Chroma). For enhanced spatial resolution, rapidly acquired images (10 Hz) were collected without binning, and the 380 nm sequence was converted to a \(\Delta F/F_0\) ratio, where \(\Delta F/F_0 = (F_0 - F)/F_0\), \(F_0\) is the recorded fluorescence, and \(F\) was obtained from the average of the initial 15 frames of the image sequence, prior to cell stimulation. These images were displayed using an inverted pseudocolor scale. For measurement of \([\text{Ca}^{2+}]_i\) during photolysis, cells were loaded with the \([\text{Ca}^{2+}]_i\)-sensitive dye Oregon Green 488 BAPTA-2 by way of diffusion through the patch pipette. The dye was excited at 488 ± 15 nm, and fluorescence emission was collected through a 525 ± 25 nm bandpass filter (Chroma). Images were acquired at 10 Hz without binning and converted to a \(\Delta F/F_0\) ratio. Photolysis of \(\beta\)-myo-Insp-P2,M3,1,2(2-thiophen-yl)-ethyl ester (caged InsP3) was achieved using a pulsed xenon arc lamp excited at 150 W. The entire excitation and emission wavelengths were contained within the bandpass of a monochrometer (T.I.L.L. Photonics). A continuous, low level strobe output of UV light (360 ± 7.5 nm) was reflected onto the plane of focus using a DM400 dichroic mirror and Super Fluor 40 ×, 1.3 NA oil immersion objective.

**Whole Cell Patch Clamp—**Patch-clamped cells were superfused with an extracellular recording solution that contained (mM): 140 NaCl, 4.7 KCl, 1.13 MgCl2, 1 CaCl2, 10 HEPES-NaOH, 10 \(\beta\)-glucose, pH 7.3. Intracellular (pipette) solution contained (mM): 140 KCl, 1.3 MgCl2, 2 Mg-ATP, 10 HEPES-NaOH, 1 \(\beta\)-hydroxyethylideneaminotriacetic acid, 0.075 fura-2 \(K^+\), pH 7.3. For flash photolysis experiments, the solution contained (mM): 0.075 Oregon Green 488 BAPTA-2 and 0.01 M NaF. Where noted, 30 \(\mu\)M HT221 was added to the pipette solution.

**RESULTS AND DISCUSSION**

**Physiological CCK Stimulation Results in PKA-dependent Phosphorylation of the Type III InsP3R—**Previous studies have shown that CCK stimulation and pharmacological activation of PKA result in phosphorylation of type III InsP3R in pancreatic acinar cells (25). However, a link between CCK stimulation and PKA activation has not yet been established. Thus, experiments were undertaken to determine whether CCK-evoked phosphorylation of type III InsP3R was mediated by PKA. Acinar cell samples containing equal protein amounts were stimulated with 50 \(\mu\)M CCK, prior to or following treatment with 30 \(\mu\)M Rb-cAMP, and 2 \(\mu\)M H89 to inhibit PKA. Additional aliquots were treated with 10 \(\mu\)M forskolin as a positive control. All drug/agonist treatments were 5 min in duration. Following immunoprecipitation of the type III InsP3R, proteins were separated electrophoretically on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with an antibody that recognizes phosphorylated substrates, specifically by detecting phosphorylated Ser/Thr residues with enhanced chemiluminescent substrate (Pierce). Where indicated, nitrocellulose membrane was stripped of primary and secondary antibodies in a solution that contained (mM): 62.5 Tris-HCl, 100 \(\beta\)-mercaptoethanol, and 69 SDS. Membranes were stripped at 50 °C for 30 min.

**Statistical Analysis—**Data was analyzed using appropriate Student’s \(t\) test and GraphPad Prism statistical software. A \(p\) value of less than 0.05 was considered statistically significant.
that inhibitors of PKA are not entirely specific for this kinase, further support for the idea that CCK stimulation induces phosphorylation through PKA includes data demonstrating that stimulation of cells with physiological levels of muscarinic agonist, which presumably activates protein kinase C, failed to result in incorporation of 32P into InsP3R, and furthermore, that direct activation of protein kinase C by phorbol ester similarly failed to induce phosphorylation of type III InsP3R (25).

