Intracellular Ca^{2+} oscillations generated via the Ca^{2+}-sensing receptor are mediated by negative feedback by PKCα at Thr[888]

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[1]*Division of Digestive Diseases, Department of Medicine, Center for Ulcer Research and Education: Digestive Diseases Research Center David Geffen School of Medicine and Molecular Biology Institute, University of California, Los Angeles, California; and* [2]*Institute of Immunology, Genetics and Metabolism, Consejo Nacional de Investigaciones Científicas y Tecnicas, Universidad de Buenos Aires, Buenos Aires, Argentina*

Young SH, Rey O, Sinnett-Smith J, Rozengurt E. Intracellular Ca^{2+} oscillations generated via the Ca^{2+}-sensing receptor are mediated by negative feedback by PKCα at Thr[888]. *Am J Physiol Cell Physiol* 306: C298–C306, 2014. First published December 11, 2013; doi:10.1152/ajpcell.00194.2013.—To clarify the mechanism(s) underlying intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations induced by an elevation in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) via the extracellular Ca^{2+}-sensing receptor (CaR), we analyzed the pattern of $[Ca^{2+}]_i$ response in multiple (2,303) individual HEK-293 cells transfected with the human CaR. An increase in the $[Ca^{2+}]_e$ from 1.5 to 3 mM produced oscillatory fluctuations in $[Ca^{2+}]_i$ in 70% of the cell population. To determine the role of PKC in the generation of $[Ca^{2+}]_i$ oscillations, cells were exposed to increasing concentrations (0.5–5 μM) of the preferential PKC inhibitor Ro-31-8220 before stimulation by extracellular Ca^{2+}. Ro-31-8220 at 3–5 μM completely eliminated the $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations and transformed the pattern to a peak and sustained plateau response. Treatment with other broad PKC inhibitors, including GFI or Gö6983, produced an identical response. Similarly, treatment with Ro-31-8220 or GFI eliminated $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in colon-derived SW-480 cells expressing the CaR. Treatment with inhibitors targeting classic PKCs, including Gö6976 and Ro-32-0432 as well as small interfering RNA-mediated knockdown of PKCα, strikingly reduced the proportion of cell displaying $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations. Furthermore, none of the cells analyzed expressing a CaR mutant in which the major PKC phosphorylation site Thr[888] was converted to alanine (CaR$_{T888A}$) showed $[Ca^{2+}]_i$ oscillations after CaR activation. Our results show that $[Ca^{2+}]_i$ oscillations induced by activation of the CaR in response to an increase in extracellular Ca^{2+} or exposure to the calcimimetic R-568 result from negative feedback involving PKCα-mediated phosphorylation of the CaR at Thr[888].

G protein-coupled receptor; single cell Ca^{2+} imaging; PKC inhibitor Ro-31-8220; PKC downregulation; mutant CaR$_{t888a}$

OSCILLATORY CHANGES IN INTRACELLULAR Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to receptor stimulation are fundamental mechanisms of signaling in excitable and nonexcitable cells implicated in the regulation of a variety of targets and processes (13), including Ca^{2+}- and calmodulin-dependent protein kinase II (45), conventional protein kinase C (PKC) isoforms (32, 37), mitochondrial function (12, 18), and nuclear transcriptional activity leading to differential gene expression (21, 22, 25, 55). Although Ca^{2+} oscillations have attracted intense interest for several decades, the underlying mechanisms regulating their amplitude, frequency, and duration remain incom-

pletely understood (13). Most models proposed to explain the mechanism by which $[Ca^{2+}]_i$ oscillations are generated in response to G protein-coupled receptor (GPCR) activation are based broadly on the periodic production of inositol 1,4,5-trisphosphate ($InsP_3$) produced by dynamic uncoupling of G_q/phospholipase C (PLC) or on the biphasic effects of $[Ca^{2+}]_i$ on the $InsP_3$ receptor (15, 47). However, definitive evidence identifying the mechanism(s) involved is available in only few instances.

The extracellular Ca^{2+}-sensing receptor (CaR), a member of the C family of GPCRs originally identified in the parathyroid gland, is expressed in many tissues and organs (4, 5), including the gastrointestinal tract (33). Accordingly, the CaR not only maintains Ca^{2+} homeostasis but also plays multiple diverse roles in the control of normal and abnormal cell function (16, 19, 34, 42). A number of studies of CaR activation in individual living cells have shown that $[Ca^{2+}]_i$ oscillates upon stimulation of CaR by an elevation in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) within a physiological range (2, 10, 35–37, 53, 54). We proposed that intracellular $[Ca^{2+}]_i$ oscillations induced by activation of the CaR in response to an increase in $[Ca^{2+}]_e$ result from negative feedback involving cyclic PKC-mediated phosphorylation of the CaR at the inhibitory residue Thr[888] (37, 54) but the PKC isoform(s) involved was not identified. Subsequently, Ward and colleagues (10) reported that $[Ca^{2+}]_i$ oscillations were still observed in a significant number of cells expressing the CaR$_{T888A}$ mutant and stimulated by a small increase in $[Ca^{2+}]_e$. These investigators concluded that phosphorylation of CaR at Thr[888] is not the exclusive determinant of CaR-induced $[Ca^{2+}]_i$ oscillations (10) and proposed the existence of other phosphorylation sites or mechanisms. In fact, additional phosphorylation sites for PKC (Ser[895]) and PKA (Ser[899]) have been suggested in the COOH-terminal region of the CaR (1, 46). These considerations prompted us to reexamine whether PKC-mediated phosphorylation of the CaR at Thr[888] is both necessary and sufficient for generating $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations or additional mechanisms, including protein kinases other than PKC isoforms and phosphorylation sites other than Thr[888], are also involved.

To identify the mechanisms leading to $[Ca^{2+}]_i$ oscillations induced by activation of the CaR by increases in $[Ca^{2+}]_e$ or exposure to the calcimimetic R-568, we analyzed the pattern of $[Ca^{2+}]_i$ response (oscillatory or nonoscillatory) evoked by submaximal concentrations of $[Ca^{2+}]_e$ in 2,303 individual HEK-293 cells transfected with wild-type or mutant human CaR. We used multiple approaches, including PKC inhibitors with different structure and specificity, prolonged exposure to

phorbol-12,13-dibutyrate (PDBu) to downregulate classic and novel PKCs, small interfering (si)RNA-mediated depletion of PKCα, and a CaR mutant in which the major PKC phosphorylation site (Thr[888]) was altered by substitution of alanine for threonine (CaR$_{T888A}$). Our results support the notion that PKC-mediated phosphorylation of the CaR at Thr[888] is necessary and sufficient for generating $[Ca^{2+}]_i$ oscillations in response to an increase in $[Ca^{2+}]_e$ and identify PKCα as the major PKC isoform essential in this process.

