Role of Ca\(^{2+}\) Feedback on Single Cell Inositol 1,4,5-Trisphosphate Oscillations Mediated by G-protein-coupled Receptors*

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The dynamics of inositol 1,4,5-trisphosphate (Ins\((1,4,5)\)P\(_3\)) production during periods of G-protein-coupled receptor-mediated Ca\(^{2+}\) oscillations have been investigated using the pleckstrin homology (PH) domain of phospholipase C (PLC) \(\delta\), tagged with enhanced green fluorescent protein (eGFP-PH\(_{PLC\delta}\)). Activation of noradrenergic \(\alpha_{1B}\) and muscarinic \(M_3\) receptors recombiantly expressed in the same Chinese hamster ovary cell indicates that Ca\(^{2+}\) responses to these G-protein-coupled receptors are stimulus strength-dependent. Thus, activation of \(\alpha_{1B}\) receptors produces transient baseline Ca\(^{2+}\) oscillations, sinusoidal Ca\(^{2+}\) oscillations, and then a steady-state plateau level of Ca\(^{2+}\) as the level of agonist stimulation increased. Activation of \(M_3\) receptors, which have a higher coupling efficiency than \(\alpha_{1B}\) receptors, produced a sustained increase in intracellular Ca\(^{2+}\) even at low levels of agonist stimulation. Confocal imaging of eGFP-PH\(_{PLC\delta}\) visualized periodic increases in Ins\((1,4,5)\)P\(_3\) production underlying the baseline Ca\(^{2+}\) oscillations. Ins\((1,4,5)\)P\(_3\) oscillations were blocked by thapsigargin but not by protein kinase C down-regulation. The net effect of increasing intracellular Ca\(^{2+}\) was stimulatory to Ins\((1,4,5)\)P\(_3\) production, and dual imaging experiments indicated that receptor-mediated Ins\((1,4,5)\)P\(_3\) production was sensitive to changes in intracellular Ca\(^{2+}\) between basal and \(-200\) nm. Together, these data suggest that \(\alpha_{1B}\) receptor-mediated Ins\((1,4,5)\)P\(_3\) oscillations result from a positive feedback effect of Ca\(^{2+}\) onto phospholipase C. The mechanisms underlying \(\alpha_{1B}\) receptor-mediated Ca\(^{2+}\) responses are therefore different from those for the metabolotropic glutamate receptor 5a, where Ins\((1,4,5)\)P\(_3\) oscillations are the primary driving force for oscillatory Ca\(^{2+}\) responses (Nash, M. S., Young, K. W., Challiss, R. A. J., and Nahorski, S. R. (2001) Nature 413, 381–382). For \(\alpha_{1B}\) receptors the Ca\(^{2+}\)-dependent Ins\((1,4,5)\)P\(_3\) production may serve to augment the existing regenerative Ca\(^{2+}\)-induced Ca\(^{2+}\)-release process; however, the sensitivity of Ca\(^{2+}\) feedback is such that only transient baseline Ca\(^{2+}\) spikes may be capable of causing Ins\((1,4,5)\)P\(_3\) oscillations.

Frequency encoding of Ca\(^{2+}\) signals in the form of oscillations provides a versatile mechanism by which a number of intracellular processes are controlled (1–3). Intracellular Ca\(^{2+}\) oscillations, which arise from periodic release from intracellular stores, can manifest as either transient oscillations from a baseline Ca\(^{2+}\) level or sinusoidal oscillations upon a raised plateau level of Ca\(^{2+}\) (see Refs. 4 and 5). Putative mechanisms by which cell surface G-protein-coupled receptors (GPCRs)1 stimulate Ca\(^{2+}\) oscillations via the intracellular messenger molecule Ins\((1,4,5)\)P\(_3\), can be broadly split into two categories, dependent on whether levels of Ins\((1,4,5)\)P\(_3\) are required to oscillate (6, 7). Thus, Ca\(^{2+}\) oscillations driven by a steady state raised level of Ins\((1,4,5)\)P\(_3\) may occur via a regenerative Ca\(^{2+}\)-induced Ca\(^{2+}\)-release process (CICR), which is an integral property of the Ins\((1,4,5)\)P\(_3\) receptor (InsP\(_3\)R) (see Ref. 5). In contrast, the sensitivity of certain oscillatory Ca\(^{2+}\) responses to feedback inhibition by protein kinase C (PKC), or regulators of G-protein signaling (RGS) proteins, suggests that Ins\((1,4,5)\)P\(_3\) levels may oscillate and hence drive Ca\(^{2+}\) oscillations (8–11). These two categories need not be mutually exclusive as, for example, Ca\(^{2+}\) may be required to recruit PKC to the plasma membrane before feedback inhibition can occur (12).