Because it has previously been reported that stimulation of acinar cells with agonist, even for short periods of time, can result in a down-regulation of InsP3R (30), the blot in Fig. 1A was stripped and reprobed with α-InsP3R, to determine the levels of receptor that were immunoprecipitated from each sample. As seen in Fig. 1B, approximately equal amounts of receptor were present in each sample, suggesting that agonist stimulation, at the time points and concentrations studied, does not result in decreased InsP3R population. These data demonstrate that physiological CCK stimulation of pancreatic acinar cells results in the PKA-mediated phosphorylation of type III InsP3R, which is consistent with the observation that CCK receptors, in addition to coupling to Gαq, also interact with Gαs (27). The current data support the idea that phosphorylation of InsP3R by PKA is an agonist-specific event and therefore may be a good candidate mechanism for contributing to the selective shaping of [Ca2+]c signals. Thus, we investigated whether the temporal characteristics of CCK-evoked [Ca2+]c signals are defined by this PKA-mediated phosphorylation.

**Time-dependent Change in the Kinetics of CCK-evoked [Ca2+]c:** Stimulation of fura-2-loaded acinar cells with ACh or CCK results in the production of [Ca2+]c oscillations with distinct temporal characteristics. ACh-evoked oscillations (50–400 nM CCh) are typically produced upon an elevated [Ca2+]c plateau, while CCK-evoked oscillations (1–50 pM CCK) typically initiate from the base-line [Ca2+]c level (Fig. 2, A and B; note the same time scale for each trace) (9). To begin to understand the mechanisms by which divergent, agonist-evoked [Ca2+]c responses are produced, we investigated the subcellular kinetics of Ca2+ waves evoked by CCK and CCh stimulation using agonist concentrations that produce [Ca2+]c oscillations and comparable peak [Ca2+]c changes. The initiation and spreading of the [Ca2+]c wave was monitored during multiple [Ca2+]c oscillations in the same train of spikes using high temporal and spatial resolution imaging. Specifically, the time needed to produce a 50% peak [Ca2+]c response, measured as the time from initiation of the response in the apical pole of the cell to a 50% peak [Ca2+]c, change in the basal pole, was determined for each oscillation of the response. Two paradigms were used to measure the kinetics of the [Ca2+]c response induced by muscarinic receptor stimulation. First, the rate of [Ca2+]c increase for the initial spike produced upon stimulation with CCh was determined. Following 3–5 min of agonist stimulation, CCh was removed for 3 min and then reapplied, and the rate of the resultant initial [Ca2+]c increase was again determined. Second, the kinetics of each [Ca2+]c rise during a train of oscillations in the continued presence of CCh were analyzed. These parameters were compared with the kinetics of responses evoked during continuous CCK stimulation investigating the rate of Ca2+ rise for both the first and fourth oscillation (time-matched, to compare with CCh stimulation).

Fig. 2C illustrates the rising phase of a [Ca2+]c response induced by stimulation of small acinar cell clusters with 250 nM CCh. Following 3–5 min of agonist stimulation and agonist wash-out, the rate of [Ca2+]c increase of a subsequent CCh-evoked response was not significantly altered compared with the control response, suggesting that the [Ca2+]c release events evoked by CCh stimulation are not modified over time. Specifically, the time required to produce a 50% peak [Ca2+]c increase, as measured to the peak of the individual response, was 2.9 ± 0.2 s for the initial and 3.0 ± 0.6 s (6 ≤ n ≤ 18; p = 0.95) for the subsequent response.

Although the initial [Ca2+]c increase produced following stimulation with CCh was chosen to allow direct comparison with CCK-evoked responses, the values obtained may not fully represent the kinetics of an individual spike during an oscillating CCh response, as these oscillations are typically produced upon an elevated [Ca2+]c plateau. Thus we measured the rate of [Ca2+]c increase, defined as the peak [Ca2+]c change in the basal pole divided by the time to produce that [Ca2+]c change for each individual oscillation produced within a train of oscillations. Using these parameters, the rate of [Ca2+]c increase for the second, third, and fourth oscillations was 102 ± 7, 114 ± 4, and 101 ± 8% of the rate of the first oscillation produced on the elevated [Ca2+]c plateau (Fig. 2E; n = 3). These data indicate that the rate of CCh-evoked Ca2+ increases are not altered over time, regardless of whether the oscillations originate from the resting base line [Ca2+]c or an elevated plateau.