MATERIALS AND METHODS

Materials. Fura 2-AM, DMEM, and HBSS were obtained from Invitrogen (Carlsbad, CA). Ro-31-8220, Ro-32-0432, GFI (bisindolylmaleimide I), GFV (bisindolylmaleimide V), Go6983, Go6976, and HEPES were obtained from Calbiochem (San Diego, CA). Kb NB 142–70 was obtained from Tocris (Ellsville, MO). LY317615 and AEB071 {3-(1H-indol-3-yl)-4-[2-(4-methylpiperazin-1-yl)quinazolin-4-yl]-1H-pyrrole-2,5-dione} were obtained from Selleck Chemicals (Houston, TX). H-89 and 539654 were obtained from EMD Millipore (Billerica, MA). R-568 was obtained from R&D Systems (Minneapolis, MN). HBSS was supplemented with 0.2 mM $CaCl_2$ and 20 mM HEPES. In experiments with R-568, bovine serum albumin was added at 0.5%. The final Ca^{2+} concentration and pH were 1.5 mM and 7.4, respectively.

Cell culture and transfection. HEK-293 cells were maintained in culture in DMEM supplemented with 10% FBS as described previously (30, 53). For experimentation, cells were plated onto 18-mm diameter glass coverslips inside 35-mm plastic dishes, where they could be dually transiently transfected with a plasmid encoding the human CaR or a mutant CaR receptor (CaR$_{T888A}$) and as a marker a plasmid encoding the sequence for DsRed (53). A line of HEK cells stably expressing the CaR, developed in our laboratory, was also used (37). SW-480 cells, a cell line derived from human colorectal adenocarcinoma in which the expression of endogenous CaR is nondetectable by RT-PCR, were transiently cotransfected with a plasmid encoding the human CaR (pCR3.1-CaR) and a plasmid encoding a red fluorescent protein (pDsRed-Express) to facilitate the identification of the transfected cells. After 16 h, the cultures were loaded with the Ca^{2+} indicator as described below.

The generation of the plasmid encoding the CaR$_{T888A}$ mutant was previously described (54). In experiments using siRNA, pooled siRNA duplexes were purchased from Invitrogen (Carlbad, CA). PKCα siRNA pools were designed to target the mRNA of PKCα (GenBank Accession No. NM_002737.2) and consist of two different duplexes oligo1 (UUCAUCUUCACCAAAUGGAGGC) and oligo2 (GCCUCCAUUUGAAGAUGAA). With the use of the reverse transfection method, the siRNA pool obtained was mixed with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol and added to 35-mm dishes. Control transfections were carried out with Dharmacon (Lafayette, CO) siCONTROL nontargeting siRNA four-oligo pool (catalog no. D-001206–13). At 3 days after transfection, cells were used for experiments and subsequent Western blot analysis to verify depletion of PKCα protein.

Immunoblotting and detection of PKCα. HEK 293 cells were lysed in 2× SDS-PAGE sample buffer (20 mM Tris·HCl, pH 6.8, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, and 10% glycerol) and boiled for 10 min. After SDS-PAGE, proteins were transferred to Immobilon-P membranes. The transfer was carried out at 100 V, 0.4 A at 4°C for 4 h using a Bio-Rad transfer apparatus. The transfer buffer consisted of 200 mM glycine, 25 mM Tris, 0.01% SDS, and 20% CH_3OH. For detection of protein, membranes were blocked using 5% nonfat dry milk in PBS (pH 7.2) and then incubated for ≥2 h with the PKCα antibody (Cell Signaling Technologies) diluted in PBS containing 3% nonfat dry milk. Primary antibody bound to immunoreactive bands was visualized by enhanced chemiluminescence detection

with horseradish peroxidase-conjugated anti-mouse, anti-rabbit antibody, and a FUJI LAS-4000 Mini Luminescent Image Analyzer.

Measurement of $[Ca^{2+}]_i$. Cells on coverslips were removed from the incubator, washed twice with saline, and then incubated in saline containing 5 μM fura 2-AM for 1 h at 37°C. Coverslips were then washed with saline and mounted in an experimental chamber (volume: 0.5 ml) perfused (1 ml/min) with heated saline at 37°C. The chamber in turn was placed on the stage of an inverted microscope (Axio Observer.A1) to which was attached a digital camera (AxioCam MRm) and operated with associated software (AxioVision, all components Carl Zeiss, Thornwood, NY). Ratio images (340 nm divided by 380 nm) were obtained in ~1 s^{-1}, and the average ratio values from small regions (10 μm²) from each cell were stored for offline analysis. Ratio values were calibrated and converted to Ca^{2+} concentrations by using a series of Ca^{2+} buffers containing fura 2 (calcium calibration kit; Molecular Probes). Data are presented as means ± SE unless otherwise stated.

Statistics. Data are reported as percentage of cells responding in a defined category (i.e., oscillating or nonoscillating cells). Bars on graphs with percentages are 95% confidence interval for a proportion (Instat, La Jolla, CA). Bars where confidence intervals were not overlapping with control confidence intervals are marked with an asterisk.

RESULTS

$[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations: dose-response relationships. To examine the mechanism(s) underlying $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations via CaR, we analyzed the pattern of $[Ca^{2+}]_i$ response in individual HEK-293 cells transfected with the human CaR, an extensively used model system in studies of CaR regulation. Cells were loaded with the fluorescent Ca^{2+} indicator fura 2-AM and incubated in the presence of medium containing 1.5 mM $[Ca^{2+}]_e$. Intracellular Ca^{2+} imaging revealed that most transfected cells exhibited a stable $[Ca^{2+}]_i$ with only a small proportion of cells (3–5%) exhibiting spontaneous oscillatory activity. In line with previous studies, an increase in the $[Ca^{2+}]_e$ from 1.5 to 3 mM produced a rapid elevation in $[Ca^{2+}]_i$ followed by striking oscillatory fluctuations in $[Ca^{2+}]_i$ (Fig. 1A). As shown in Fig. 1, this pattern was observed in 74% of the cell population ($n = 756$ cells). Most other cells (24%) displayed a rapid peak and plateau response (Fig. 1B), which in some cases was preceded or followed by $[Ca^{2+}]_i$ spikes of diminishing amplitude (Fig. 1C). Cells were classified as nonoscillatory "plateau" if the amplitude of the secondary peaks was <10% of the amplitude of the predominant peak. The plateau response was also seen with very small increases in $[Ca^{2+}]_e$ as shown in the lower trace of Fig. 1B. Similar results were obtained in HEK-293 cells transiently transfected with a plasmid encoding the human CaR or in cells stably expressing the CaR. In contrast, an increase in $[Ca^{2+}]_e$ from 1.5 to 3 mM did not induce any $[Ca^{2+}]_i$ oscillations in nontransfected HEK-293 cells or in cells transfected with vector.