The recent introduction of the pleckstrin homology (PH) domain of phospholipase C-\(\delta\), tagged with enhanced green fluorescent protein (eGFP-PH\(_{PLC\delta}\)) to detect Ins\((1,4,5)\)P\(_3\) in single cells (13–15) has enabled the profile of agonist-stimulated Ins\((1,4,5)\)P\(_3\) production to be investigated directly. This method has indeed detected Ins\((1,4,5)\)P\(_3\) oscillations in ATP-stimulated Madin-Darby canine kidney cells (14) and metabolotropic glutamate receptor 5a (mGluR5a)-stimulated CHO cells (10, 12). Ins\((1,4,5)\)P\(_3\) oscillations mediated by mGluR5a activation arise from a PKC-dependent dynamic uncoupling of the receptor from its signaling pathway (9, 10, 12) giving rise to synchronous Ins\((1,4,5)\)P\(_3\) and Ca\(^{2+}\) oscillations. In contrast, however, muscarinic \(M_3\) receptor-driven sinusoidal Ca\(^{2+}\) oscillations in CHO cells were associated with a modest steady-state increase in Ins\((1,4,5)\)P\(_3\) (10).

This current study has investigated the cellular dynamics of Ins\((1,4,5)\)P\(_3\) production and Ca\(^{2+}\) signaling in individual CHO cells in response to noradrenergic \(\alpha_{1B}\) and muscarinic \(M_3\) receptor stimulation, using the mGluR5a model for PKC-dependent dynamic receptor uncoupling as a comparison. Our results provide the first direct evidence that Ca\(^{2+}\) oscillations may stimulate transient increases in Ins\((1,4,5)\)P\(_3\) via a positive feedback effect on phospholipase C (PLC). Moreover, although PKC can regulate receptor activity, it appears not to be directly involved in the Ins\((1,4,5)\)P\(_3\) oscillations initiated by \(\alpha_{1B}\) recep-

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1 The abbreviations used are: GPCR, G-protein coupled receptor; CICR, Ca\(^{2+}\) induced Ca\(^{2+}\)-release; eGFP-PH\(_{PLC\delta}\); PH domain of PLC\(_\delta\); tagged with enhanced green fluorescent protein; Ins\((1,4,5)\)P\(_3\), inositol 1,4,5-trisphosphate; InsP\(_3\)R, inositol 1,4,5-trisphosphate receptor; MCH, methacholine; NA, noradrenaline; PKC, protein kinase C; PDBu, phorbol dibutyrate; PLC, phospholipase C; RFU, relative fluorescent units; PH\(_{PLC\delta}\), pleckstrin homology; CHO, Chinese hamster ovary; KHB, Krebs-Henseleit buffer; RGS, regulators of G-protein signaling.
tors. Furthermore, the Ca$^{2+}$ sensitivity of PLC isoforms in CHO cells is such that only Ca$^{2+}$ oscillations of the transient base-line spiking nature would be capable of stimulating this positive feedback. In comparison, in the same cells, mGlur5a-mediated Ins(1,4,5)P$_3$ oscillations appear to contain elements of both positive Ca$^{2+}$ feedback and dynamic PKC-mediated inhibition, dependent on the agonist concentration. The role of this Ca$^{2+}$ feedback-induced Ins(1,4,5)P$_3$ production may be to enhance the regenerative CICR process by increasing the number of Ins(1,4,5)P$_3$-bound InsP$_3$Rs available for activation.