Although the rate of [Ca2+]c increase for CCh-evoked responses remained constant during stimulation, the CCK-evoked [Ca2+]c, rise slowed markedly during the period of stimulation, such that the time needed to produce a 50% peak [Ca2+]c, increase was altered from 4.6 ± 0.8 s to 7.1 ± 0.5 s (Fig. 2D; 4 ≤ n ≤ 7; p = 0.03) for the first and fourth oscillation, respectively. As compared with the initial CCK-evoked oscillation, the rate of [Ca2+]c rise for the subsequent three oscillations decreased to 67 ± 8, 58 ± 9, and 43 ± 7%, respectively, of the initial oscillation (Fig. 2F; 4 ≤ n ≤ 5; p = <0.04).

Although there are numerous factors that could potentially contribute to the slowing of CCK-induced oscillations, this event is consistent with our previous work which showed that phosphorylation of Ca2+-release channels can "dampen" Ca2+ release (26). Thus we investigated whether an agonist-specific phosphorylation event could contribute to the slowing and shaping of the Ca2+ signals and, ultimately, to the generation of agonist-specific patterns of oscillations.

**PKA Activation Results in a Slowing of the Kinetics of Agonist and InsP2-induced [Ca2+]c Responses:** To test whether PKA-mediated phosphorylation of InsP3R contributes to the
The time to 50% peak \([\text{Ca}^{2+}]\) versus control. For the experiments shown in Fig. 3A, B, typical pattern of \([\text{Ca}^{2+}]\), oscillations evoked by stimulation of fura-2-loaded acinar cells with physiological concentrations of CCh or CCK. Note that ACh stimulation results in frequent oscillations superimposed upon an elevated \([\text{Ca}^{2+}]\), plateau, whereas CCK stimulation results in less frequent oscillations from the baseline \([\text{Ca}^{2+}]\), level. C and D, representative raw kinetic traces of the initial \([\text{Ca}^{2+}]\), increase produced by agonist stimulation (solid line) and the response produced following 5 min of agonist stimulation (dotted line). For the CCh kinetics, an initial \([\text{Ca}^{2+}]\), increase was used for both time points, as this is kinetically most similar to the CCK-evoked \([\text{Ca}^{2+}]\), increase. For clarity, only kinetics from a region of interest in the basal pole of the cell are displayed. E and F, pooled data for experiments represented in panels C and D, respectively. The rate of \([\text{Ca}^{2+}]\), increase was determined for the four consecutive \([\text{Ca}^{2+}]\), oscillations produced following initiation of the response for cells stimulated with CCh or the four oscillations including the initial oscillation for cells stimulated with CCK and is expressed as percent of initial oscillation rate. An asterisk denotes statistically significant difference versus control. For the experiments shown in panels C–F, images were acquired at 10 Hz.

Fig. 2. Kinetics of CCK-evoked oscillations change over time. A and B, typical pattern of \([\text{Ca}^{2+}]\), oscillations evoked by stimulation of fura-2-loaded acinar cells with physiological concentrations of CCh or CCK. Note that ACh stimulation results in frequent oscillations superimposed upon an elevated \([\text{Ca}^{2+}]\), plateau, whereas CCK stimulation results in less frequent oscillations from the baseline \([\text{Ca}^{2+}]\), level. C and D, representative raw kinetic traces of the initial \([\text{Ca}^{2+}]\), increase produced by agonist stimulation (solid line) and the response produced following 5 min of agonist stimulation (dotted line). For the CCh kinetics, an initial \([\text{Ca}^{2+}]\), increase was used for both time points, as this is kinetically most similar to the CCK-evoked \([\text{Ca}^{2+}]\), increase. For clarity, only kinetics from a region of interest in the basal pole of the cell are displayed. E and F, pooled data for experiments represented in panels C and D, respectively. The rate of \([\text{Ca}^{2+}]\), increase was determined for the four consecutive \([\text{Ca}^{2+}]\), oscillations produced following initiation of the response for cells stimulated with CCh or the four oscillations including the initial oscillation for cells stimulated with CCK and is expressed as percent of initial oscillation rate. An asterisk denotes statistically significant difference versus control. For the experiments shown in panels C–F, images were acquired at 10 Hz.