We also analyzed the pattern of $[Ca^{2+}]_i$ response in individual HEK-293 cells transfected with the human CaR but challenged with different concentrations of extracellular Ca^{2+}. The results are displayed in Fig. 2. An increase in the $[Ca^{2+}]_e$ from 1.5 mM to either 2.25 mM or 3 mM produced an elevation in $[Ca^{2+}]_i$ followed by oscillatory fluctuations in $[Ca^{2+}]_i$ in most responding cells in the populations (Fig. 2A). In contrast, the majority of responding cells (90%) displayed a rapid peak and plateau response when the $[Ca^{2+}]_e$ was elevated from 1.5 mM
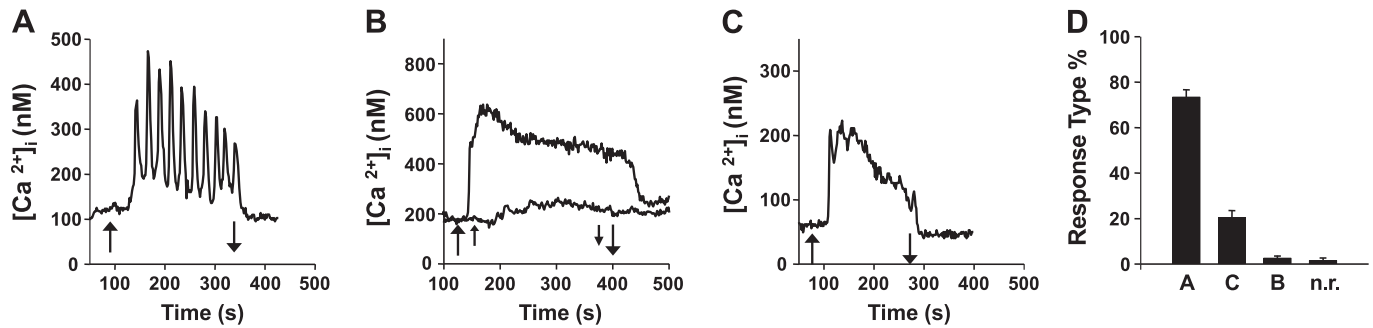
Fig. 1. Patterns of intracellular of Ca^{2+} signaling in Ca^{2+}-sensing receptor (CaR)HEK-293 cells. Starting from a resting concentration of 1.5 mM $CaCl_2$, CaRHEK-293 cells were perfused with a solution containing 3 mM $CaCl_2$ (upward arrow) for 200–300 s as indicated before return to perfusion containing 1.5 mM $CaCl_2$ (downward arrow). A: typical tracing of an oscillatory response induced by increasing extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$). B: peak and plateau response without intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations. Bottom trace: perfusion with 2.25 mM $CaCl_2$. C: in some cases the pattern shown in B was preceded by $[Ca^{2+}]_i$ spikes of diminishing amplitude. D: percentage of cells with each type of response (756 cells; n.r., not responding). In subsequent figures, the results are presented as the percentage of oscillating and nonoscillating cells in the population.

to either 5 or 10 mM (Fig. 2A). The results indicate that a small increase (0.75–1.5 mM) in $[Ca^{2+}]_e$ elicits oscillatory fluctuations in $[Ca^{2+}]_i$ via the CaR whereas a large increase (3.5–8.5 mM) in $[Ca^{2+}]_e$ induces a peak and plateau response in most cells.

To further examine the pattern of $[Ca^{2+}]_i$ response in response to increasing $[Ca^{2+}]_e$ we also determined the average frequency of Ca^{2+}-induced oscillations (Fig. 2B) and the amplitude of the peak in the cells that responded in a nonoscillatory manner (Fig. 2C). The average frequency of Ca^{2+}-induced oscillations mediated by the CaR increased from 2 ± 0.1 min^{-1} (SE) to 3.3 ± 0.2 min^{-1} (SE) when the $[Ca^{2+}]_e$ was increased from 1.5 to 2.25 and 3 mM, respectively (Fig. 2B). A further increase in $[Ca^{2+}]_e$ to 5 or 10 mM induced a slight increase in the frequency of oscillations in the few cells that responded with this pattern. The amplitude of the $[Ca^{2+}]_i$ peak of cells responding with a nonoscillatory pattern showed a striking dose-response relationship, as illustrated in Fig. 2C. The results displayed in Fig. 2 indicate that the pattern of $[Ca^{2+}]_i$ response in individual HEK-293 cells transfected with the human CaR is sharply dose dependent. A small (physiological) increase in the concentration of extracellular Ca^{2+} elicited oscillatory $[Ca^{2+}]_i$ fluctuations in most responding cells whereas a large increase in extracellular Ca^{2+}, outside the range of homeostatic changes, resulted in a peak and plateau response in the vast majority of the cells. Consequently, we

focused our subsequent experiments in defining the mechanism of the oscillatory response via the CaR.

Broad-spectrum PKC inhibitors eliminate $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations. The multigene PKC family consists of classic (α, βI, βII, and γ), novel (δ, ϵ, η, and θ) and atypical (ζ and ι) isoforms (11). To determine the role of PKC in the generation of $[Ca^{2+}]_i$ oscillations via the CaR, cells were exposed to increasing concentrations (0.5–5 μM) of the potent, cell-permeable PKC family inhibitor Ro-31-8220, also known as bisindolylmaleimide IX (52), for 1 h before stimulation by an increase in extracellular Ca^{2+}. Exposure to Ro-31-8220 strikingly decreased the proportion of single responding cells displaying $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations and produced a corresponding increase in the fraction of cells responding with a nonoscillatory pattern in a concentration-dependent manner (Fig. 3). Treatment with Ro-31-8220, at a concentration as low as 0.5 μM, reduced the proportion of cells exhibiting oscillations from 70% in the control to ~15% in the treated cells. Cell exposure to Ro-31-8220 at higher concentrations (3–5 μM) completely eliminated the $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations and transformed the pattern to a peak and sustained plateau response. It is noteworthy that the total number of cells responding to an elevation of $[Ca^{2+}]_e$ was not altered by prior exposure to Ro-31-8220, at any of the concentrations tested (Fig. 3, triangles).
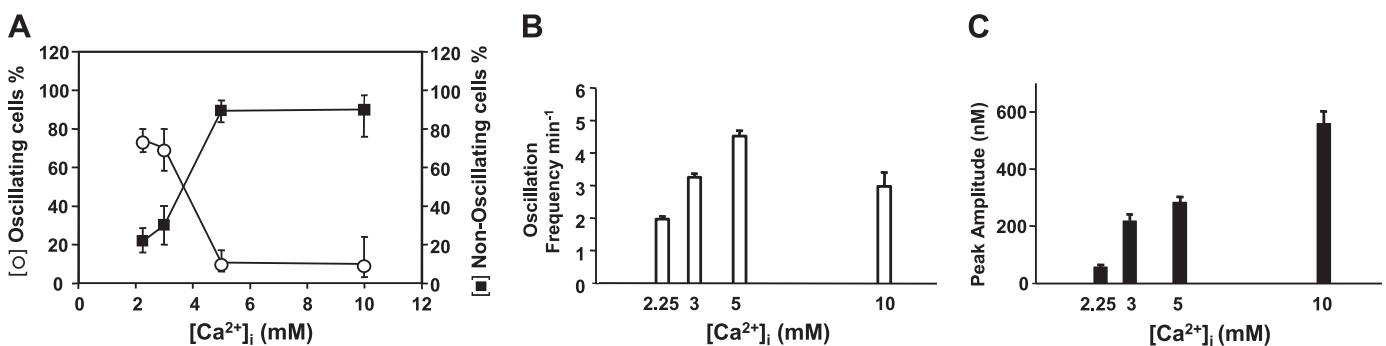