MATERIALS AND METHODS

Cell Culture and Plasmid Transfection—CHO cells stably expressing noradrenergic $\alpha_1_B$ (2.3 pmol/mg protein) and muscarinic $M_3$ receptors (3.4 pmol/mg protein) (CHO$\alpha_1_B$/$M_3$) were maintained in minimum Eagle’s medium-v (Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 2.5 µg/ml fungizone, 400 µg/ml G418, and 300 units/ml hygromycin. Metabotropic glutamate receptor 5a expressing CHO cells (CHO-lac-mGlur5a) were created using the LacSwitch II-inducible expression system (Stratagene) as described previously (12). Maximal levels of mGlur5a expression were achieved by preincubation with 100 µM isopropyl-1-thio-β-D-galactopyranoside (12). Cells were plated to near confluence on coverslips before being transfected with 1 µg of plasmid DNA plus 3 µl of FuGENE 6 (Roche Applied Science) per coverslip (according to manufacturer’s instructions). Cells were used 48 h after transfection. For experiments examining mGlur5a transiently expressed in CHO$\alpha_1_B$/$M_3$ cells, 0.5 µg of mGlur5a in pDNA3 (Invitrogen) was co-transfected with 0.5 µg of eGFP-PHPLC from 3 µl of FuGENE 6 per coverslip. The medium was changed after 24 h to prevent accumulation of extracellular glutamate, and cells were used after a further 24 h. The eGFP-PHPLC construct was a kind gift from Prof. Tobias Meyer.

Single Cell Imaging of Ins(1,4,5)P$_3$ and Ca$^{2+}$—Coverslips were mounted on the stage of an Olympus IX70 inverted epifluorescence microscope and perfused at 37°C with Krebs-Henseleit buffer (in mM: NaCl 118, KCl 4.7, MgSO$_4$ 1.2, CaCl$_2$ 1.3, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 4.2, HEPES 10, glucose 11.7, pH 7.4). Images of cells after excitation at 488 nm were collected using an Olympus FV500 laser scanning confocal microscope at a scan rate of 1.5–2.5 Hz. Increases in cellular fluorescence were detected by measuring the translocation of eGFP-PHPLC$_{CHO}$ from the plasma membrane to the cytosol. This was done by creating a cytosolic region of interest and plotting the average pixel intensity in that region versus time. Data are expressed in relative fluorescent units (RFU) by subtraction of background fluorescence followed by dividing the fluorescent intensity at a given time by the initial fluorescence within each region of interest (RFU$_i$). Images involving co-detection of Ins(1,4,5)P$_3$ and Ca$^{2+}$ were made by transfecting CHO$\alpha_1_B$/$M_3$ cells with eGFP-PHPLC$_{CHO}$ as above and then loading the cells with fura-2 (2 µM fura-2AM, 1 h). The cells were then excited at 340 and 380 nm (for fura-2) and 488 nm (for eGFP) using a Spectra master II monochromator (PerkinElmer Life Sciences). Images were collected via a cooled CCD camera using the Merlin2000 data acquisition system (PerkinElmer Life Sciences) at a sample rate of 0.7 Hz. Changes in cytosolic eGFP-PHPLC$_{CHO}$ were measured as described above. Simultaneously, changes in cytosolic Ca$^{2+}$ (Ca$^{2+}_{cyt}$) were measured by converting the 340/380 ratio of fluorescence (after background subtraction) to approximate [Ca$^{2+}$]$_i$ using the method of Grynkiewicz et al. (16). The minimal and maximal fluorescence ratios (R$_{min}$ and R$_{max}$) were obtained from a sample set of CHO cells using 5 µM ionomycin and 6 mM EGTA (for R$_{max}$) followed by 10 mM CaCl$_2$ (for R$_{min}$).

RESULTS

Receptor-dependent Ca$^{2+}$ Oscillations in CHO Cells—The temporal patterns of intracellular Ca$^{2+}$ signals in CHO cells co-expressing noradrenergic $\alpha_1_B$ and muscarinic $M_3$ receptors were compared with those elicited by the metabotropic glutamate receptor mGlur5a. The $\alpha_1_B$ receptor was capable of stimulating Ca$^{2+}$ oscillations over a range of agonist concentrations. Thus, increasing the concentration of noradrenaline (NA) from 10$^{-8}$ to 3 $\times$ 10$^{-7}$ M produced base-line Ca$^{2+}$ oscillations, the frequency of which increased with the stimulus strength (Fig. 1A). At NA concentrations above 10$^{-7}$ M a steady-state plateau level of Ca$^{2+}$ was observed (Fig. 1A). In contrast, although oscillatory Ca$^{2+}$ responses were occasionally observed at low agonist doses (data not shown) (10), in most cells examined, concentrations of methacholine (MCH) of 10$^{-8}$ M and above produced a peak and plateau Ca$^{2+}$ response (Fig. 1B). Oscillatory Ca$^{2+}$ signals in CHO-lac-mGlur5a cells, occurring via cyclical changes in Ins(1,4,5)P$_3$ production (12), differed from those observed with either NA or MCH in CHO$\alpha_1_B$/$M_3$ cells, in that they were largely of constant frequency and did not saturate with increasing agonist concentration. Thus, at the lowest concentration of glutamate investigated (L-Glu, 10$^{-6}$ M), base-line Ca$^{2+}$ transients could be observed (Fig. 1C). Increasing the concentration of L-Glu to 3 $\times$ 10$^{-6}$ M increased the frequency of these base-line oscillations, but further increasing the frequency of L-Glu even up to 3 $\times$ 10$^{-4}$ M had no additional effects on the Ca$^{2+}$ signal (Fig. 1C).