slowing and shaping of \([\text{Ca}^{2+}]\), signals, we pharmacologically manipulated PKA in an attempt to alter the kinetics of a \([\text{Ca}^{2+}]\), signal produced by stimulation with an agonist that normally does not induce phosphorylation. We therefore investigated whether PKA activation could alter the kinetics of a response to muscarinic stimulation, such that the rate of \([\text{Ca}^{2+}]\), rise would resemble that of a CCK response. Stimulation of cells with CCh (250 nM) produced a \([\text{Ca}^{2+}]\), rise that initiated in the apical pole and spread as a wave throughout the cell, as seen in the pseudocolor image series in Fig. 3A. Treatment of cells with 100 μM Bt2cAMP and 10 μM forskolin for 5 min to maximally activate PKA resulted in the slowing of the \([\text{Ca}^{2+}]\), rise of a subsequent response to CCh. Representative line traces in Fig. 3B, showing the rising phase of the CCh-evoked response prior to and following PKA activation, as well as the pooled data in Fig. 3C demonstrate that activation of PKA resulted in a significant slowing of the \([\text{Ca}^{2+}]\), rise induced by CCh stimulation. The time needed to reach a 50% peak \([\text{Ca}^{2+}]\), change increased from 2.9 ± 0.2 s to 6.6 ± 1.4 s following activation of PKA (6 ≤ n ≤ 18; p = 0.0003; Fig. 3C). The time to 50% peak \([\text{Ca}^{2+}]\), increase following PKA activation is comparable with that of a time matched CCK oscillation (7.1 ± 0.5 s; n = 4). These data indicate that pharmacologically induced PKA phosphorylation can alter the kinetics of a response to a non-PKA-activating agonist, such that the rate of \([\text{Ca}^{2+}]\), rise in the presence of activated PKA resembles that of a response produced by CCK stimulation.

To further address the hypothesis that the mechanism that regulates the kinetics of CCK-induced oscillations is at the level of the InsP3R, we investigated the effect of PKA-mediated activation on the time course of InsP3-evoked \([\text{Ca}^{2+}]\), signals in whole cell patch-clamped cells. Although we have previously demonstrated that PKA activation markedly attenuates the amplitude of \([\text{Ca}^{2+}]\), signals generated by threshold levels of InsP3 (26), the effect of PKA phosphorylation on larger InsP3-evoked \([\text{Ca}^{2+}]\), signals, reminiscent of those produced by agonist stimulation, has not been determined. We therefore utilized continuous strobe photolysis of caged InsP3 to generate a \([\text{Ca}^{2+}]\) signal with kinetics similar to that induced by agonist and determined the effect of PKA-mediated phosphorylation on these responses. Cells were loaded with Oregon Green BAPTA-2 and 10 μM caged InsP3 through the patch pipette. InsP3 was subsequently released by a continuous strobe discharge of a Xenon flash lamp. Photorelease of InsP3 resulted in the initiation of a \([\text{Ca}^{2+}]\), signal in the apical region, which spread as a wave throughout the cell with similar spatiotem-
poral properties to that observed during a CCh-evoked re-
sponse (Fig. 4A). Treatment of cells with Bt2cAMP and forsko-
lin for 5 min prior to photolysis resulted in a slowing of the
subsequent response to strobe photolysis of caged InsP3, with
the time required to produce a 50% peak $[Ca^{2+}]_c$ change in
creasing to 310 ± 44% of the time-matched control response
($n = 3; p = 0.0002$; Fig. 4, A and B). Additionally, the peak
$[Ca^{2+}]_c$ change in the apical and basal regions decreased fol-
lowing activation of PKA, reaching 95 ± 3% of time-matched
control in the apical region ($n = 4; p = 0.03$) and 92 ± 2% of
control in the basal region ($n = 4; p = 0.0004$; Fig. 4C).