Fig. 2. Fraction of CaRHEK-293 cells showing oscillations decreases with increasing external calcium concentration. Cells were studied after increasing the $[Ca^{2+}]_e$ to 2.25, 3, 5, or 10 mM from the resting concentration of 1.5 mM. A: percentage of oscillating and nonoscillating cells. B: $[Ca^{2+}]_i$ oscillation frequency as a function of extracellular Ca^{2+} concentration. C: amplitude of the peak $[Ca^{2+}]_i$ in cells showing nonoscillatory pattern in response to an increase in extracellular Ca^{2+} concentration. Graphs reflect analysis based on 495 cells.
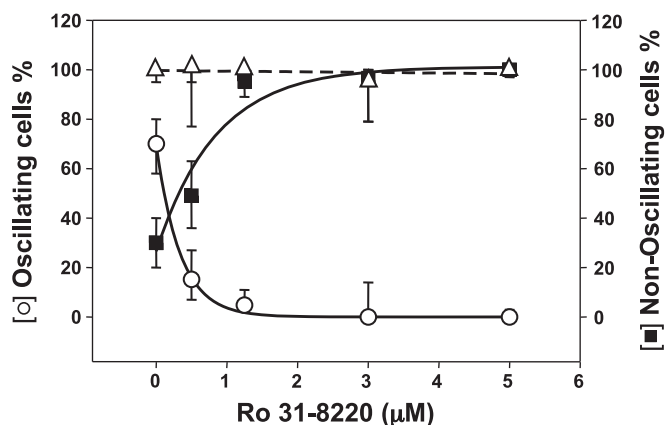
Fig. 3. Exposure to the PKC inhibitor Ro-31-8220 eliminates $[Ca^{2+}]_i$ oscillations in a dose-dependent manner. CaRHEK-293 cells were pretreated for 1 h with increasing concentrations of the PKC inhibitor Ro-31-8220. The cultures were then perfused with a solution containing 3 mM $CaCl_2$. Results show the percentage of cells showing $[Ca^{2+}]_i$ oscillations (○) or displaying a nonoscillatory response (■, type B + type C of Fig. 1). Also shown is the percentage of total responding cells (patterns A + B + C) in the population exposed to increasing concentrations of Ro-31-8220 (△).

We next determined whether the conversion of the $[Ca^{2+}]_i$ response from oscillatory to nonoscillatory elicited by an elevation of $[Ca^{2+}]_e$ can be also induced by exposure to other inhibitors of the PKC family. Treatment with increasing concentrations of GFI (also known as GF 109203X or bisindolylmaleimide I), a different PKC inhibitor (48), produced a similar conversion of the response pattern from $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations to peak and sustained plateau. At a concentration of 3.5 μM, treatment with GFI completely eliminated the oscillatory pattern of response in the CaRHEK-293 population (Fig. 4A). In contrast, treatment with an inactive analog, GFV (also known as bisindolylmaleimide V), did not have any discernable effect. Similar results obtained in HEK-293 cells transiently transfected with a plasmid encoding the human CaR or in cells stably expressing the CaR. To further substantiate the results obtained with Ro-31-8220 and GFI, we also determined the effect of Gö6983, another inhibitor of all isoforms of the PKC family (17). As shown in Fig. 4, A and B, exposure of CaRHEK-293 cells to Gö6983 completely eliminated the $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in these cells and transformed the pattern to a peak and sustained plateau response. To verify the PKC inhibitory activity of Ro-31-8220, GFI, and Gö6983 in CaRHEK-293 cells, at the concentrations used here, we determined whether exposure to these compounds prevented $[Ca^{2+}]_e$-evoked phosphorylation of MARCKS, a well-established substrate of PKCs (40). Treatment with these inhibitors completely suppressed $[Ca^{2+}]_e$-evoked MARCKS phosphorylation in CaRHEK-293 (data not shown).

As pharmacological inhibitors such as Ro-31-8220, GFI, or Gö6983 may have targets other than PKC, we also decreased the protein expression of PKCs via phorbol ester-mediated downregulation. Specifically, long-term exposure of cells to phorbol esters leads to a marked and progressive reduction in the level of conventional PKCs (PKCs α, β, and γ) and novel PKCs (δ, ε, η, and θ), which require diacylglycerol (DAG) for their activation but does not affect the level of protein expression of the atypical isoforms of PKC. For this approach, CaRHEK-293 cells were treated for 48 h with 100 nM PDBu

and subsequently stimulated by an elevation of $[Ca^{2+}]_e$. As shown in Fig. 4, A and B, PKC downregulation strikingly decreased the proportion of single cells displaying $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations and produced a corresponding increase in the fraction of cells responding with a nonoscillatory pattern. Collectively, these results substantiated the notion that PKC activity is essential for the generation of $[Ca^{2+}]_e$-induced $[Ca^{2+}]_i$ oscillations.

Broad-spectrum PKC inhibitors eliminate $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in SW-480 cells. The CaR is increasingly implicated in the regulation of multiple cellular functions in the gastrointestinal tract. Using colon-derived cells expressing the CaR, including SW-480, we showed that an elevation of $[Ca^{2+}]_e$ promoted striking intracellular Ca^{2+} oscillations in these cells (35). To determine the role of PKC in $[Ca^{2+}]_i$ oscillations elicited in response to CaR stimulation in human colon-derived epithelial cells, SW-480 cells were transiently cotransfected with a plasmid encoding the human CaR (pCR3.1-CaR) and a plasmid encoding a red fluorescent protein (pDsRed-Express) to facilitate the identification of the transfected cells. In agreement with our previous results, single cell imaging of fura-2 loaded SW-480 cells expressing the CaR revealed that a rise in $[Ca^{2+}]_e$ from 1.5 to 3.0 mM stimulated an increase in $[Ca^{2+}]_i$ in most cells in the population (84%; n = 64 cells). Further analysis of SW-480 cells expressing the CaR indicated that a rise in $[Ca^{2+}]_e$ induced $[Ca^{2+}]_i$ oscilla-
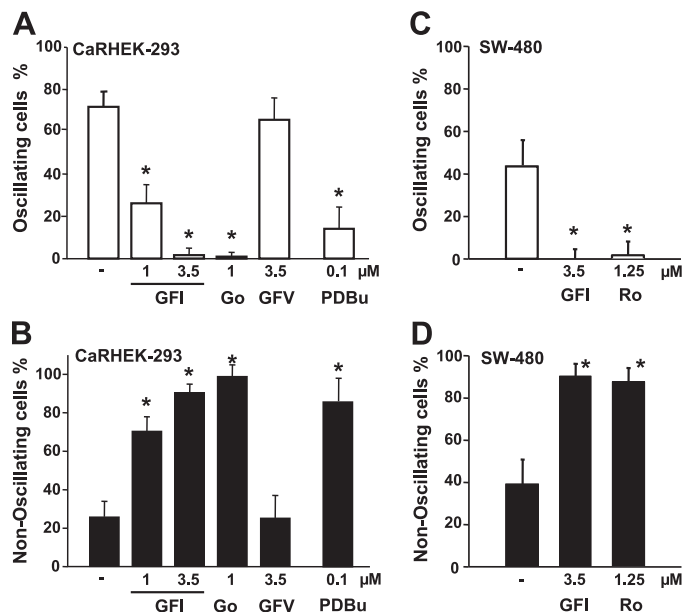