Receptor-dependent Ins(1,4,5)P$_3$ Oscillations in CHO Cells—Simultaneous single cell measurements of Ins(1,4,5)P$_3$ production (using eGFP-PHPLC$_{CHO}$) and Ca$^{2+}$ mobilization (using fura-2) demonstrated that although the $\alpha_1_B$ and $M_3$ receptors are co-expressed at similar levels in the CHO$\alpha_1_B$/$M_3$ cells, the muscarinic $M_3$ receptor stimulates ~10-fold more Ins(1,4,5)P$_3$ production at maximal agonist concentrations (Fig. 2A). In contrast, due to the large degree of amplification between Ins(1,4,5)P$_3$ production and subsequent Ca$^{2+}$ mobilization (17), the peak Ca$^{2+}$ responses produced by NA and MCH were not significantly different (Fig. 2A, inset, measured simultaneously...
from the same cells as Fig. 2A, main panel). When temporal changes in Ca\(^{2+}\) and Ins(1,4,5)P\(_3\) were co-imaged, small oscillatory changes in Ins(1,4,5)P\(_3\) were observed during periods of NA-induced base-line Ca\(^{2+}\) spiking (Fig. 2B). However, due to the limited sensitivity of this method for detecting cytoplasmic changes in eGFP-PHPLC, further experiments were conducted using confocal microscopy.

Confocal imaging more clearly revealed the presence of an oscillatory Ins(1,4,5)P\(_3\) response to low concentrations of NA in CHO\(_{1B/M3}\) cells. This was most prevalent with 10\(^{-7}\) M NA, which was around the threshold level for Ins(1,4,5)P\(_3\) detection (Fig. 3B). Oscillatory Ins(1,4,5)P\(_3\) signals in response to 10\(^{-7}\) M NA were characterized by a larger initial peak of Ins(1,4,5)P\(_3\) production (increase in F/F\(_{0}\) value of 0.40 ± 0.05 RFU, mean of 47 cells), followed by smaller transient increases of relatively consistent height (0.15 ± 0.01 RFU increase, 141 oscillations in 47 cells, measured during periods of base-line Ins(1,4,5)P\(_3\) oscillations). The average frequency of the Ins(1,4,5)P\(_3\) oscillations in response to 10\(^{-7}\) M NA was 0.042 ± 0.003 Hz, or 1 peak every 23.6 s (47 cells). Increasing the concentration of NA altered the temporal dynamics of response, such that at 10\(^{-4}\) M NA a peak and plateau increase in Ins(1,4,5)P\(_3\) was observed (0.64 ± 0.09 RFU initial peak increase, and 0.35 ± 0.04 RFU increase after 120 s, 80–86 cells) (Fig. 3A). As already described above, the M\(_3\) receptor is more efficiently coupled to Ins(1,4,5)P\(_3\) production. In confocal measurements, the most common temporal profile of Ins(1,4,5)P\(_3\) production in response to MCH was a robust peak and plateau response (Fig. 3C). At 10\(^{-4}\) M MCH this corresponded to a 6.17 ± 0.28 RFU initial increase in F/F\(_{0}\), dropping to 4.93 ± 0.24 RFU increase after 120 s (39 cells). It should be noted that the greater level of stimulation observed, compared with the co-imaging data, is due to the lower initial cytosolic fluorescence in the confocal section. On rare occasions, small oscillatory responses were also observed with MCH stimulation (Fig. 3D). In three cells in which M\(_3\) receptor-mediated Ins(1,4,5)P\(_3\) oscillations were observed, the initial peak increase (0.16 ± 0.01 RFU) was smaller than that observed with 10\(^{-7}\) M NA, as were the subsequent peaks (0.10 ± 0.01 RFU increase, 9 oscillations in three cells). The average frequency of these oscillations was 0.032 ± 0.001 Hz, or 1 every 31.2 s.