The ability of PKA activation to slow the rate of $[Ca^{2+}]_c$
increase of the InsP3-induced response, as well as to decrease
the peak $[Ca^{2+}]_c$ change, suggests that the target of the phos-
phorylation event is indeed the InsP3R. As further confirma-
tion of this idea, we investigated whether this modulation could
be prevented by inhibiting the action of PKA. We have previ-
ously shown that the regulatory subunit of PKA is targeted
specifically to the type III InsP3R in acinar cells (26) Thus,
disruption of PKA localization is predicted to selectively inter-
fere with phosphorylation of this receptor subtype. We utilized
peptide HT31, which disrupts the binding of PKA to A-kinase
anchoring protein scaffolds, as a specific tool to perturb the
type III InsP3R interaction with PKA without globally altering
PKA activity (31, 32). This peptide has been shown to markedly
reduce the effectiveness of PKA regulation of a number of ion

Fig. 3. Activation of PKA alters the kinetics of CCh-induced $[Ca^{2+}]_c$ response. A, image sequences of a fura-2-loaded cell stimulated with 250 nM CCh prior to and following a 5-min treatment with 100 μM Bt2cAMP and 10 μM forskolin. Images are displayed as ΔF/ΔFo of the 380 nm sequence using an inverted pseudocolor scale. B, representative kinetics of the images shown in A showing the slowed kinetics of the CCh response following activation of PKA. Kinetics were produced from a region of interest in the basal pole of the cell. C, time to 50% peak $[Ca^{2+}]_c$ increase for all experiments performed as described in A. Time to 50% peak $[Ca^{2+}]_c$ increase for the time-matched CCh response as well as the response to 5 pM CCK following 5 min of stimulation are shown for comparison. All images were acquired at 10 Hz.
channels, including voltage-gated Ca\(^{2+}\) channels and CFTR (33, 34).

HT31 (30 µM) was introduced into cells through the patch pipette and InsP\(_3\) was again photoreleased in the absence and presence of PKA activation. Under these conditions, Bt2cAMP and forskolin treatment had no effect on the kinetics of InsP\(_3\)-induced [Ca\(^{2+}\)]\(_c\) release (Fig. 4B). In the presence of HT31, release of InsP\(_3\) following PKA activation produced a [Ca\(^{2+}\)]\(_c\) rise with a time to 50% peak that was not significantly different from control (117 ± 19% of control; n = 3; p = 0.3; Fig. 4C). Additionally, the decrease in the amplitude of the [Ca\(^{2+}\)]\(_c\) rise in the apical and basal regions following activation of PKA was abolished in the presence of HT31. The peak [Ca\(^{2+}\)]\(_c\) in the apical region following PKA activation in the presence of HT31 was found to be 102 ± 2% of control (n = 6; p = 0.9), whereas in the basal region it was 101 ± 3% of control (n = 6; p = 0.7; Fig. 4C). Thus, pharmacological activation of PKA leads to a decrease in the rate and magnitude of InsP\(_3\)-evoked [Ca\(^{2+}\)]\(_c\) release.
PKA Phosphorylation of Type III InsP₃R Is Involved in the Production of a Physiological CCK Response—Although the current data demonstrate that stimulation of acinar cells with physiological concentrations of CCK results in the PKA-mediated phosphorylation of type III InsP₃R and that this phosphorylation alters the kinetics of a CCh- and InsP₃-evoked [Ca²⁺]ᵢ response, there are as yet no data suggesting that this phosphorylation is necessary for the production of a physiological CCK response. We therefore determined the requirement for a PKA-mediated phosphorylation event at the level of the type III InsP₃R during the production of a physiological CCK response, again utilizing HT31 as a selective inhibitor of PKA phosphorylation of type III InsP₃R. Specifically, the temporal pattern of agonist-evoked [Ca²⁺]ᵢ responses was determined in the presence and absence of type III receptor phosphorylation.