Fig. 4. Effect of PKC inhibitors GFI and Gö6983 on $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in CaRHEK-293 and SW-480 cells. A and B: CaRHEK-293 cells were treated with GF 109203X (GFI) at either 1 μM or 3.5 μM, GFV at 3.5 μM, and Gö6983 at 1 μM (Go) for 1 h, as indicated. Some cultures were treated with 100 nM phorbol-12,13-dibutyrate (PDBu) for 48 h. In all cases, cells were stimulated by raising $[Ca^{2+}]_e$ to 3.0 mM. A: bars represent the percentage of cells showing $[Ca^{2+}]_i$ oscillations. B: percentage of cells showing a nonoscillatory response. C and D: effect of PKC inhibitors GFI and Ro-31-8220 on $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in SW-480 cells expressing the CaR. SW-480 cells expressing the CaR were treated with the PKC inhibitors GF 109203X (GFI) at 3.5 μM or Ro-31-8220 (Ro) at 1 μM for 1 h before stimulation by raising $[Ca^{2+}]_e$ to 3.0 mM. C: bars represent the percentage of cells showing $[Ca^{2+}]_i$ oscillations. D: percentage of cells showing a nonoscillatory response. *Confidence intervals were not overlapping with control confidence intervals.

tions in 44% of the population. Analysis of individual cells revealed that treatment with either Ro-31-8220 at 1.25μM ($n = 57$ cells) or GFI at 3.5 μM ($n = 83$ cells) completely eliminated the $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in CaR-expressing SW-480 cells and transformed the pattern to a nonoscillatory response (Fig. 4, *C* and *D*). It is noteworthy that the total number of cells responding to an elevation of $[Ca^{2+}]_e$ was not altered by prior exposure to either Ro-31-8220 or GFI at the concentrations tested. The results presented in Figs. 3 and 4 substantiated the notion that PKC activity is essential for the generation of $[Ca^{2+}]_i$ oscillations through the CaR in different cell types.

Preferential inhibitors of classic PKCs eliminate $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations. Our next objective was to identify the PKC isoform(s) involved in the negative feedback required for the generation of the $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations. To determine whether classic PKCs mediate feedback inhibition of CaR signaling, we used Gö6976, which is a preferential inhibitor of these PKC isoforms. For example, Gö6976 potently inhibits PKCα (IC_{50} = 2.3 nM) but does not inhibit PKCδ, -ε, and -ζ isozymes, even at micromolar concentrations (27). Treatment of CaRHEK-293 cells with Gö6976 (1–3 μM) for 1 h eliminated the $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations and transformed the pattern to a peak and sustained plateau response in a dose-dependent manner (Fig. 5). Similarly, cell exposure to the PKC inhibitor Ro-32-0432 (at 1.0–2.5 μM), which exhibits a 10-fold selectivity for PKCα (50), eliminated the $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations and transformed the pattern to a peak and sustained plateau response (Fig. 5). In contrast, inhibitors preferentially targeting PKCβ (e.g., 539654 and Ly 3176155), at concentrations that were inhibitory in other systems (7, 8), had little effect on $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations (Fig. 5.)

AEB071 is a novel and potent inhibitor of classical and novel PKC isotypes (44). This agent has robust inhibitory activity on PKCα, PKCβ, and PKCθ and lesser inhibitory activity on PKCδ, PKCε, and PKCη. AEB071 did not inhibit ~200 other kinases involved in cell signaling (44). As shown in Fig. 5, exposure of CaRHEK-293 cells to AEB071 at 2.5 μM, markedly reduced $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in these cells and transformed the pattern of $[Ca^{2+}]_i$ signaling in these cells to a peak and sustained plateau response. We also verified that AEB071 prevented MARCKS phophorylation, a marker of PKC activity, in response to an increase in $[Ca^{2+}]_e$ in CaRHEK-293 cells (data not shown).

The members of the PKD family are major downstream targets of PKCs in many cell types (41). Given that PKCs are upstream kinases in PKD activation and Gö6976 directly inhibits PKDs, we also examined the effect of a direct inhibitor of the PKD family on $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations. As shown in Fig. 5, exposure to kb NB142–70 did not produce any significant change in $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations. In addition to PKCs, it has been suggested that PKA also phosphorylates the CaR. We found that the potent PKA inhibitor H-89 at 20 μM did not produce any change in the proportion of cells displaying $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations (Fig. 5). The results imply that neither PKD nor PKA are implicated in generating the negative feedback required for $[Ca^{2+}]_i$ oscillatory responses induced by activation of the CaR in response to $[Ca^{2+}]_e$. In contrast, inhibition of classic PKC isoforms, especially PKCα, virtually eliminated $[Ca^{2+}]_i$ oscillations in
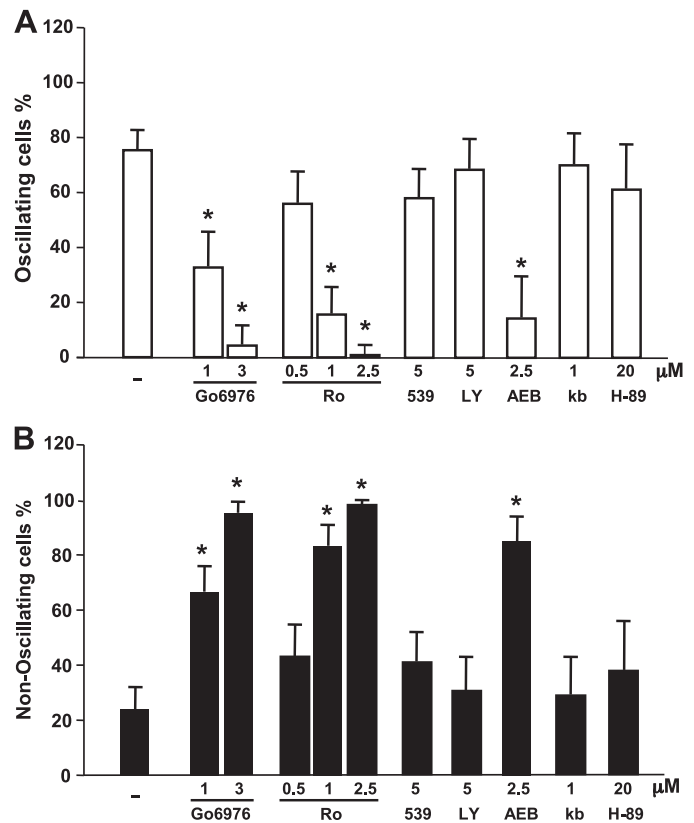