**Role of Ca\(^{2+}\) and PKC in Single Cell Ins(1,4,5)P\(_3\) Oscillations**—NA-induced Ins(1,4,5)P\(_3\) and Ca\(^{2+}\) oscillations appeared dependent on both mobilization of Ca\(^{2+}\) from intracellular stores (as they were inhibited by addition of the smooth endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) pump inhibitor, thapsigargin (5 × 10\(^{-6}\) M), Fig. 4B), and protein kinase activity (as they were inhibited by staurosporine (10\(^{-6}\) M), Fig. 4C). In both cases, these inhibitors produced raised steady-state levels of Ins(1,4,5)P\(_3\) and [Ca\(^{2+}\)]\(_i\) in CHO\(_{1B/M3}\) cells stimulated with 10\(^{-7}\) M NA (Fig. 4).

To investigate further the role of [Ca\(^{2+}\)]\(_i\) in NA-mediated IP\(_3\) production, CHO\(_{1B/M3}\) cells were pretreated with thapsigargin (2 × 10\(^{-6}\) M) in the absence of extracellular Ca\(^{2+}\) (nominally Ca\(^{2+}\) free) for 5 min. Cells were then stimulated with NA (10\(^{-4}\) M for 200 s) and then washed in normal KHB (containing 1.3 mM CaCl\(_2\)) for 10 min, before being stimulated with NA (10\(^{-4}\) M for 200 s) again. In the absence of extracellular Ca\(^{2+}\), NA produced an initial peak 0.59 ± 0.07 RFU increase in
The effect of thapsigargin suggested that a positive feedback effect of Ca$^{2+}$ on PLC activity could be the mechanism behind the observed NA-mediated Ins(1,4,5)P$_3$ oscillations. To determine the dynamic range of this Ca$^{2+}$-sensitive Ins(1,4,5)P$_3$ production, Ca$^{2+}$ and Ins(1,4,5)P$_3$ levels in response to $10^{-4}$ M NA were measured simultaneously, while extracellular Ca$^{2+}$ was stepped from 1.3 to 1.0, 0.3, and then back to 1.3 mM (Fig. 6). In this set of experiments, $10^{-4}$ M NA in the presence of 1.3 mM extracellular Ca$^{2+}$ produced peak and plateau Ins(1,4,5)P$_3$ and Ca$^{2+}$ responses. Although changing extracellular Ca$^{2+}$ to 1.0 mM caused the plateau level of [Ca$^{2+}$], to drop from 332 ± 26 to 243 ± 27 nM, no alteration in the level of Ins(1,4,5)P$_3$ production was observed (Fig. 6). However, further stepwise decreases in extracellular Ca$^{2+}$ to 0.3 mM and nominally Ca$^{2+}$-free resulted in a decline in NA-mediated Ins(1,4,5)P$_3$ production toward prestimulated levels. The exact point at which decreases in [Ca$^{2+}$] began to affect Ins(1,4,5)P$_3$ levels varied between 70 and 170 nM depending on the cell examined (mean 120 ± 7 nM, 21 cells). On the return of 1.3 mM Ca$^{2+}$ to the perfusing buffer, Ins(1,4,5)P$_3$ levels increased with rising Ca$^{2+}$ (Fig. 6). However, again Ins(1,4,5)P$_3$ production was not sensitive to the full dynamic range of intracellular Ca$^{2+}$. Thus, depending on the individual cell, Ins(1,4,5)P$_3$ levels increased with [Ca$^{2+}$], up to 83–260 nM (mean 184 ± 15 nM, 18 cells). After this point, further increases in intracellular Ca$^{2+}$ did not additionally enhance NA-mediated Ins(1,4,5)P$_3$ production. Stimulation of PLC activity by MCH ($10^{-5}$ M) was similarly sensitive to changes in [Ca$^{2+}$] (data not shown).