Fura-2-loaded acinar cells were whole cell patch-clamped and stimulated with a range of CCK concentrations that, in control patch-clamped cells, resulted in [Ca²⁺]ᵢ oscillations. Under control conditions, stimulation with 5–10 pM CCK resulted in oscillations in [Ca²⁺]ᵢ that were indistinguishable from oscillations produced in intact cells (Fig. 5A, n = 3). These oscillations consisted of the characteristic pattern of base-line [Ca²⁺]ᵢ spikes. However, in the presence of HT31, stimulation with 5 pM CCK no longer resulted in the production of base-line spikes but rather in irregular oscillations produced upon an elevated [Ca²⁺]ᵢ plateau (Fig. 5B, n = 3). Additionally, stimulation with 10 pM CCK in the presence of HT31 produced a peak-and-plateau response (Fig. 5C, n = 3). These data suggest that PKA-mediated phosphorylation of type III InsP₃R plays a major role in shaping the normal pattern of CCK-evoked [Ca²⁺]ᵢ oscillations.

To further support our hypothesis that PKA-mediated phosphorylation of type III receptors is an agonist-specific event required for the generation of CCK-evoked responses but not muscarinic responses, we determined the pattern of CCh-evoked oscillations when the ability of PKA to phosphorylate the type III receptor is prevented. As shown in Fig. 5D, stimulation of acinar cells with CCh in the presence of HT31 resulted in the generation of a typical CCh response with frequent, regular oscillations superimposed on an elevated [Ca²⁺]ᵢ plateau. Images were acquired at 1 Hz.

PKA-mediated phosphorylation of InsP₃R is a general mechanism by which [Ca²⁺]ᵢ oscillations can be shaped—The data presented thus far suggest that the production of a physiological CCK response involves the selective phosphorylation of type III InsP₃R by PKA. However, the question remains as to whether phosphorylation of InsP₃R is a general mechanism by which the pattern of agonist-evoked oscillations can be modulated in pancreatic acinar cells. Thus, we investigated whether PKA-mediated phosphorylation is involved in the generation of [Ca²⁺]ᵢ oscillations in response to another agonist, bombesin, which produces oscillations that are essentially indistinguishable from those produced by CCK stimulation (Fig. 6A). We hypothesized that if PKA-mediated phosphorylation was an important event underpinning the specific CCK-induced oscillatory pattern, then the nearly identical bombesin-induced signal would result from stimulation of similar pathways.

Identical procedures used to demonstrate phosphorylation of InsP₃R upon CCK stimulation were used to study the phosphorylation state of the type III InsP₃R following bombesin stimulation. Stimulation of acinar cells with concentrations of bombesin that elicited [Ca²⁺]ᵢ oscillations resulted in phosphorylation of the type III InsP₃R, as detected by immunoprecipitation and immunoblotting (Fig. 6B). In addition, preincubation of cells with PKA inhibitors resulted in a decreased level of phosphorylation following stimulation with bombesin, indicating a role for PKA activation in the bombesin-induced phosphorylation of the type III receptor. As described in Fig. 1, incubation...
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In summary, this study has demonstrated that in pancreatic acinar cells stimulation with CCK and bombesin results in phosphorylation of the type III InsP3R in a manner dependent on PKA activation. This appears to be an integral event controlling the pattern of CCK-evoked [Ca2+]c signals, as disruption of PKA localization markedly altered the normal temporal pattern of the agonist-evoked [Ca2+]c oscillations. Thus, type III InsP3R phosphoregulation may in part exert its effect through the alteration of InsP3R sensitivity (26), ultimately leading to a slowing of the kinetics of the agonist evoked response. These data are consistent with type III InsP3R phosphoregulation as part of a mechanism by which the pattern of [Ca2+]c spiking in a variety of non-excitable cells, thus having wide ranging consequences for the control of physiological end points sensitive to specific spatiotemporal patterns of [Ca2+]c changes.

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