Fig. 5. Inhibitors with selectivity for classic PKCs eliminated $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations. CaRHEK-293 cells were treated for 1 h with the preferential inhibitors of classic PKCs Gö6976 (Go) and Ro-32-0432 (Ro) or inhibitors preferentially targeting PKC β, 539654 (539), Ly 3176155 (LY); an inhibitor for both classic and novel PKCs AEB071 (AEB), PKA (H-89), or PKD [kb NB 140–70 (kb)] at the indicated concentrations. Cells were then stimulated by raising $[Ca^{2+}]_e$ to 3.0 mM. *A*: open bars show percentage of cells showing oscillations. *B*: closed bars show percentage of cells with nonoscillatory responses. *Confidence intervals were not overlapping with control confidence intervals.

response to CaR activation in every cell in the population of CaR-transfected cells. The results suggest that PKCα activity is essential for the generation of sinusoidal $[Ca^{2+}]_i$ oscillations via CaR activation.

Knockdown of PKCα with siRNA reduces $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in all cells. To examine whether PKCα is the predominant isoform that mediates the negative feedback required for the generation of $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations, we depleted its expression in CaRHEK-293 via transient transfection of siRNA targeting PKCα and tested $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in the resulting PKCα-deficient cells. Initially, we verified that transfection of CaRHEK-293 cells with the siRNAs targeting PKCα caused a marked decrease in the level of protein expression of this PKC isoform (Fig. 6*B*). In contrast, transfection of the nontargeted siRNA did not produce any decrease in the level of PKCα expression in CaRHEK-293 cells (Fig. 6*B*). The salient feature of the results is that siRNA-mediated knockdown of PKCα in CaRHEK-293 cells strikingly reduced the proportion of cells displaying $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations, compared with cells transfected with nontargeted siRNA (Fig. 6, *B*, *C*, and *D*). These results identify PKCα as the predominant isoform of the PKC family that mediates the negative feedback re-
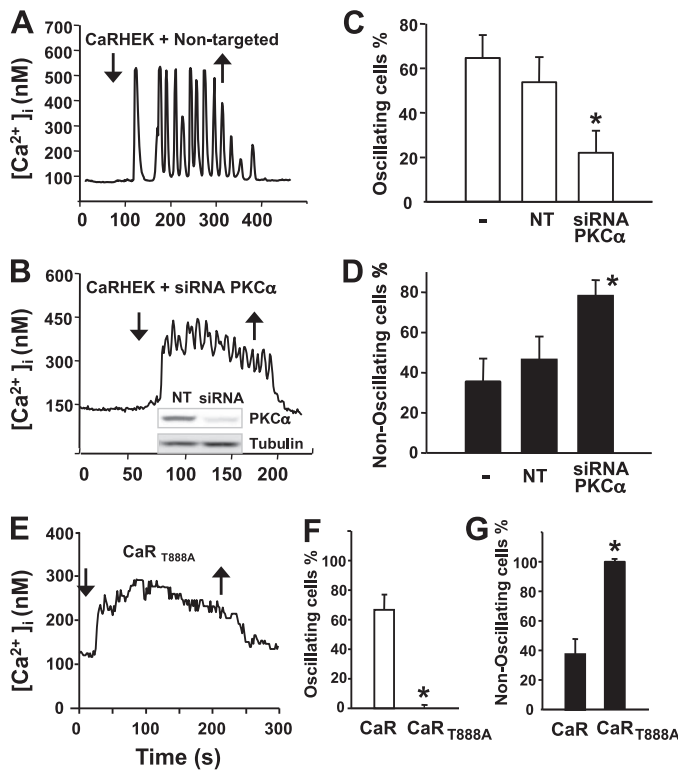
Fig. 6. *A–D*: knockdown of PKCα reduced $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in CaRHEK-293 cells. From a resting $[Ca^{2+}]_e$ of 1.5 mM, $[Ca^{2+}]_e$ was increased to 3.0 mM (downward arrows). Upward arrows show return to 1.5 mM $[Ca^{2+}]_e$. *A*: typical individual tracing of $[Ca^{2+}]_i$ vs. time from a CaRHEK-293 cell transfected with nontargeted oligonucleotides. *B*: typical $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ response in a cell transfected with siRNA targeting PKCα. *Inset*: cells lysates were analyzed by SDS-PAGE and immunoblotting with a PKCα polyclonal antibody and total β-tubulin to verify equal gel loading. *C* and *D*: data summary for all 187 analyzed cells in control (-), nontargeted (NT), and siRNA PKCα (siRNA PKCα) conditions. *E–G*: HEK-293 cells expressing CaR_T888A displayed nonoscillatory $[Ca^{2+}]_i$ responses even in response to a small increase in $[Ca^{2+}]_e$. *E*: CaRHEK-293 cells expressing wild-type CaR and HEK-293 cells expressing CaR_T888A were stimulated by raising $[Ca^{2+}]_e$ to 2.25 mM (downward arrow). Upward arrow shows return to 1.5 mM $[Ca^{2+}]_e$. *F* and *G*: bars represent the data summary for 225 cells. *Confidence intervals were not overlapping with control confidence intervals.

quired for the generation of $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in CaRHEK-293 cells.

Mutation of Thr888 of the CaR eliminates $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations in all cells. Motif-based profile-scanning programs recognized the sequence surrounding Thr[888] (FKVAARA<u>T</u>LRRSNVSRKRSS) as the best PKCα-putative phosphorylation site in the amino acid sequence of the CaR. Accordingly, previous results indicated that mutation of Thr[888] to a nonphosphorylatable amino acid either eliminated (12) or reduced (10) the number of cells displaying $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations. If CaR-mediated $[Ca^{2+}]_i$ oscillations are generated by the periodic phosphorylation of Thr[888] by PKCα, mutation of Thr[888] to a nonphosphorylatable amino acid should abolish $[Ca^{2+}]_i$ oscillations mediated by this receptor in all the cells in the population. To test this prediction, we extended our previous studies and analyzed multiple cells expressing a CaR in which Thr[888] was mutated to alanine (T888A). As shown in Fig. 6 E, HEK-293 cells expressing CaR_T888A did not show $[Ca^{2+}]_i$ oscillations after CaR activation by increases in $[Ca^{2+}]_e$ to 2.25 mM, while cells with the wild-type CaR

continued to show oscillations. A smaller increment in $[Ca^{2+}]_e$ (0.75 mM) was chosen to minimize the possibility that cells with CaR_T888A showed only increased sensitivity to $[Ca^{2+}]_e$. The $[Ca^{2+}]_i$ response consisted of a rapid rise in $[Ca^{2+}]_i$ followed by a sustained phase of elevated $[Ca^{2+}]_i$. Critically, of 225 responding cells analyzed from independent preparations that responded to an increase in $[Ca^{2+}]_e$ to 2.25 mM, all cells showed this behavior, i.e., none exhibited $[Ca^{2+}]_i$ oscillations (Fig. 6, *E-G*).