Although the above data strongly suggests that Ca$^{2+}$ feedback could be the driving force for NA-induced Ins(1,4,5)P$_3$ oscillations, the observation that both Ca$^{2+}$ and Ins(1,4,5)P$_3$...
oscillations could be inhibited by staurosporine (Fig. 4C) also suggested a role for protein kinases. As PKC-mediated negative feedback dynamically controls mGluR5a oscillations (9, 10, 12), a potential role for PKC was also investigated. In addition to the effect on NA-mediated oscillatory responses (Fig. 4C), staurosporine (10^{-6}\text{M}) also increased levels of Ins(1,4,5)P_3 production in response to maximal concentrations of NA (10^{-4}\text{M}, 35 cells) (Fig. 7A). In contrast, MCH-induced Ins(1,4,5)P_3 production in response to 10^{-6} and 10^{-8}\text{M} MCH appeared insensitive to staurosporine (Fig. 7, B and C, 94 and 40 cells, respectively).

As staurosporine is a relatively broad spectrum inhibitor of protein kinases, the role of PKC in NA-induced Ins(1,4,5)P_3 oscillations was further examined by pretreating CHO_{α_1B/M3} cells for 20–24 h with the phorbol ester PDBu (1 \mu\text{M}), as part of an established protocol to down-regulate PKC isoforms (6). This treatment enhanced Ins(1,4,5)P_3 production in response to both low and high concentrations of NA (Fig. 8, A and C). Thus the mean peak increase in Ins(1,4,5)P_3 production in response to 10^{-7}\text{M} NA in PDBu-treated cells was 1.16 ± 0.17 RFU, followed by a raised plateau level of 1.05 ± 0.17 RFU (29 cells). This represents a 5-fold increase in Ins(1,4,5)P_3 production compared with non-PDBu-treated cells (see above) (Fig. 8, A and B). Due to this enhanced stimulus strength, no oscillations in Ins(1,4,5)P_3 were observed with applications of 10^{-7}\text{M} NA. Ins(1,4,5)P_3 production in response to 10^{-7}\text{M} NA was similarly affected. In this case, after PDBu treatment, the mean peak increase in Ins(1,4,5)P_3 production was 3.98 ± 0.02 and 2.50 ± 0.16 RFU, respectively, under control conditions (50 cells), and peak and plateau increases of 3.51 ± 0.20 and 2.99 ± 0.17 RFU, respectively, in cells pretreated with 10^{-6}\text{M} PDBu for 24 h (48 cells).

**Differential Ins(1,4,5)P_3 Oscillations Induced by Noradrenergic α_{1B} and mGlu5a Receptors—** To compare the Ca^{2+} feedback-mediated Ins(1,4,5)P_3 oscillations observed with α_{1B} receptor activation with the PKC feedback-mediated mGluR5a oscillations described previously (10, 12), the mGluR5a was transiently transfected into CHO_{α_1B/M3} cells. Stimulation of mGluR5a-transfected CHO_{α_1B/M3} cells with L-Glu appeared to stimulate two distinct types of Ins(1,4,5)P_3 oscillation (Fig. 9). Low concentrations of L-Glu (3 × 10^{-6}\text{M}) produced small transient oscillations in Ins(1,4,5)P_3 production of relatively constant magnitude. The amount of Ins(1,4,5)P_3 produced by each transient was similar to that observed with low levels of NA stimulation (Figs. 3 and 4) and consisted of a 0.18 ± 0.02 RFU transient initial peak, followed by further transient increases of 0.17 ± 0.01 RFU (70 oscillations from 22 to 25 cells) (Fig. 9). These transient increases occurred with a frequency of 0.024 ± 0.002 Hz, or one every 41.7 s (24). However, and in contrast to the effect of increasing levels of α_{1B} receptor stimulation, oscillations in Ins(1,4,5)P_3 production were still maintained at higher levels of mGluR5a activation.
Thus, Ca\(^{2+}\) oscillations may be generated by a steady-state increase in In(1,4,5)P\(_3\) via a regenerative CICR process, or Ca\(^{2+}\) oscillations may follow cyclical changes in In(1,4,5)P\(_3\) which are entrained by a negative feedback loop targeting In(1,4,5)P\(_3\) production. Recent experiments have begun to shed some light on the specific mechanisms underlying this important signaling phenomenon (22). For example, feedback regulation of In(1,4,5)P\(_3\) production may occur via ROS proteins (11), with the receptor-G-protein-ROS complex regulating In(1,4,5)P\(_3\) oscillations and hence the resulting Ca\(^{2+}\) signal. Crucially, direct single cell measurements of In(1,4,5)P\(_3\) production with eGFP-PHPLC\(_{D51}\) as a biosensor have emphasized a role for oscillations in In(1,4,5)P\(_3\) production. By using this construct, coincident oscillations in In(1,4,5)P\(_3\) and Ca\(^{2+}\) have been observed in ATP-stimulated canine kidney epithelial cells (14). Furthermore, our own work has demonstrated that oscillations in In(1,4,5)P\(_3\) appear to be crucial for mGluR5a-induced Ca\(^{2+}\) oscillations in CHO cells in which the mGlu5a receptor is under the control of an inducible expression system (10, 12). In this case it appears that the sensitivity of mGluR5a to phosphorylation, and hence inhibition, by PKC (9) is a key element to the negative feedback pathway.