$[Ca^{2+}]_i$ oscillations in response to R-568: dependence on PKC activity. Phenylalkylamines, including R-568, are positive allosteric modulators (calcimimetics) that activate CaR by increasing the apparent affinity of CaR for $[Ca^{2+}]_e$ through binding to different sites from the orthosteric agonists (20, 31). A previous study showed that calcimimetics induce $[Ca^{2+}]_i$ oscillations in the presence of a threshold concentration of extracellular Ca^{2+} (28), but the role of PKC in mediating the oscillatory response was not examined. Here, we determined whether R-568, like $[Ca^{2+}]_e$, also induces different patterns of $[Ca^{2+}]_i$ response (i.e., oscillatory and nonoscillatory) at different concentrations. To examine this possibility, we monitored $[Ca^{2+}]_i$ in individual CaRHEK-293 cells that were exposed to increasing concentrations of R-568 (10–500 nM). As shown in Fig. 7A, exposure of the cells to 50 nM R-568 induced striking $[Ca^{2+}]_i$ oscillations in most responding cells in the population (~90%). A 10-fold increase in the concentration of R-568 fold to 500 nM (Fig. 7B) did not change the pattern of the response, as most cells in that population responded with robust $[Ca^{2+}]_i$ oscillations, characterized by an average frequency of 5.2 ± 0.2 min⁻¹ (Fig. 7C). The effect of R-568 was mediated through the CaR since this compound did not induce any detectable change in $[Ca^{2+}]_i$ in HEK cells that did not express the CaR.

The results demonstrating an important difference between $[Ca^{2+}]_e$ and R-568 in their dose-dependent effects on $[Ca^{2+}]_i$ oscillations prompted us to determine whether R-568-induced $[Ca^{2+}]_i$ oscillations also depend critically on PKC-mediated feedback. We found, for the first time, that treatment of the CaRHEK-293 cells with broad-spectrum PKC inhibitors, including GFI and Go6983, strikingly decreased the proportion of cells displaying R-568-evoked $[Ca^{2+}]_i$ oscillations and produced a corresponding increase in the fraction of cells responding with a nonoscillatory pattern, e.g., peak and plateau (Fig. 7, *D* and *E*). Our results imply that R-568 and small increase in the extracellular Ca^{2+} concentration induce $[Ca^{2+}]_i$ oscillations via a similar mechanism involving PKC.

DISCUSSION

Multiple lines of evidence indicate that the CaR plays a critical role in maintaining Ca^{2+} homeostasis in the organism (5). It is increasingly recognized that the CaR also plays multiple additional roles in the control of normal and abnormal cell function (16, 19, 34, 38, 42), including pancreatic insulin secretion (43), inflammasome activation (24, 39), β-catenin signaling (34), epithelial cell proliferation (35), metastatic cancer dissemination (3), and stem cell differentiation (38). Accordingly, the mechanisms of CaR signaling are attracting intense interest in cell regulation.

Previous studies using HEK-293 and epithelial colon cells led us to propose a model to explain the mechanism by which the CaR triggers Ca^{2+} oscillations in response to an increase in
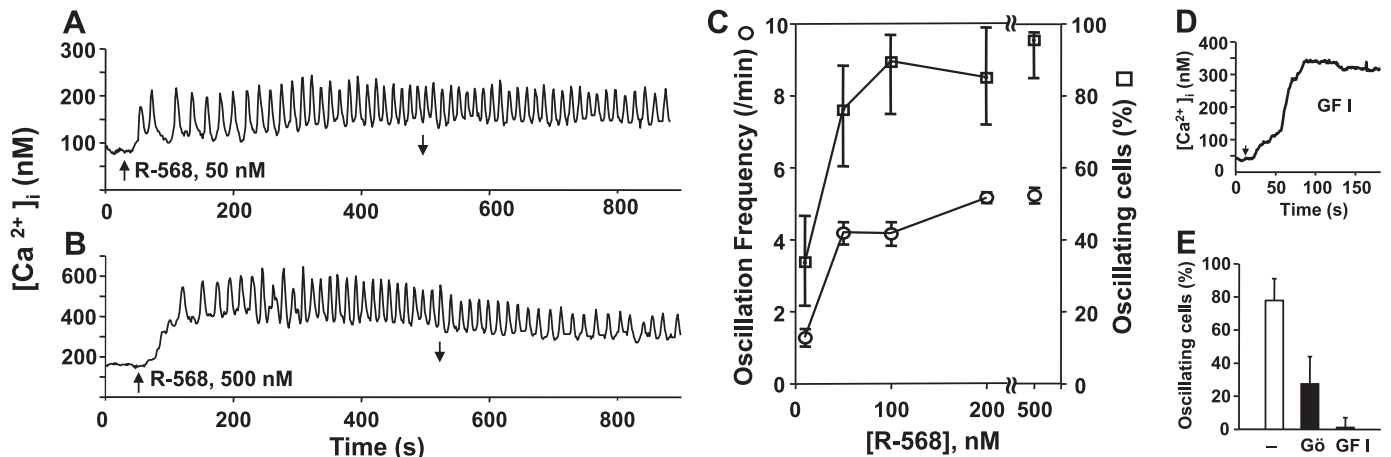
Fig. 7. Exposure to the calcimimetic R-568 induced [Ca^{2+}]$_i$ oscillations in CaRHEK-293 cells. *A*: perfusion with saline containing R-568 at 50 nM was started at the upward arrow. R-568 was removed at the time marked by a downward arrow. *B*: perfusion with R-568 at 500 nM. *C*: percentage of cells oscillating, and the oscillation frequency are plotted at R-568 concentrations of 10, 50, 100, 200, and 500 nM. Data from 145 cells. *D*: response to 50 nM R-568 (marked by upward arrow) after pretreatment with the PKC inhibitor GFI (1 h, 3.5 μM). *E*: both PKC inhibitors Gö6983 (1 h, 1 μM) and GFI inhibited the oscillatory response to 50 nM R-568 (black bars) compared with control (white bar). Data are from 101 cells.