Here, we have extended these studies to other PLC-linkedGPCRs, and we have observed an oscillatory In(1,4,5)P\(_3\) response to stimulation of \(\alpha_{1B}\) receptors with low concentrations of NA. Despite the small magnitude of In(1,4,5)P\(_3\) response, simultaneous measurements of Ca\(^{2+}\) signals demonstrated that In(1,4,5)P\(_3\) and Ca\(^{2+}\) oscillate synchronously. Further investigation of the NA-induced In(1,4,5)P\(_3\) signal indicated that responses were regulated by changes in [Ca\(^{2+}\)]\(_i\). Intracellular Ca\(^{2+}\) mobilization was important, as In(1,4,5)P\(_3\) oscillations were blocked by treating cells with thapsigargin. Furthermore, thapsigargin pretreatment and removal of extracellular Ca\(^{2+}\) lowered NA-induced In(1,4,5)P\(_3\) production relative to a control response in 1.3 mM extracellular Ca\(^{2+}\), suggesting a positive feedback role for Ca\(^{2+}\) on receptor-mediated In(1,4,5)P\(_3\) production. Dual imaging experiments indicated that receptor-stimulated In(1,4,5)P\(_3\) production was not sensitive to the full dynamic range of changes in [Ca\(^{2+}\)]\(_i\), observed in individual cells. Thus NA-mediated In(1,4,5)P\(_3\) production appeared to be enhanced only by changes in [Ca\(^{2+}\)]\(_i\), between basal and ~200 nM. This limited Ca\(^{2+}\) sensitivity suggests that only transient base-line Ca\(^{2+}\) oscillations may be capable of producing an oscillatory In(1,4,5)P\(_3\) response. Sinusoidal Ca\(^{2+}\) oscillations, as they occur on a raised plateau level of intracellular Ca\(^{2+}\), may be beyond the Ca\(^{2+}\)-sensitive range and hence be unable to stimulate In(1,4,5)P\(_3\) oscillations.

PDBu-mediated down-regulation of PKC was shown to enhance NA-mediated In(1,4,5)P\(_3\) production in CHO\(\alpha_{1B}\)/M\(_3\) cells. Importantly, both In(1,4,5)P\(_3\) and Ca\(^{2+}\) oscillations were still observed in PKC down-regulated cells, albeit at concentrations of NA that were sub-threshold in untreated cells. Thus PKC-mediated feedback inhibition did not appear essential for the oscillatory responses observed. Furthermore, although PKC-mediated phosphorylation of \(\alpha_{1B}\) receptors has been demonstrated using phorbol esters to activate PKC, it is notable that these sites differ from those phosphorylated in response to agonist stimulation of the receptor (23). In addition, biochemical studies suggest that G-protein-coupled receptor kinases, and not PKC, are responsible for homologous phosphorylation and desensitization of the \(\alpha_{1B}\) receptor (23–25). It would therefore appear unlikely that NA-induced In(1,4,5)P\(_3\) oscillations occur via a dynamic receptor phosphorylation-uncoupling mechanism mediated by PKC. Although this differentiates the response from those observed with activation of mGluR5a (10, 12), it should be noted that PKC-mediated phosphorylation...
reactions clearly alter NA-induced Ins(1,4,5)P₃ production (see Figs. 4, 7, and 8). Although this may reflect an action of PKC on other components of the Ins(1,4,5)P₃ signaling cascade (23), it may also reflect an alteration in the basal phosphorylation state of the α₁n receptor. Thus, treatment of rat-1 cells with staurosporine or Ro 31-8220 has been shown to reduce basal phosphorylation of recombinant α₁n receptors (26). The addition of staurosporine, or pretreatment with PDBu, in this study may therefore enhance receptor signaling by reducing basal rather than agonist-mediated phosphorylation of the α₁n receptor. Indeed it is interesting to speculate that the poor efficacy of Na⁺ that has been reported in CHO cells (28, 32, 33), which is comparable with the results presented in this current study. The results presented here suggest that for α₁B and M₄ receptor activation in CHO cells, changes in intracellular Ca²⁺ regulate agonist-mediated responses and produce Ins(1,4,5)P₃ oscillations. Whether the feedback effect of Ca²⁺ reflects enhancement of Go₃-mediated PLC-β isoforms or direct Ca²⁺ activation of PLC-δ is not clear. Direct activation of PLC-δ might be the mechanism by which Ca²⁺ entry, in the absence of activated G-proteins, stimulated Ins(1,4,5)P₃ production in Purkinje cells (34). However, PLC-δ activity appears sensitive to a greater range of Ca²⁺ concentration (0.1–10 μM) in permeabilized HL-60 cells (35) than PLC-β. Hence, the limited sensitivity of PLC activity observed in this current study appears more in keeping with an effect on PLC-β isoforms. It is of interest to note from Fig. 5, A and B, that the secondary, plateau phase of Ins(1,4,5)P₃ production appears particularly sensitive to regulation by Ca²⁺. Whether this indicates recruitment of a Ca²⁺-sensitive PLC, not present during initial periods of agonist stimulation, is as yet unclear.