[Ca^{2+}]$_e$ In this model, [Ca^{2+}]$_e$-induced CaR activation stimulates PLCβ, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to produce two second messengers: InsP$_3$ and DAG. InsP$_3$ binds to its receptor in the endoplasmic reticulum (ER) and induces a conformational change that leads to the mobilization of Ca^{2+} from the ER stores whereas DAG and Ca^{2+} activate classic PKCs. Activated cPKCs then phosphorylate the CaR at the inhibitory Thr^{888} providing the negative feedback needed to cause periodic InsP$_3$ production and sinusoidal [Ca^{2+}]$_i$ oscillations (37, 54). However, other phosphorylation sites and/or mechanisms underlying the generation of oscillatory response have been suggested (10). Consequently, here we expanded our previous studies to determine whether PKC-mediated phosphorylation of the CaR at Thr^{888} is both necessary and sufficient for generating [Ca^{2+}]$_e$-evoked [Ca^{2+}]$_i$ oscillations or additional mechanisms, including protein kinases other than PKC and phosphorylation sites other than Thr^{888}, are also involved. Furthermore, we also examined the role of PKC in the generation of [Ca^{2+}]$_i$ oscillations in response to R-568, a positive allosteric modulator of the CaR.

In the present study we continued to exploit HEK-293 cells as a model system to elucidate CaR-signaling mechanisms. We found that a small (physiological) increase in the concentration of extracellular Ca^{2+} (0.75–1.5 mM) elicited oscillatory [Ca^{2+}]$_i$ fluctuations in most responding cells whereas a large increase in extracellular Ca^{2+}, outside the range of homeostatic changes (e.g., 3.5–8.5 mM), resulted in a peak and plateau response in the vast majority of the cells. We conclude that the oscillatory pattern of response is of physiological interest, and accordingly, we extended previous studies defining the mechanism of the oscillatory response via the CaR. Based on the analysis of multiple (2,303) single cells, we conclude that [Ca^{2+}]$_i$ oscillations induced by activation of the CaR in response to a small increase (0.75–1.5 mM) in extracellular Ca^{2+} result from negative feedback involving PKCα-mediated phosphorylation of the CaR at Thr^{888}. Our evidence is as follows: *1*) treatment with inhibitors that suppress the activity of all isoforms of the PKC family, including Ro-31-8220, GFI, and Gö6983, or downregulation of classic and novel PKC isoforms by prolonged treatment with PDBu completely eliminated [Ca^{2+}]$_e$-evoked [Ca^{2+}]$_i$ oscillations and transformed the pattern to a peak and sustained plateau response; *2*) similar results were obtained with human colon-derived SW-480 epithelial cells, transfected with a plasmid encoding the human CaR; *3*) treatment of HEK 293 cells expressing CaR with either Gö6976, a potent inhibitor of classic PKCs (27), or Ro-32-0432, which exhibits a 10-fold selectivity for PKCα (50), also eliminated the [Ca^{2+}]$_e$-evoked [Ca^{2+}]$_i$ oscillations and transformed the pattern to a peak and sustained plateau response; *4*) crucially, siRNA-mediated knockdown of PKCα in CaR-HEK-293 cells strikingly reduced the proportion of cell displaying [Ca^{2+}]$_e$-evoked [Ca^{2+}]$_i$ oscillations, compared with cells transfected with nontargeted siRNA; these results identified, for the first time, PKCα as the PKC isoform necessary for the generation of [Ca^{2+}]$_e$-evoked [Ca^{2+}]$_i$ oscillations; and *5*) analysis of cells expressing a CaR mutant in which Thr^{888} was converted to alanine (CaR$_{T888A}$) demonstrated that all cells responded displaying a peak and sustained plateau response rather than [Ca^{2+}]$_i$ oscillations, even when in this case the cells were challenged with a small increase in [Ca^{2+}]$_e$ to compensate for an increase in agonist sensitivity of this mutant. Collectively, our results support a model that envisages [Ca^{2+}]$_i$ oscillations induced by activation of the CaR in response to a physiological increase in [Ca^{2+}]$_e$ as a consequence of negative feedback mediated by PKCα-mediated phosphorylation of the CaR at Thr^{888}.

In accord with this model, the structure of PKCα is ideally suited to mediate periodic phosphorylation of the CaR at Thr^{888} in response to repetitive spikes of [Ca^{2+}]$_i$. It is well established that the regulatory region of PKCα contains a C2 domain that binds Ca^{2+} and mediates PKCα membrane translocation (9). Indeed, Newton and colleagues (49) demonstrated that oscillations in [Ca^{2+}]$_i$ induce oscillations in PKCα activity, emphasizing the role of Ca^{2+} binding to the C2 domain in the control of the activity and membrane localization of PKCα in the cell. Our own previous results demonstrated that [Ca^{2+}]$_e$-evoked [Ca^{2+}]$_i$ oscillations in HEK-293 cells expressing CaR

are associated with periodic InsP$_3$ production and oscillatory translocations of PKCα to the plasma membrane (37). All these findings support a critical role of PKCα in mediating [Ca^{2+}]$_i$ oscillations via its C2 domain. CaR also generates [Ca^{2+}]$_i$ oscillations in response to aromatic amino acids in the presence of a threshold [Ca^{2+}]$_e$ that are characterized by lower frequency Ca^{2+} spikes that return to the baseline levels (53). Amino acid-induced [Ca^{2+}]$_i$ oscillations via the CaR are thought to be generated by periodic changes in TRPC1 activity (36) and thus differ from [Ca^{2+}]$_e$-evoked [Ca^{2+}]$_i$ oscillations mediated by PKCα.

There is considerable pharmacological interest in the development of allosteric modulators of CaR function. Although these agents also elicit [Ca^{2+}]$_i$ oscillations, the mechanism(s) involved has not been elucidated. Here, we show that the positive allosteric modulator R-568 induced [Ca^{2+}]$_i$ oscillations even at high concentrations. Our results demonstrate that [Ca^{2+}]$_i$ oscillations induced by R-568 were dependent on PKC activity. Thus we propose that allosteric modulators by increasing the affinity of the CaR for [Ca^{2+}]$_e$ induce [Ca^{2+}]$_i$ oscillations via periodic feedback inhibition by PKC.

Interestingly, a mutation in Thr^{888} has been recently discovered in human patients with autosomal dominant hypocalcemia (23), demonstrating the functional importance of PKC-mediated phosphorylation of this site and the generation of [Ca^{2+}]$_i$ oscillations in vivo. Members of the PKC family have been the subject of intense interest as potential drug targets in a variety of diseases (14, 26, 51). For example, AEB071 is being tested as a therapeutic agent in a variety of clinical settings, including autoimmune diseases (14) and ocular melanoma (51). However, the results of clinical trials using PKC inhibitors have not been successful, largely owing to inadequate therapeutic effects and/or unanticipated adverse reactions (29). In view of the results presented here, it is important to consider that administration of inhibitors of PKCα in vivo will cause an increased responsiveness of the CaR to [Ca^{2+}]$_e$ potentially leading to alterations in Ca^{2+} homeostasis.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.H.Y. and O.R. performed experiments; S.H.Y., O.R., and J.S.-S. analyzed data; S.H.Y., O.R., J.S.-S., and E.R. interpreted results of experiments; S.H.Y. and J.S.-S. prepared figures; S.H.Y. and E.R. drafted manuscript; S.H.Y., O.R., J.S.-S., and E.R. edited and revised manuscript; E.R. conception and design of research; E.R. approved final version of manuscript.

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