The question therefore arises as to the role of Ca²⁺ feedback-mediated Ins(1,4,5)P₃ oscillations in the highly regulated Ca²⁺ release process. Activation of InsP₃Rs is under the dual control of Ins(1,4,5)P₃ and Ca²⁺ (reviewed in Refs. 36 and 37), and the temporal nature of these interactions is highly important. Thus, Ins(1,4,5)P₃ binding uncovers the stimulatory Ca²⁺-binding site of InsP₃Rs, allowing Ca²⁺ to facilitate further Ca²⁺ mobilization. In contrast, Ca²⁺ binding to InsP₃Rs in the absence of bound Ins(1,4,5)P₃ inhibits the receptor (38). Therefore, increasing the levels of Ins(1,4,5)P₃, either via positive feedback onto PLC or indeed via dynamic receptor regulation (10, 12), may partially overcome this "lateral inhibition" of InsP₃Rs and hence further increase Ca²⁺ release. It should be noted that this does not represent an uncontrolled positive feedback loop onto PLC activation. The limited sensitivity of PLC activity to Ca²⁺ implies that further Ca²⁺ increases will still cause lateral inhibition and hence prevent Ins(1,4,5)P₃ from releasing Ca²⁺. The intrinsic inactivation of the InsP₃R and dissociation of Ins(1,4,5)P₃ (39) reduces [Ca²⁺], and hence Ins(1,4,5)P₃ production. The delay between Ca²⁺ oscillations, which is controlled by the strength of agonist stimulus, is likely to reflect the amount of Ins(1,4,5)P₃ being produced in combination with some intrinsic property of the regenerative CICR process such as intracellular store refilling.

In summary, Ca²⁺ oscillations in CHO cells are capable of stimulating transient increases in Ins(1,4,5)P₃ via a positive feedback effect on PLC. Due to the demonstrated sensitivity of PLC activity to Ca²⁺, only Ca²⁺ increases of a transient baseline spiking nature would be expected to cause positive feedback. Hence sinusoidal Ca²⁺ oscillations may occur in the absence of any oscillatory Ins(1,4,5)P₃ signal (12). Although Ca²⁺ feedback-mediated Ins(1,4,5)P₃ oscillations appear smaller than those caused by dynamic receptor uncoupling, this work demonstrates that both positive and negative feedback pathways can lead to the same result, namely an oscillatory Ins(1,4,5)P₃ response. Positive feedback onto PLC is likely to occur in all situations where Ca²⁺ is oscillating in the appropriate range, and there is sufficient Go₃-mediated activation of PLC. In contrast, PKC-mediated dynamic receptor uncoupling is likely to occur only where the receptor is a suitable substrate for PKC.

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InS₃ Oscillations
Role of Ca²⁺ Feedback on Single Cell Inositol 1,4,5-Trisphosphate Oscillations Mediated by G-protein-coupled Receptors
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